GLOBAL EDITION

BROCK BIOLOGY OF MICROORGANISMS

SIXTEENTH EDITION

Madigan • Bender • Buckley • Sattley • Stahl

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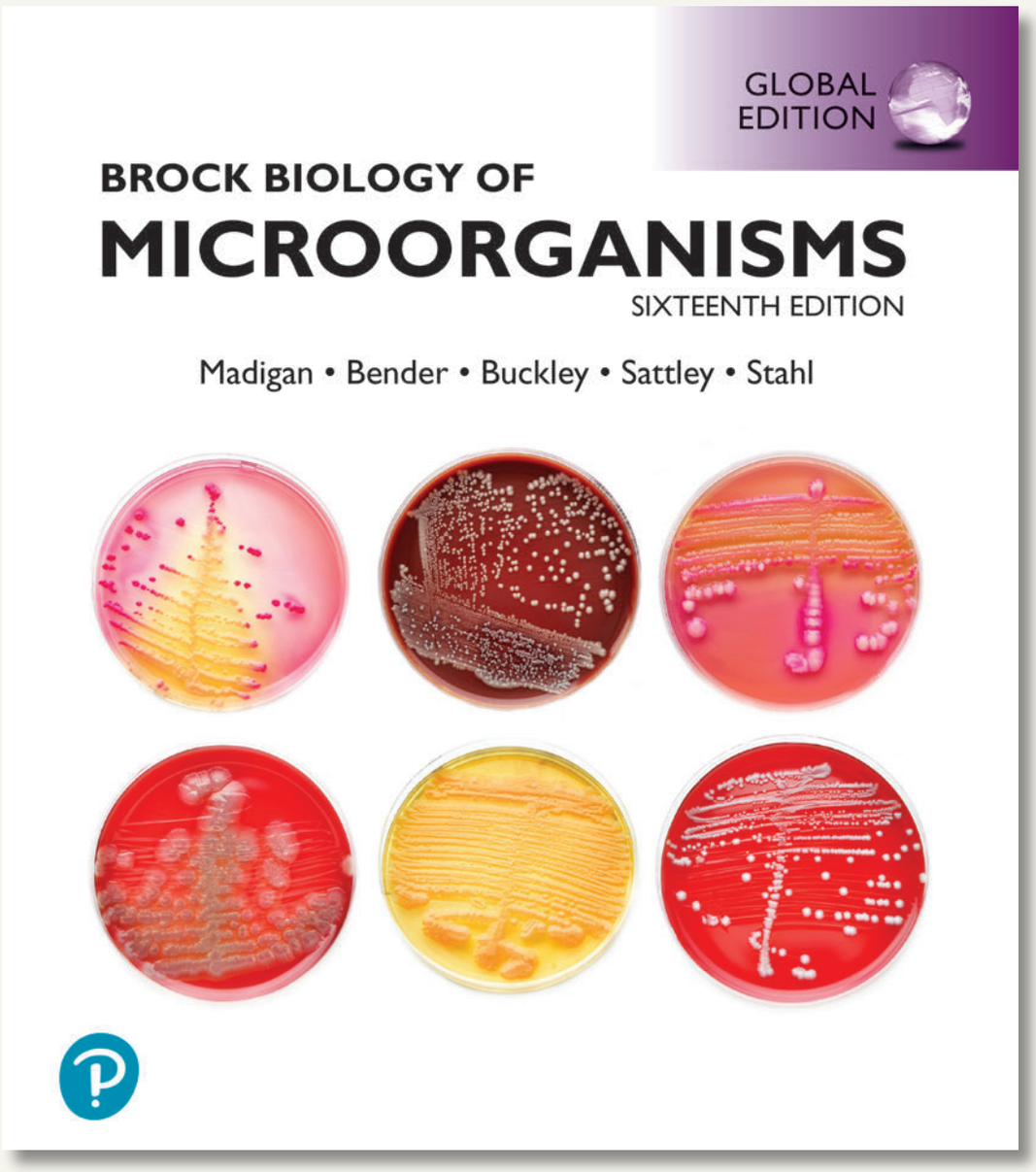
Authoritative. Accurate. Accessible.

Brock Biology of Microorganisms **is the leading microbiology text for majors, setting the standard for impeccable scholarship, accuracy, a visually stunning art program, and the use of cutting-edge research to illustrate basic concepts.**



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Making Connections Across

UPDATED! Each chapter is carefully cross-referenced to connect students with related material found earlier (◀) or later (▶) in the book.

I • Bacterial Cell Division

Prokaryotic cell division is preceded by chromosome replication and the synthesis of new cell wall material in a way that defines cell shape. Cell division is orchestrated in a carefully controlled fashion by protein complexes whose activities can be visualized by powerful light microscopic techniques.

Most cells divide by binary fission (◀ Section 4.6 and Figure 4.8), and this process occurs in a defined series of steps such that each daughter cell obtains a copy of the genome. During the division cycle, the cell must also produce new peptidoglycan and cytoskeleton elements to prevent bursting from osmotic forces. This cytoskeleton gives the cell its distinct morphology (◀ Figure 1.8). To successfully orchestrate all of these events, various regulatory cascades are put into play. In this first part of the chapter we focus on the molecular mechanisms employed by two well-studied gram-negative bacteria, *Escherichia coli* and *Caulobacter crescentus*, and introduce advanced microscopic techniques that have revealed the major molecular events that underlie cell division and cell morphology.

NEW! Key Concept statements at the start of each key topic of a chapter give students a big-picture view of the content to come before they dive in and immerse themselves in the details.

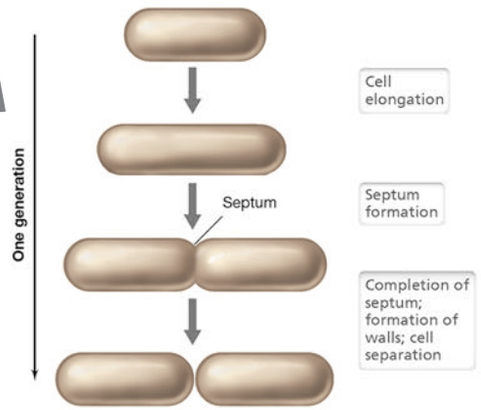


Figure 4.8 Binary fission in a rod-shaped bacterium. Cell numbers (and all components of the cells) double every generation.

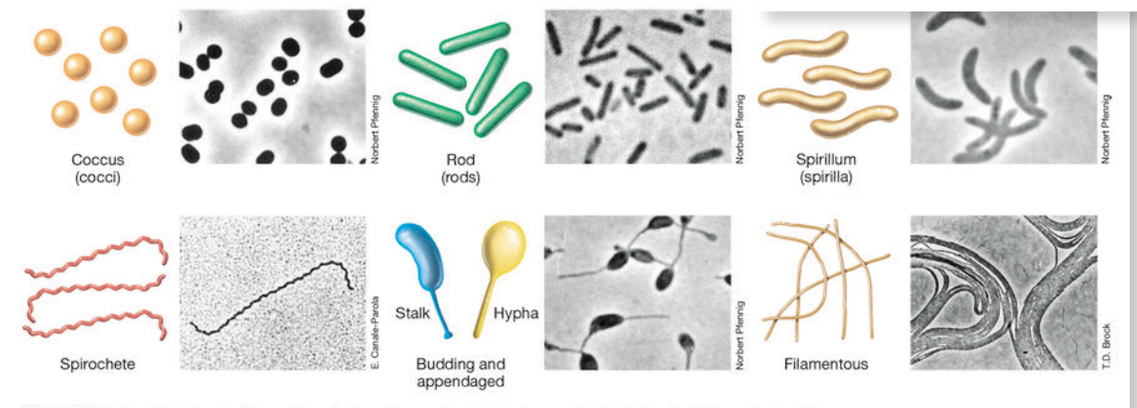
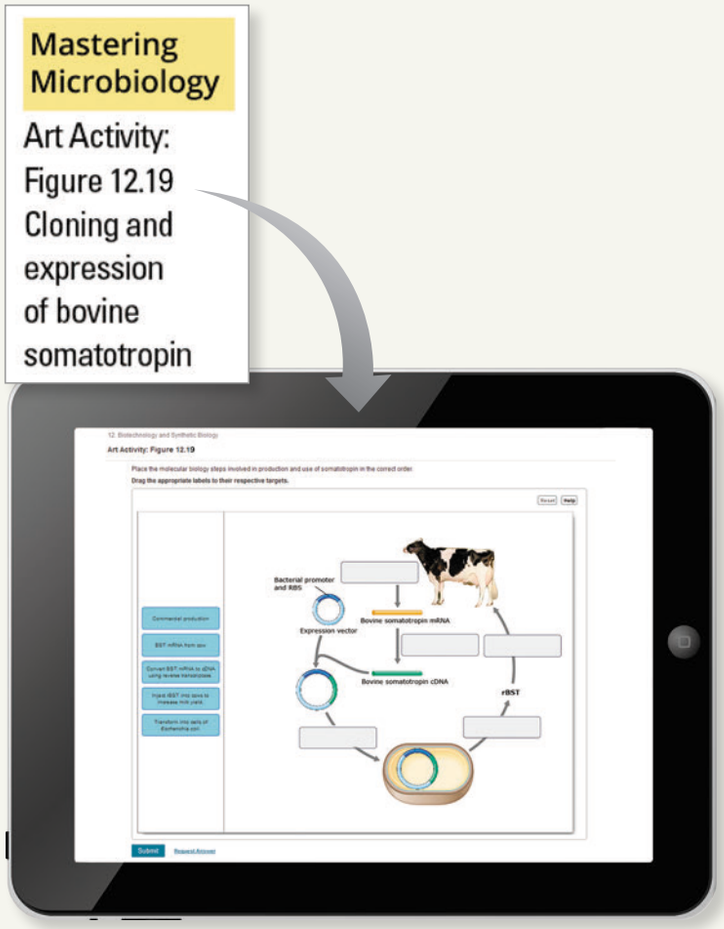


Figure 1.8 Cell morphologies. Beside each drawing is a phase-contrast photomicrograph of cells showing that morphology. Coccus (cell diameter in photomicrograph, 1.5 μm); rod (1 μm); spirillum (1 μm); spirochete (0.25 μm); budding (1.2 μm); filamentous (0.8 μm). All photomicrographs are of species of *Bacteria*. Not all of these morphologies are known among the *Archaea*, but cocci, rods, and spirilla are common.

Concepts in Microbiology

NEW! Marginal annotations highlight some of the best material available for instructors to assign in Mastering Microbiology, guiding students along their journey with insightful materials that support and strengthen the learning experience.



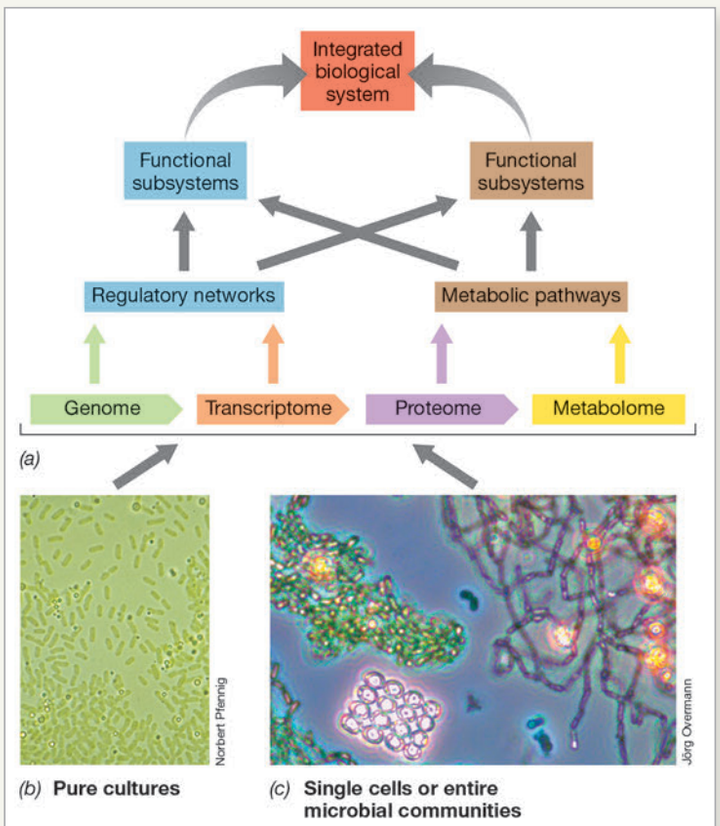


Figure 10.29 The components of systems biology. *(a)* The results of various "omics" analyses are combined and successively integrated into higher-level views of the entire biology of a pure culture, such as *(b)* that of the green sulfur bacterium *Chlorobium*; or of a mixed microbial community, such as *(c)* that of phototrophic sulfur bacteria obtained from a lake; or of a single cell isolated from a microbial community (see Figure 10.30).

Genomics, and the various "omics" it has spawned, is woven into every chapter of the text, providing students with concrete examples of how powerful tools have allowed microbiologists to probe deeper and farther into the microbial world than ever before.

Cutting-Edge Content

MICROBIOLOGYNOW

When Antibiotics Fail, Bacteriophage Therapy to the Rescue

Acquiring an antibiotic-resistant infection or "superbug" is one of medicine's biggest nightmares. What can medical practitioners do to treat the patient? Besides drugs, viruses known as bacteriophages have been recruited to specifically target and kill bacteria.

Despite microbiologists' tinkering with using bacteriophages as antimicrobials for decades, their actual application in medicine has been minimal. However, the emergence of antibiotic resistance has led to renewed focus on using these tiny microbes as therapeutic agents. The photo above shows Ella Balasa (right side of photo), a microbiologist who has cystic fibrosis. Cystic fibrosis is a genetic disease that results in a buildup of thick mucus in the lungs. This mucus allows bacteria to flourish in the lungs, which results in infections and subsequent lung damage that can be fatal. Ella had been treated numerous times with strong antibiotics specific for a respiratory infection caused by the bacterial pathogen *Pseudomonas aeruginosa*, but the microbial cells had become unresponsive to the drugs. At the time of this photo, the recurrent infection had decreased her lung function to the point where she required constant supplemental oxygen.

As an alternative treatment route, Dr. Benjamin Chan (on the left) took mucus from Ella's lungs infected with *P. aeruginosa* and isolated a bacteriophage that specifically killed the pathogen (see zones of clearing on Petri plate). This bacteriophage was propagated and then poured into a device so that Ella could inhale the therapy. The result of her treatment? Amazingly, the bacteriophage therapy along with a mixture of antibiotics resulted in the infection clearing a few weeks later!

While bacteriophage therapy is l
specificity and the ability of the path
become resistant to viral infection, t
success stories illustrate the future
therapy when all other options fail.

Source: Kortright, K.E., B.K. Chan, J.
Phage therapy: A renewed approach
bacteria. *Cell Host Microbe* 25(2): 21

NEW! Thirty-four Microbiology Now chapter opening vignettes were composed for this edition, each designed to introduce a chapter's theme through a recent discovery in the field of microbiology. These exciting accounts will draw students into the chapter and show how the chapter content connects with real-world problems.

NEW! Several new Explore the Microbial World features provide fascinating stories that highlight how important chapter concepts have evolved from research in the microbial world.

Explore the Microbial World

Pattern Recognition Receptors of Hydrothermal Vent Tube Worms Facilitate Endosymbiosis

Invertebrates and plants lack adaptive immunity but have a well-developed innate immune response to a wide variety of pathogens. As discussed in Section 26.6, virtually all multicellular organisms respond to pathogen invasion by recognizing signature molecules found on pathogen surfaces. These molecules contain conserved, repetitive structures called pathogen-associated molecular patterns (PAMPs) that include molecules such as the lipopolysaccharide (LPS) and flagellin of gram-negative bacteria, the peptidoglycan of gram-positive bacteria, and the double-stranded RNA of certain viruses. The term *microbe-associated molecular pattern*, or MAMP, is also commonly used to describe signature molecules found on microorganisms. However, "MAMP" is a broader term than "PAMP" because it includes components found on microorganisms that are not pathogenic.

Unlike PAMPs, which are exploited specifically for innate defense against pathogens, MAMPs found on nonpathogenic bacteria can serve an entirely different purpose—that of facilitating, through host pattern recognition receptors (PRRs), a mutualistic partnership rather than a confrontation between a host and the bacteria that colonize it. As we learned in Chapter 23, a wide variety of plants and animals maintain symbiotic relationships with microorganisms. There we discussed the association of tube worms that develop near hydrothermal vents in the deep sea with autotrophic, sulfur-oxidizing bacteria (SOB) that inhabit their *trophosome*, a spongy internal organ that comprises most of the volume of the 1- to 2-m-long worms (Figure 1). These SOB form an endosymbiosis with the worms in which the bacteria provide all organic carbon requirements for their animal host in exchange for a steady supply of essential metabolites, in particular H_2S, O_2, and CO_2. H_2S is the energy source for the SOB; they oxidize it to S^0 and then SO_4^{2-} and respire the electrons to generate a proton motive force that drives ATP synthesis. Oxygen (O_2) is required as a terminal acceptor of electrons that have traversed the electron transport chain. CO_2 is the carbon source and is incorporated into bacterial cell material by way of the Calvin cycle, the major means of autotrophy in chemolithotrophic bacteria.

This fascinating association raises the question of how it is established. Specifically, how does the tube worm populate its trophosome with SOB to the exclusion of other, potentially pathogenic, bacteria? The answer appears to be closely linked to MAMPs associated with the endosymbiotic SOB. Although host PRRs are typically used to recognize and eliminate pathogens, the study of tube worms and other animals that harbor endosymbiotic microbes shows a broader functionality for PRRs in that they can also interact beneficially with MAMPs to selectively populate a host with nonpathogenic symbionts.

The tube worm trophosome contains a large number of specialized host immune cells called *bacteriocytes*, and it is within these cells that the bacterial symbionts take up residence. The tube-worm bacteriocytes express high levels of PRRs that recognize MAMPs, such as specific cell surface lipoproteins associated with SOB. This positive interaction locates the bacteria to the trophosome and, with a steady supply of simple nutrients from the hydrothermal vent system delivered by blood circulating in the worm, stimulates colonization and growth of the symbionts in their animal host.

As this example illustrates, in addition to providing a rapid response to pathogen challenge, innate immune mechanisms—specifically, the interaction of PRRs with MAMPs—may also serve the primary role in governing host–symbiont interactions and the establishment of endosymbiotic relationships. Given the critical role innate immune mechanisms play in maintaining the animal–bacterial symbiosis within the tube-worm trophosome, it is likely that similar tightly choreographed molecular mechanisms constitute a host–microbe "dialogue" that helps to maintain balanced communities of beneficial microbiota in virtually all animals, including humans.

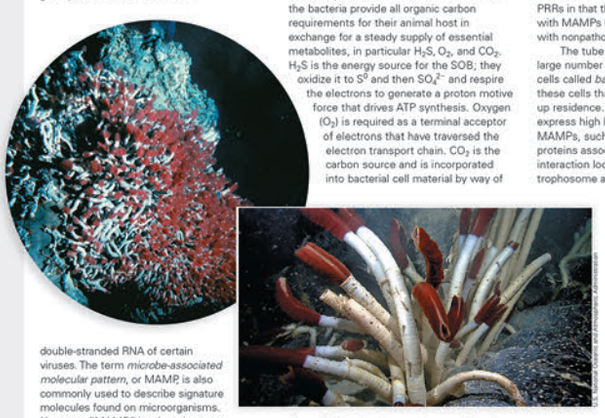


Figure 1 Hydrothermal vent tube worms harboring endosymbiotic sulfur-oxidizing bacteria. Top: A "black smoker" hydrothermal vent community containing several tube worms that obtain organic carbon from sulfur-oxidizing chemolithotrophic bacteria (SOB) living within them. Bottom: A close-up view of tube worms; each worm is 1–2 m long. The red area on the top of each worm, called the plume, is where O_2 and H_2S are taken in to be fed to the worm's SOB endosymbionts residing in the trophosome.

NEW! A section on immunotherapy highlights exciting advancements in the use of genetic engineering and molecular immunology to treat cancer.

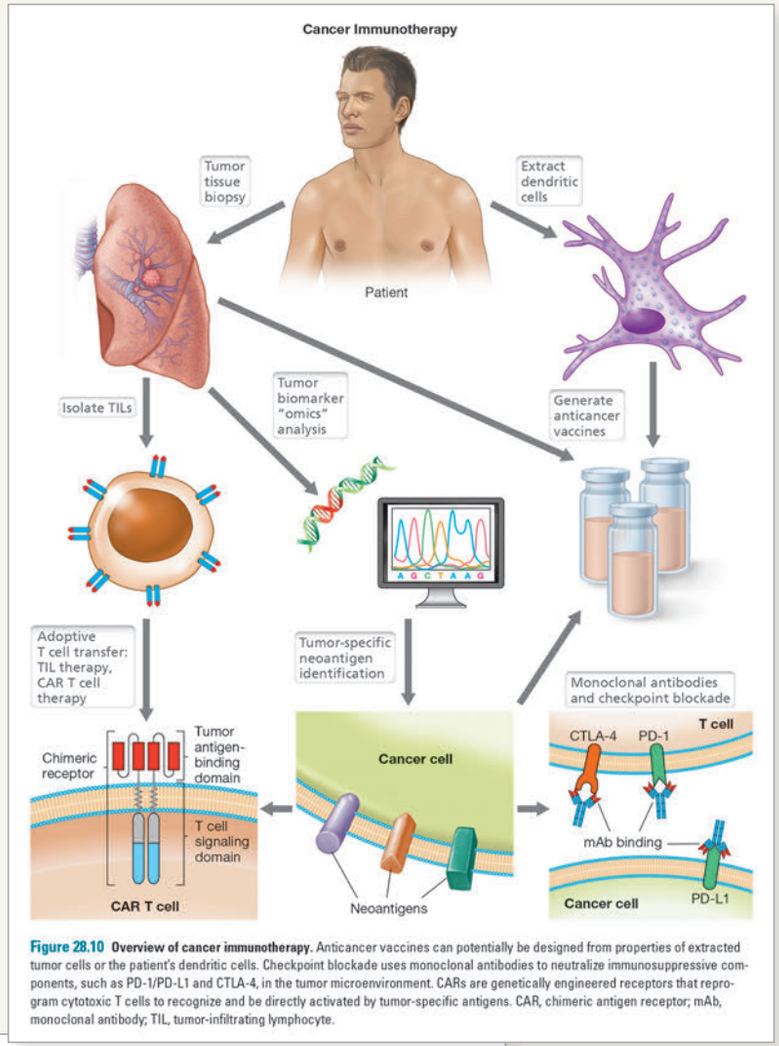


Figure 28.10 Overview of cancer immunotherapy. Anticancer vaccines can potentially be designed from properties of extracted tumor cells or the patient's dendritic cells. Checkpoint blockade uses monoclonal antibodies to neutralize immunosuppressive components, such as PD-1/PD-L1 and CTLA-4, in the tumor microenvironment. CARs are genetically engineered receptors that reprogram cytotoxic T cells to recognize and be directly activated by tumor-specific antigens. CAR, chimeric antigen receptor; mAb, monoclonal antibody; TIL, tumor-infiltrating lymphocyte.

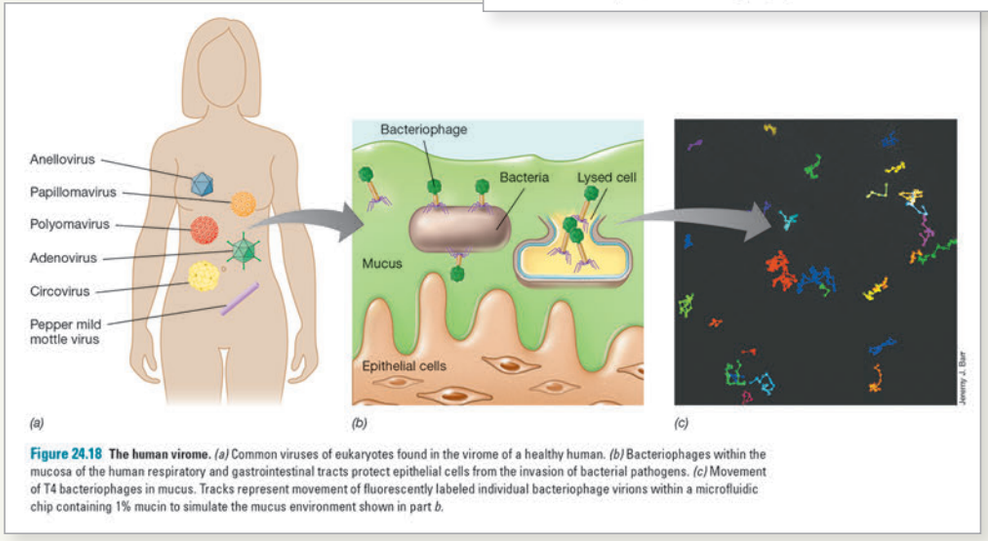
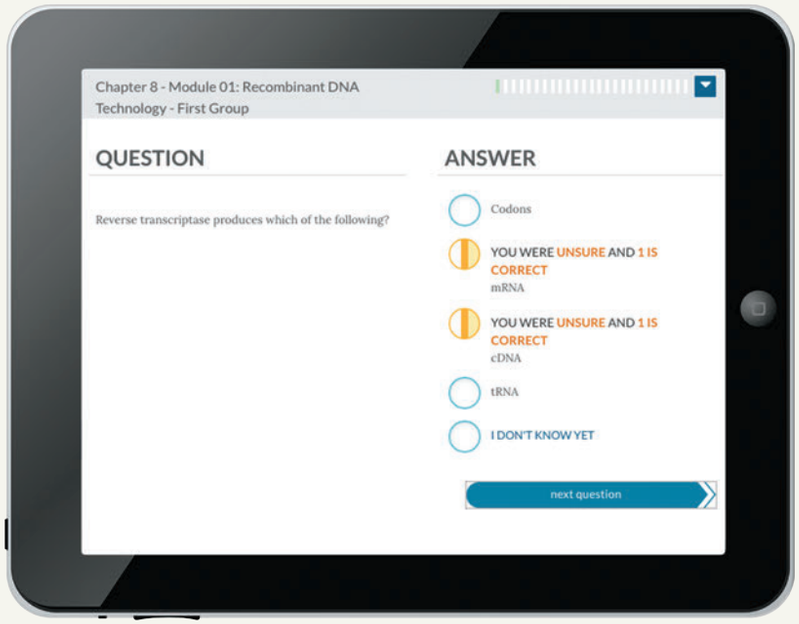


Figure 24.18 The human virome. *(a)* Common viruses of eukaryotes found in the virome of a healthy human. *(b)* Bacteriophages within the mucosa of the human respiratory and gastrointestinal tracts protect epithelial cells from the invasion of bacterial pathogens. *(c)* Movement of T4 bacteriophages in mucus. Tracks represent movement of fluorescently labeled individual bacteriophage virions within a microfluidic chip containing 1% mucin to simulate the mucus environment shown in part *b*.

NEW! The chapter on the human microbiome now includes a new section on the human virome, describing how metagenomics is aiding the discovery and isolation of many new viruses. Extensive coverage is provided of the impact of early life events on the development of the newborn gut microbiome and of recent successes in probiotic therapy for preventing newborn intestinal diseases.

Empower Each Learner



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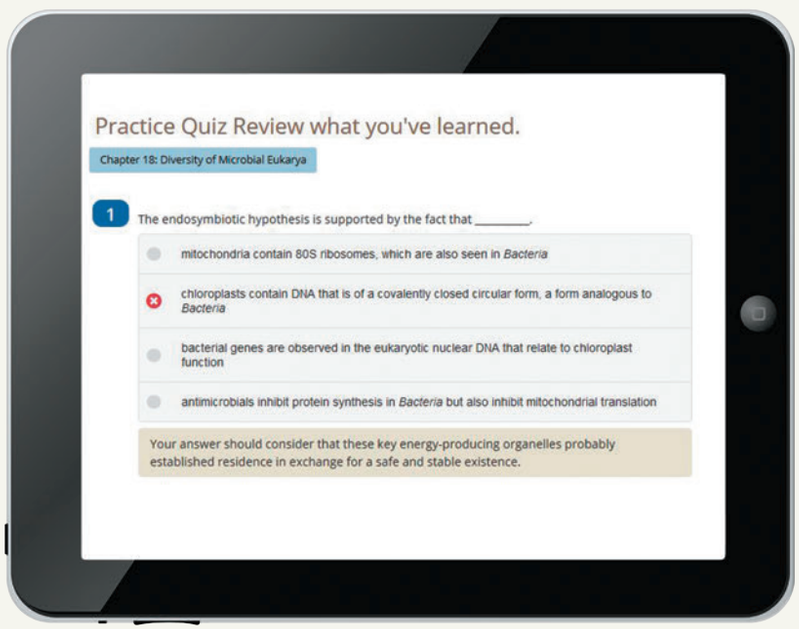
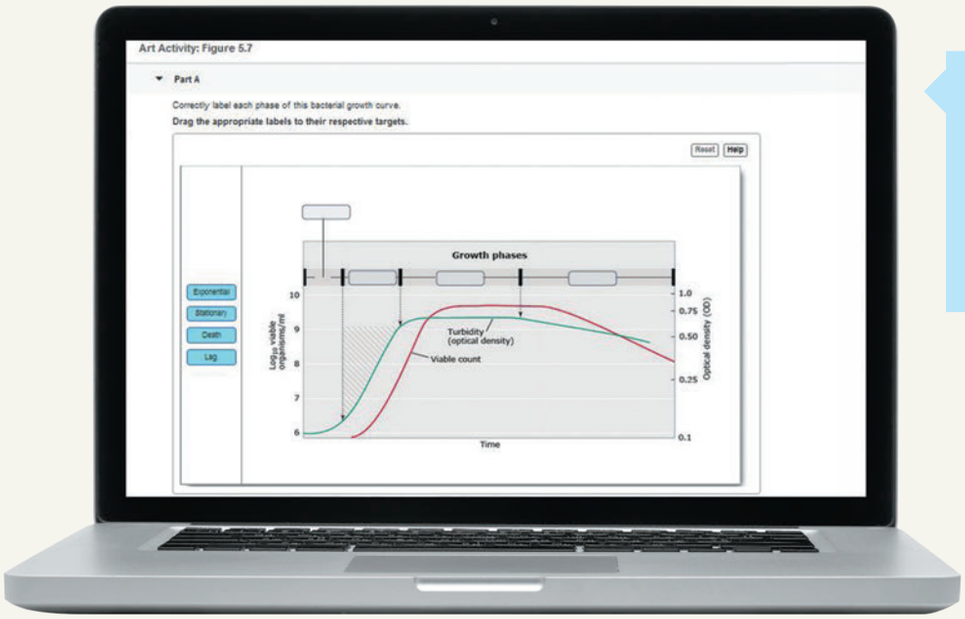


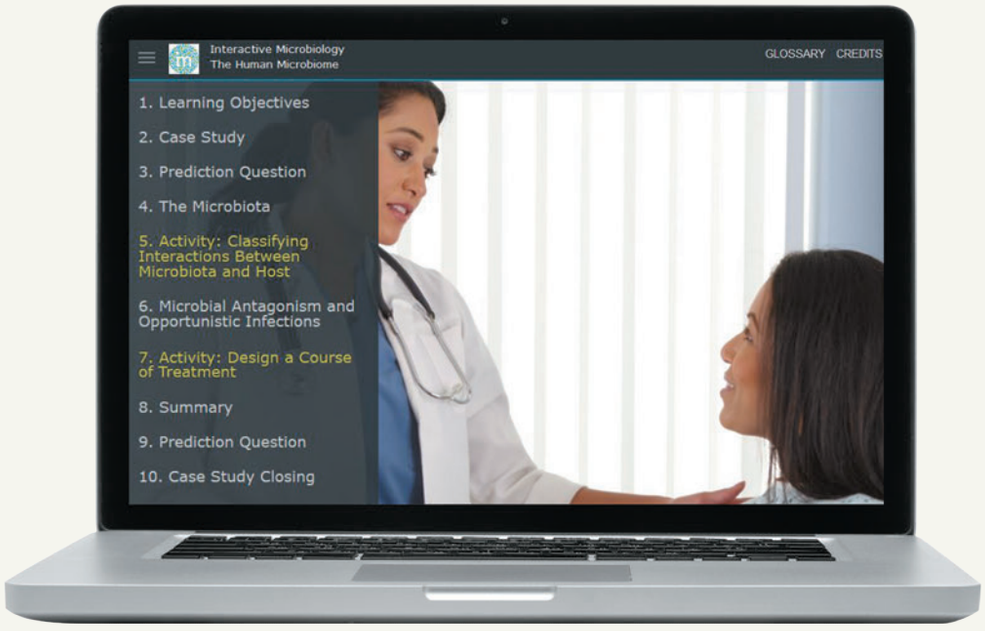
Mastering is so much more than homework. The **Pearson Study Area** allows students to access multiple study tools in one place. They don't need to search Google to find study tools that align with their course materials. Students have access to practice quizzes, videos and animations, vocabulary tools, and more.

with Mastering Microbiology



Get students engaged with content by assigning a variety of questions in Mastering Microbiology. These include:

- Reading Questions
- Art-Based Activities
- Coaching Activities and more



Interactive Microbiology is a dynamic suite of interactive tutorials and animations that teach key microbiology concepts including Operons, Biofilms and Quorum Sensing, Complement, Human Microbiota, and Antibiotic Resistance. Interactive Microbiology actively engages students with each topic, enabling them to learn from manipulating variables, predicting outcomes, and answering formative and summative assessment questions. Each tutorial presents the concept within a real healthcare scenario in order to emphasize problem solving and interest students from the beginning.

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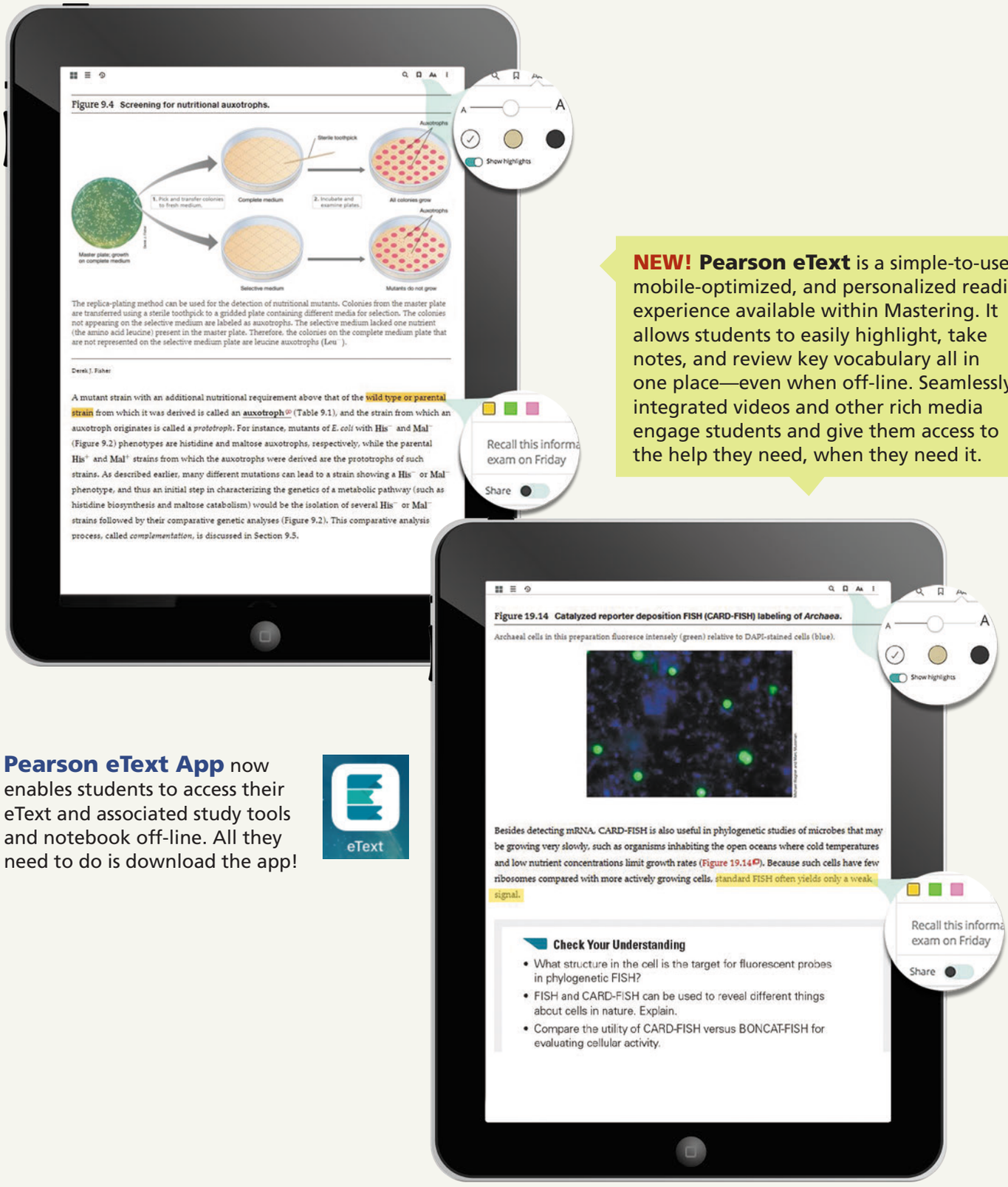

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Pearson eText App now enables students to access their eText and associated study tools and notebook off-line. All they need to do is download the app!

BROCK BIOLOGY OF MICROORGANISMS

SIXTEENTH EDITION
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MICHAEL T. MADIGAN
Southern Illinois University Carbondale

KELLY S. BENDER
Southern Illinois University Carbondale

DANIEL H. BUCKLEY
Cornell University

W. MATTHEW SATTLEY
Indiana Wesleyan University

DAVID A. STAHL
University of Washington Seattle

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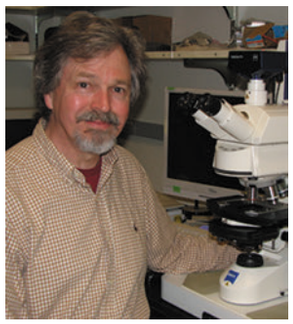
About the Authors

Michael T. Madigan received his B.S. in Biology and Education from Wisconsin State University–Stevens Point (1971) and his M.S. (1974) and Ph.D. (1976) in Bacteriology from the University of Wisconsin–Madison in the laboratory of Thomas Brock. Following a postdoc at Indiana University with Howard Gest, Mike moved to Southern Illinois University Carbondale, where he taught courses in introductory microbiology and bacterial diversity as a professor of microbiology for 33 years. In 1988 Mike was selected as the Outstanding Teacher in the College of Science and in 1993, the Outstanding Researcher. In 2001 he received the SIUC Outstanding Scholar Award and Distinguished Professor title. In 2003 Mike received the Carski Award for Distinguished Undergraduate Teaching from the American Society for Microbiology (ASM), and he is an elected Fellow of the American Academy of Microbiology (ASM) and the American Association for the Advancement of Science (AAAS). He has also been recognized by the American Red Cross as a major volunteer blood donor for the 24 gallons of blood he has donated since 1967. Mike's research is focused on phototrophic bacteria that inhabit extreme environments, and for the past 20 years his emphasis has been Antarctic microbiology. Mike has co-edited a major treatise on phototrophic bacteria and served for 10 years as chief editor of the journal *Archives of Microbiology.* He currently serves on the editorial board of the journals *Environmental Microbiology* and *Antonie van Leeuwenhoek*. Mike's other interests include forestry, swimming, reading, and caring for his dogs and horses. He lives on a small farm near a quiet lake with his wife, Nancy, three dogs (Kato, Nut, and Merlyn), and three horses (Eddie, Georgie, and Roscoe).

Kelly S. Bender received her B.S. in Biology from Southeast Missouri State University (1999) and her Ph.D. (2003) in Molecular Biology, Microbiology, and Biochemistry from Southern Illinois University Carbondale. Her dissertation research focused on the genetics of perchlorate-reducing bacteria. During her postdoctoral fellowship, Kelly worked on the genetic regulation of sulfate-reducing bacteria in the laboratory of Judy Wall at the University of Missouri–Columbia. She also completed a transatlantic biotechnology fellowship at Uppsala University in Sweden researching regulatory small RNAs in bacteria. In 2006, Kelly returned to her alma mater, Southern Illinois University Carbondale, as an Assistant Professor in the Department of Microbiology and in 2012 was tenured and promoted to Associate Professor. She has served as Chair of the SIUC Department of Microbiology since 2018. Her lab studies a range of topics including regulation in sulfate-reducing bacteria, the microbial community dynamics of sites impacted by acid mine drainage, and diversity of phototrophic heliobacteria. Kelly teaches courses in introductory microbiology and microbial diversity, has served on numerous federal grant review panels, and is an active member of the American Society for Microbiology (ASM). Her other interests include spending time with her daughter, Violet, and husband, Dick.

Daniel H. Buckley is a Professor at Cornell University in the School of Integrative Plant Science and the Department of Microbiology. He earned his B.S. in Microbiology (1994) at the University of Rochester and his Ph.D. in Microbiology (2000) at Michigan State University. His graduate research in the laboratory of Thomas M. Schmidt explored environmental factors that influence microbial diversity in soils. Dan then received a National Science Foundation Postdoctoral Fellowship to work with Pieter T. Visscher, University of Connecticut, investigating linkages between microbial diversity and biogeochemistry within microbial mats and stromatolites. Dan moved to Cornell in 2003 where he investigates the ecology and evolution of the diverse microorganisms that live in soils. He has taught both introductory and advanced courses in microbiology, microbial diversity, and microbial genomics. He received a National Science Foundation Faculty Early Career Development (CAREER) award in 2005 for excellence in integrating research and education, and served as Co-Director of the MBL Microbial Diversity summer course in Woods Hole, Massachusetts (2009–2013). He currently serves on the editorial boards of *Applied and Environmental Microbiology* and *Environmental Microbiology*. Dan lives in Ithaca, New York, with his wife, Merry, and sons, Finn and Colin.

W. Matthew Sattley received his B.A. in Biology in 1998 from Blackburn College (Illinois) and his Ph.D. (2006) in Molecular Biology, Microbiology, and Biochemistry from Southern Illinois University Carbondale. His graduate studies focused on the microbiology of sulfur cycling and other biogeochemical processes in permanently ice-covered lakes of Antarctica. In his postdoctoral research at Washington University in Saint Louis, he studied the physiology and genomics of anoxygenic phototrophic bacteria in Robert Blankenship's laboratory. Matt then accepted a faculty appointment to the Department of Biology at MidAmerica Nazarene University (Kansas), where he supervised undergraduate research and taught courses in microbiology, environmental science, and cell biology. In 2010, Matt transitioned to the Division of Natural Sciences at Indiana Wesleyan University (IWU), where he is a Professor of Biology and has served as the Director of the Hodson Research Institute, a faculty-led summer research program for undergraduate students in the Natural Sciences. Matt's research group investigates the ecology, diversity, and genomics of bacteria that inhabit extreme environments, and in 2017, he was the recipient of IWU's Outstanding Scholarship Award. Matt is a member of the American Society for Microbiology (including its Indiana Branch) and the Indiana Academy of Science. Matt lives in Marion, Indiana, with his wife, Ann, and sons, Josiah and Samuel. Outside of teaching and research, Matt enjoys playing drums, reading, motorcycling, and baseball.



David A. Stahl received his B.S. degree in Microbiology from the University of Washington, Seattle, and completed graduate studies in microbial phylogeny and evolution with Carl Woese in the Department of Microbiology at the University of Illinois at Urbana–Champaign. Subsequent work as a postdoctoral fellow with Norman Pace, then at the National Jewish Hospital in Colorado, involved early applications of 16S rRNA-based sequence analysis to the study of natural microbial communities. In 1984 Dave joined the faculty at the University of Illinois with appointments in Veterinary Medicine, Microbiology, and Civil Engineering. In 1994 he moved to the Department of Civil Engineering at Northwestern University, and in 2000 returned to the University of Washington as professor in the Departments of Civil and Environmental Engineering and Microbiology. Dave is known for his work in microbial evolution, ecology, and systematics, and received the 1999 Bergey Award and the 2006 ASM Procter & Gamble Award in Applied and Environmental Microbiology. Dave is an elected fellow of the American Academy of Microbiology and a member of the National Academy of Engineering. His main research interests surround the biogeochemistry of nitrogen and sulfur and the microbial communities that sustain the associated nutrient cycles. His laboratory was the first to culture ammonia-oxidizing *Archaea,* a group believed to be the key mediators of this process in the nitrogen cycle. Dave has taught several courses in environmental microbiology, was one of the founding editors of the journal *Environmental Microbiology*, and has served on many advisory committees. Outside the lab, Dave enjoys hiking, bicycling, spending time with family, reading a good science fiction book, and—with his wife, Lin—renovating an old farmhouse on Bainbridge Island.

Dedications

Michael T. Madigan

dedicates this book to the 10^{31} (more or less) microbial cells on and within Earth that maintain our planet in a habitable state. Keep up the good work, guys.

Kelly S. Bender

dedicates this book to the memory of her grandmother, Alberta, whose biggest regret in life was not being able to attend school past the fifth grade.

Daniel H. Buckley

dedicates this book to his father, Ron, who taught me ingenuity and persistence.

W. Matthew Sattley

dedicates this book to the memory of his father, Steven, and to his mother, Patrice, for demonstrating the benefits of working hard and seeking knowledge.

David A. Stahl

dedicates this book to his wife, Lin. My love, and one that helps me keep the important things in perspective.

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Preface

Welcome to the best learning resource in microbiology education today: the visually stunning 16th Edition of *Brock Biology of Microorganisms* (*BBOM*). The 16th Edition is the most student-friendly and accessible edition yet and presents the most exciting and recent picture of the science of microbiology available today. For three generations, both students and instructors alike have praised the accuracy, authority, consistency, and teachability of *BBOM* for exploring the principles of microbiology in a readable, connected, and visually appealing way.

Both students and instructors will benefit from at least four important strengths of the 16th Edition: (1) our approach of using cutting-edge research to solidify basic concepts; (2) the seamless integration of molecular and ecological microbiology with coverage of evolution, diversity, the immune system, and infectious diseases; (3) the spectacular art program complemented with striking and compelling photos; and (4) the wide assortment of teaching and learning tools that accompany the book itself. With an extremely strong author team that employs experts in each major theme, *BBOM* 16th Edition leads the way in presenting the essential principles of microbiology that students need to master today.

What's New in the 16th Edition?

The 16th Edition guides students through the six major themes of microbiology as outlined by the American Society for Microbiology Conference on Undergraduate Education (ASMCUE): Evolution, Cell Structure and Function, Metabolic Pathways, Information Flow and Genetics, Microbial Systems, and the Impact of Microorganisms. With new and revised artwork complemented by over 60 new photos, *BBOM* 16th Edition (16e) presents microbiology as the visual science it is. Thirty-four new MicrobiologyNow chapter-opening vignettes were composed for this edition, each designed to introduce a chapter's theme through a recent discovery in the field of microbiology. These exciting accounts will naturally draw students into the chapter and show how the chapter's content connects with real-world problems. Several new Explore the Microbial World features were also developed for this edition, each designed to give students a feel for exciting special topics in microbiology and to fuel their scientific curiosity.

Genomics, and all of the various "omics" it has spawned, support content in every chapter of *BBOM* 16e, reflecting the reality of how omics has transformed all of biology, especially microbiology. The result is a robust and modern treatment of microbiology that guides students through the maze of omics with concrete examples of how these powerful tools have allowed microbiologists to probe deeper and farther into the microbial world than ever before.

To reinforce the learning experience, the 16e debuts a new pedagogical aid called Key Concepts. These brief summaries of each chapter part are written in clear and straightforward language that give students a heads-up as to what is coming in the following sections. Complementing the Key Concepts, each numbered section is summarized in the chapter review and accompanied by a review question that links concept review with concept mastery.

BBOM 16e is supported by Mastering Microbiology, Pearson's online homework, tutorial, and assessment system that assists students in pacing their learning and keeps instructors current on class performance. Mastering Microbiology includes a new feature, Dynamic Study Modules, which adapt to the student's performance in real time to help each student's study of course topics. Students build the confidence they need to deepen their understanding, participate meaningfully, and perform better in and out of class. Other highlights include chapter-specific reading quizzes, MicroLab Tutorials, MicrobiologyNow coaching activities, Clinical Case and MicroCareer coaching activities, animation quizzes, MCAT Prep questions, and many additional study and assessment tools. Collectively, the content and presentation of *BBOM* 16e, coupled with the powerful learning tools of Mastering Microbiology, create an unparalleled educational experience in microbiology.

Revision Highlights

UNIT 1 The Foundations of Microbiology

Chapter 1

- The microbial world is introduced in an exciting and novel way by weaving together core concepts in microbiology with the historical events that led to their discovery. The foundations of microbiology are revealed through introductions to microscopy, laboratory cultivation, microbial evolution, and the molecular principles that unify all life.
- Some highlights: Vibrant new images help connect students with the diverse and numerous ways in which microbiology impacts our world. Coverage of cell size and morphology is introduced here rather than in Chapter 2 in order to draw students into the microscopic world early on and introduce them to actual microbes and their properties.

Chapter 2

- In the microbial world, cellular structures are tightly linked to cell functions, and Chapter 2 offers a complete guide to the features that define and differentiate microbial cells and their functions. Updated coverage of nutrient transport here rather than in the growth chapter places this critical cellular activity firmly within the context of the cell envelope.
- Some highlights: Electron cryotomography has provided new insight into cell biology and is incorporated in new views of peptidoglycan structure, S-layers, and diversity in cell envelope organization. Vivid new illustrations developed from cutting-edge

microscopic images of the flagellum, the archaellum, and the rotating proteins that confer gliding motility provide a fresh new look at how these structures move prokaryotic cells about their environments.

Chapter 3

- This chapter remains focused on the fundamentals of metabolism and has been revised to simplify metabolic concepts and emphasize the modularity of metabolism. The chapter starts with the essential principles and then provides examples of their application while guiding the student though the major metabolic processes that define microbial life.
- Some highlights: New art provides greater clarity and realism in understanding electron transport reactions, making this process easier to understand and easier to teach. Modularity of metabolism and the importance of the proton motive force receive greater emphasis by providing simple examples of chemolithotrophy and phototrophy to reinforce the student's understanding of energy conservation as a unifying concept in biology. Updates to fermentation clarify and distinguish this process from anaerobic respiration, and an overview of autotrophy and nitrogen fixation emphasize the connectivity between anabolic and catabolic processes in the cell.

Chapter 4

- This chapter on microbial growth and its control moves up one slot from the previous edition to better prepare students for dealing with concepts in molecular biology and genetics where microbial growth plays a central role.
- Some highlights: The essentials of microbial nutrition and laboratory culture are introduced here with a segue to counting methods and quantitative aspects of microbial growth. The dynamics of microbial growth are emphasized with exciting new coverage of the biofilm mode of growth and alternatives to binary fission. The latter includes organisms that display budding division such as *Caulobacter*—the prime model for developmental studies of bacteria—and bacteria that grow by hyphal extensions characteristic of filamentous bacteria such as *Streptomyces*, a major producer of antibiotics.

Chapter 5

- This introduction to virology moves up from its position in Unit 2 in the previous edition to round out the foundations of microbiology theme of Unit 1. This move gives earlier visibility to the importance of viruses as microbes, clearly explains how they differ from cells, and lays the necessary groundwork for dealing with the genetics, genomics, and molecular biology that follows in Unit 2.
- Some highlights: Emphasis remains on the basic principles of virology including how viruses and cells can be viewed as both similar and different and how methods for replicating viruses resemble those for growing cells. Bacteriophage T4 is used as a model lytic virus, and coverage of eukaryotic viruses is expanded beyond just animal viruses to include some major viruses of plants. This highly visual chapter is embellished with over a dozen new photos of exciting, newly discovered viruses along with supporting art that underscores the fundamentals of virology.

UNIT 2 Molecular Biology and Genetics

Chapter 6

- Moved forward two slots from its position in the previous edition to better fit as the kick-off to Unit 2, this chapter lays the necessary groundwork in molecular biology for tackling microbial genetics and genomics and the fast-moving fields of synthetic biology, molecular microbial ecology and diversity, the human microbiome, and diagnostic microbiology.
- Some highlights: Reorganized coverage of DNA supercoiling precedes new and more realistic depictions of the seminal processes of replication, transcription, and translation. New coverage of transcriptional processes in *Archaea* and their relationship to those in *Eukarya* and updated coverage of protein secretion round out this essential primer in microbe molecular biology that every student needs to master.

Chapter 7

- Because microbes must coordinate cellular processes to optimize their chances for survival and reproduction, Chapter 7 is central to Unit 2 in describing how prokaryotic cells control the seminal processes of replication, transcription, and translation. Microbial regulatory systems are highly diverse and sometimes tiered, but an appreciation for how control systems work is key to understanding metabolic diversity, pathogenesis, and synthetic biology.
- Some highlights: Reorganized and expanded coverage of gene expression in *Bacteria* and *Archaea* including activation and repression/derepression as well as chemotaxis and global controls. New coverage of two-component systems for regulating nitrogen assimilation and updated coverage of the phosphate regulon, heat shock response, and riboswitch activity exemplify the comprehensive nature of this chapter.

Chapter 8

- This chapter continues the molecular theme of Unit 2 by building on the major topics of Chapters 4, 6, and 7 in the context of the mechanisms that underlie microbial growth and differentiation. Knowledge of the molecular biology of microbial growth is central to mastering the biology of microbial populations and is keenly relevant to the topics of antibiotic efficacy, antibiotic resistance and persistence, and infectious disease microbiology in general.
- Some highlights: New high-resolution time-course images highlight the molecular processes of growth and cell shape determination. We expand coverage of biofilm formation and the signaling molecule cyclic-di-GMP in *Bacteria* and provide new coverage of biofilm formation in *Archaea*. The chapter also includes new coverage of endospore germination and phenotypic heterogeneity to encompass more topics within the evolving field of microbial growth from a molecular perspective.

Chapter 9

- This chapter rounds out Unit 2 by discussing the foundation for microbial diversity—how microbes undergo genetic change while still maintaining genomic integrity. This essential primer of microbial genetics also lays the groundwork for tackling the hot areas of

microbial omics and synthetic biology and provides the fundamental background necessary to comprehend the most recent concepts of microbial evolution that will unfold in later chapters.
- Some highlights: New and updated visual depictions of DNA exchange between microbes as well as updated coverage on natural competence and the role of pili in DNA uptake. Reorganized and new coverage of barriers to DNA transfer including CRISPR, the important bacterial and archaeal "immune system" whose applications are revolutionizing biology and clinical medicine.

UNIT 3: Genomics, Synthetic Biology, and Evolution

Chapter 10

- Because the genome is the blueprint for all biological traits, this chapter kicks off Unit 3 by discussing not only microbial genomics, but also methods to assay large pools of biological molecules. Various omics studies can be combined to provide a detailed picture of the vast range of capabilities possessed by a specific microbe or groups of microbes, which is essential to the topics of genetic engineering, synthetic biology, and microbial ecology.
- Some highlights: New and exciting coverage of functional genomics and high-throughput techniques to determine the role of individual genes. Reorganized and updated coverage of microbial genome content, proteomic applications, and systems biology highlight the ever-advancing field of omics.

Chapter 11

- This chapter continues the theme of Unit 3 by focusing on the unique genomes of viruses and the diverse mechanisms by which viral genomes are replicated. Knowledge of the molecular biology underlying viral replication is central not only to understanding how viruses infect their hosts and how they persist, but also for developing new clinical strategies for treating viral diseases of humans and other animals.
- Some highlights: New coverage of viral taxonomy precedes updated coverage of viruses that infect *Archaea*. Reorganized topics of bacteriophage genome replication and regulation of lysogeny in lambda directly link to foundational material in Chapter 5.

Chapter 12

- This high-energy chapter entitled "Biotechnology and Synthetic Biology" covers the essential tools of twenty-first-century biotechnology and describes how they have been applied to yield game-changing medical and other commercial products from the activities of genetically engineered microbes. Expanded coverage is provided of the rapidly advancing fields of synthetic biology and CRISPR genome editing—the latest revolutions to hit biology since discovery of the polymerase chain reaction (PCR). Text and art have been updated throughout.
- Some highlights: New coverage of how biobricks contribute to the construction of synthetic pathways and synthetic cells; the use of recombineering to revolutionize molecular cloning; genetically engineered delivery of human therapeutic agents; refactoring metabolic pathways; targeted microbial delivery of human drugs; and how gene drives could finally conquer malaria.

Chapter 13

- This chapter on microbial evolution was moved from the diversity unit into Unit 3 to emphasize its now closer ties to the unit theme of genomics. In addition to origin of life coverage, the chapter now focuses on how evolution affects the genome and ultimately the biology of the organism. The chapter ends with streamlined coverage of microbial systematics and the definition of a microbial species as a prelude to coverage of microbial diversity in Unit 4.
- Some highlights: New and expanded coverage of the evolution of both cells and viruses, including new art on cellular origins from hydrothermal systems and early bioenergetics; more extensive discussion of the mechanisms of microbial evolution from a genomic perspective, including genomic changes that occur during both vertical and horizontal gene transmission; broadened coverage of experimental evolution and genome dynamics.

UNIT 4 Microbial Diversity

Chapter 14

- Recent years have seen a flurry of fundamental new discoveries about how anaerobic organisms conserve energy. Chapter 14 has been updated to integrate information from new discoveries that lie at the heart of diverse metabolic pathways, including the discovery of electron bifurcation and energy-converting hydrogenases.
- Chapter 14 now includes a new introductory section that summarizes foundational principles of microbial physiology. This new section boils the diversity of the microbial world down into a few key principles that students can follow throughout the chapter. In addition, the chapter includes new art illustrating electron bifurcation, as well as electron flow in organisms such as sulfate reducers and methanogens. Old favorites throughout the chapter are also updated to account for recent discoveries in the field.

Chapter 15

- Chapter 15 has been reorganized and updated to emphasize relationships between metabolic and ecological diversity. New photos have been added to emphasize the morphological diversity of anoxygenic phototrophs and to demonstrate how microorganisms work together to modify their environments.

Chapter 16

- Chapter 16 has new coverage of difficult-to-cultivate bacteria, such as *Acidobacteria*, *Planctomycetes*, and *Fusobacteria*. The widespread application of metagenomic techniques have revealed that these *Bacteria* are of considerable importance in a range of habitats, including the human microbiome, but have only recently been obtained in laboratory culture.

Chapter 17

- Metagenomics has contributed greatly to our knowledge of archaeal diversity. Chapter 17 now exploits this and unveils the TACK, DPANN, and Asgard *Archaea*, some of which are the closest known relatives of the eukaryotes. We also update the diversity of mechanisms of methanogenesis in the archaeal domain.

Chapter 18

- Along with major updates on eukaryotic phylogeny, a new section is devoted to the haptophytes, including the globally and ecologically important coccolithophore *Emiliania huxleyi*. Coccolithophores play a major role in regulating global climate, illustrating the power that microbes exert over our biosphere.

UNIT 5 Microbial Ecology and Environmental Microbiology

Chapter 19

- The chapter begins a unit on ecology and environmental microbiology. The modern tools of the microbial ecologist are described with examples of how each has helped sculpt the science.
- Some highlights: A new method to visualize protein synthesis in single cells allows study of microbial activity in the environment. Metabolomics exploits new methods in mass spectrometry to unravel the complex metabolic interactions sustaining microbial communities. Nanosensor technologies are revealing how microbes alter the chemical landscape of three-dimensional surfaces. A new section explores multi-omics, which combines multiple state-of-the-art analytical tools to more fully characterize microbial communities.

Chapter 20

- The properties and microbial diversity of major microbial ecosystems including soils and aquatic systems are compared and contrasted in exciting ways.
- Some highlights: Expansive coverage of surface-attached microbial communities and how those communities are responding to plastic pollution of the environment. New understanding of the ecology of iron-oxidizing bacteria revealed by the isolation of new members of this biogeochemically significant group. The discovery in deep ocean sediments of novel *Archaea* that link this domain with *Eukarya*. Extensive coverage of marine viruses, their abundance and diversity, and how they alter the physiology of organisms they infect. Humans traveling to 10,000-meter depths in the oceans discover the most pressure-tolerant bacterium known.

Chapter 21

- Extensive coverage of the major nutrient cycles in nature and the microbes that catalyze them are presented in a fashion that allows the cycles to be taught as individual entities or as interrelated metabolic loops.
- Some highlights: Expanded coverage of the biogeochemistry of sulfur compounds highlights the importance of volatile microbial products such as dimethyl sulfide for cloud formation. Advances in the biochemistry of extracellular electron transfer add new understanding to how the ecology and diversity of microorganisms drive the biogeochemical cycling of iron and manganese. The mystery of how methane is generated (typically a strictly anoxic process) in highly oxygenated ocean surface waters is solved by discoveries in the phosphorus cycle described in a new Explore the Microbial World.

Chapter 22

- This chapter on the built environment shows how humans create new microbial habitats through construction of buildings, supporting infrastructure, and habitat modification, and which microbes take advantage of these habitats and why.
- Some highlights: The microbial metabolism of biologically produced and manufactured chlorinated organics has been expanded, as has the basis for the bioremediation of major chemical pollutants. How microbes are responding to the mountains of plastics contaminating the environment and the discovery of novel bacteria capable of degrading plastic bottles are described. New technology that improves the efficiency of wastewater treatment using granular sludge technology is presented, and the microbial response to the excessive use of common household cleansers is considered.

Chapter 23

- A chapter devoted to nonhuman microbial symbioses describes the major microbial partners that live in symbiotic associations with other microbes, with plants, and with animals other than humans.
- Some highlights: Newly revised section on symbioses between microorganisms addresses the ecological significance of phototroph switching in lichens and how certain bacterial species use electrically conductive structures to form intimate symbiotic associations. Several updates include how insect symbionts are used to combat transmission of major viral diseases of humans and how defensive chemicals produced by symbionts protect insects from predation. Detailed coverage is given to the elaborate "cross-talk" between microbe and animal needed to establish the squid light organ.

UNIT 6 Microbe–Human Interactions and the Immune System

Chapter 24

- A chapter on the human microbiome launches the unit on microbe–human interactions and the immune system by introducing and updating advances in our understanding of the microbes that inhabit the human body and their relationship to health and disease.
- Some highlights: The discovery of ultrasmall bacteria in the mouth parasitizing other bacteria brings a new twist to the microbial ecology of the oral cavity. A new section on the human virome describes how metagenomics is driving the discovery and isolation of interesting new viruses. Extensive coverage is devoted to the impact of early-life events on the development of the newborn gut microbiome and of recent successes in probiotic therapy for preventing newborn intestinal diseases.

Chapter 25

- Beginning with this chapter, the book shifts its focus to pathogenic microorganisms, the immune system, and disease. Part I of this chapter addresses microbial adherence, colonization and invasion, and pathogenicity, including important sections on virulence and virulence attenuation. Part II highlights key enzymes and toxins produced by microbes that contribute to pathogenesis.

- Some highlights: The updated text includes expanded coverage of bacterial adhesins supported by a new, two-part figure that highlights new discoveries in staphylococcal adherence. Revised coverage of virulence attenuation includes new artwork to show how this principle can be exploited for development of effective vaccines. An updated discussion of botulinum toxins reflects new findings and clearly presents both the neurotoxic mechanism and the surprising clinical utility of these extremely potent substances.

Chapter 26

- Chapter 26 opens with an overview of the immune system and the body's first-line barriers to infection. This is followed by a brief discussion of hematopoiesis before focusing on innate immune responses to pathogen invasion. The chapter provides a natural progression into adaptive immune responses covered in Chapter 27.
- Some highlights: In addition to a new chapter opener highlighting breakthroughs that link Alzheimer's disease to microbial infection, this chapter contains heavily edited text that includes a more comprehensive discussion of leukocyte diversity and an all-new description of the role of amyloid-β protein as an innate defense in the brain. Other highlights include expanded coverage of interferons and the role of natural killer cells as the primary effectors of antibody-dependent cell-mediated cytotoxicity. Finally, a fascinating new Explore the Microbial World highlights the role of pattern recognition receptors in establishing host–microbe mutualisms using hydrothermal vent tube worms as an example.

Chapter 27

- Chapter 27 begins with an essential discussion of the principles that define adaptive immunity: specificity, immune memory, lymphocyte selection, and immune tolerance. This is followed by sections that discuss the functional mechanisms of the key cells and proteins (immunoglobulins, major histocompatibility complexes, and T cell receptors) that drive adaptive immunity.
- Some highlights: The text has been heavily edited throughout, and this has produced a clearer and more informative presentation of B and T lymphocyte selection and tolerance, including a new discussion of T-dependent versus T-independent antigens. In addition, a new section dedicated to T cell activation and anergy clearly presents the important concept of the second signal required for T cell activation.

Chapter 28

- The newly reorganized Chapters 28 and 29 have emerged from materials presented in Chapter 28 of the 15th edition. Treating immune disorders and antimicrobial therapy (Chapter 28) separately from clinical diagnostic methods (Chapter 29) has produced a more teachable format, making these topics more accessible for students and easier for the instructor to plan course assignments.
- Some highlights: The text progresses smoothly from immune disorders and deficiencies to methods used to train and hone the immune response for disease prevention and treatment. New coverage of mRNA and plant-based vaccines shares the latest innovations in vaccinology. An all-new section on immunotherapy, supported by vibrant new artwork, highlights exciting advancements in the use of genetic engineering and molecular immunology to treat cancer.

UNIT 7 Infectious Diseases

Chapter 29

- To bring better focus to the material, this chapter is now solely dedicated to the clinical microbiology laboratory and includes information on lab safety, healthcare-associated infections, and a wide array of both culture-dependent and culture-independent techniques used to diagnose infectious diseases.
- Some highlights: The chapter launches with the description of an exciting new method of diagnosing tuberculosis—humanity's most notorious scourge. The text has been edited throughout for better organization and clarity, and art modifications help clarify complex diagnostic techniques. Updated terminology includes an introduction to point-of-care diagnostics.

Chapter 30

- This chapter introduces the topics and terminology of the science of epidemiology and public health. Historical and modern examples throughout emphasize key concepts such as emerging (and reemerging) diseases, epidemics and pandemics, and the public health threat associated with the development and use of weaponized microorganisms.
- Some highlights: incorporation of the most up-to-date statistics available on disease incidence and outbreaks throughout the text and in figures and tables, as well as an all-new section supported by photos on the emergence of the important healthcare-associated pathogen *Clostridioides* (*Clostridium*) *difficile*.

Chapter 31

- This is the first of four highly visual chapters that take an ecological approach to pathogenic microorganisms by considering infectious diseases based on their modes of transmission. Bacterial and viral diseases transmitted person to person by way of airborne particles, direct contact, or sexual contact are the focus here.
- Some highlights: Statistical data regarding key emerging and reemerging diseases, including measles, pertussis, influenza, hepatitis, HIV/AIDS, gonorrhea, and syphilis have been updated to reflect the most recent data available; an all-new discussion with supporting photo of the neglected tropical disease yaws helps impart knowledge and awareness of this lingering scourge.

Chapter 32

- In this chapter we examine pathogens transmitted to humans through either an animal vector or soil-contaminated wounds or objects. Many of these diseases have high morbidity and mortality rates, and in most cases, effective vaccines are not yet available.
- Some highlights: The text and figures include the most up-to-date statistics for diseases throughout the chapter, including rabies, hantavirus, spotted fever rickettsiosis, ehrlichiosis and anaplasmosis, Lyme disease, and the major tropical hemorrhagic fevers. In addition, the text now includes updated discussions of the emergence of key tickborne diseases in the United States and coverage of new strategies against dengue fever, including description of a new vaccine and the use of the bacterial endosymbiont *Wolbachia* to control the dengue virus–infected mosquito population.

Chapter 33

- Pathogens in contaminated water or food are easily transmitted to humans, with waterborne diseases being especially common in developing countries lacking adequate water treatment facilities. This chapter highlights the most prevalent water- and foodborne diseases and emphasizes the importance of clean water and proper food preparation and preservation in preventing these physically uncomfortable and occasionally fatal illnesses.
- Some highlights: Updated statistics have been incorporated for all major water- and foodborne diseases, including *Campylobacter* infections, which have now overtaken salmonellosis as the leading cause of bacterial food infection in the United States. New discussions cover recently elucidated norovirus pathology and new food safety developments, including the use of eBeam technology and bacteriophage sprays. A new overview figure of cholera infection integrates photos with artwork to emphasize key aspects of this devastating and all too common disease.

Chapter 34

- Eukaryotic pathogens present a special challenge to medicine because, on a cellular level, they are not that different from our own cells. Thus, it can be difficult to find selective targets for chemotherapeutic drugs. Yet the microbes highlighted in this highly visual chapter cause some of the most devastating and prevalent diseases today.
- Some highlights: New color photos adorn the chapter, including two stunning fluorescent micrographs of *Entamoeba histolytica*, the causative agent of amebic dysentery. Broader coverage of distinctive features of several diseases, including cyclosporiasis, toxoplasmosis, and malaria, has been seamlessly incorporated. All statistics have been updated with the most recent surveillance data to yield a global picture of fungal and parasitic diseases.

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Susanne Erdmann, *University of New South Wales (Australia)*
Belinda Ferrari, *University of New South Wales (Australia)*
Derek J. Fisher, *Southern Illinois University*
Jason Flowers, *Murraysmith (Seattle, WA*)
Bruce Fouke, *University of Illinois Urbana–Champaign*
Andreas Giesen, *Royal HaskoningDHV (The Netherlands)*
Mariana Gomes de Pinho, *Universidade Nova de Lisboa (Portugal)*
Hans-Peter Grossart, *Leibniz-Institute of Freshwater Ecology and Inland Fisheries (Germany)*
Ricardo Guerrero, *University of Barcelona (Spain)*
Jennifer Hatchel, *College of Coastal Georgia*
Roland Hatzenpichler, *Montana State University*
Jennifer Hess, *Aquinas College*
Donald Hilvert, *ETH Zürich (Switzerland)*
Jay Hodgson, *Georgia Southern University*
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Michael Kühl, *University of Copenhagen (Denmark)*
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Jessica Mark Welch, *Marine Biological Laboratory, Woods Hole*
Francis Martin, *Lab of Excellence ARBRE, INRA-Nancy (France)*
Sean McAllister, *University of Delaware*
Margaret McFall-Ngai, *University of Hawaii at Manoa*
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Marie Pizzorno, *Bucknell University*
Joe Pogliano, *University of California, San Diego*
Martin Polz, *Massachusetts Institute of Technology*
Tessa Quax, *University of Freiburg (Germany)*
Katherine Ralston, *University of California*
Ines Rauschenbach, *Rutgers University*
Tara Renbarger, *Indiana Wesleyan University*
Niels Peter Revsbech, *Aarhus University (Denmark)*
Ned Ruby, *University of Hawaii at Manoa*
Bernhard Schink, *University of Konstanz (Germany)*
Susan Schlimpert, *John Innes Centre (England)*
Andrey Shkoporov, *University College Cork (Ireland)*
Simon Silver, *University of Illinois Chicago*
Janice Speshock, *Tarleton State University*
Emily Stowe, *Bucknell University*
Shunichi Takahashi, *National Institute for Basic Biology (Japan)*
Francisco Tenllado, *Centro de Investigaciones Biológicas (Spain)*
Andreas Teske, *University of North Carolina*
David Valentine, *University of California, Santa Barbara*
Mikel Valle, *Asociación Centro de Investigación Cooperativa en Biociencias (Spain)*
Nicholas Verola, *Verola Studio (Vero Beach, FL)*
Marilyn B. Vogel, *Auburn University*
Judy Wall, *University of Missouri*
Dave Ward, *Montana State University*
Bishop Wash, *The Richards Group (Dallas TX)*
Jillian L. Waters, *Max Planck Institute for Developmental Biology (Germany)*
Nicki Watson, *Massachusetts Institute of Technology*
Gunter Wegener, *Max Planck Institute of Marine Microbiology (Germany)*
Mari Winkler, *University of Washington*
Gerry Wright, *McMaster University*
Vamsi Yadavalli, *Virginia Commonwealth University*
Jeremy R. Young, *University College London (England)*
Carl Zeiss AG *(Jena, Germany)*
Jizhong (Joe) Zhou, *University of Oklahoma*
Steve Zinder, *Cornell University*

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Michael T. Madigan (madigan@siu.edu)
Kelly S. Bender (bender@siu.edu)
Daniel H. Buckley (dbuckley@cornell.edu)
W. Matthew Sattley (matthew.sattley@indwes.edu)
David A. Stahl (dastahl@uw.edu)

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Contributors

Fiona Baird, *Griffith University (Australia)*
Aurélien Carlier, *Universiteit Gent (Belgium)*
Lara Grollo, *Australian Catholic University (Australia)*
HE Jianzhong, *National University of Singapore (Singapore)*
Wendy Ying Ying Liu, *Quest International University (Malaysia)*
Joy Pang, *Singapore Institute of Technology (Singapore)*
Jörg Simon, *Technical University of Darmstadt (Germany)*
Wei-Qin Zhuang, *University of Auckland (New Zealand)*

Reviewers

Oluwapelumi Adeyemi, *University of Leeds (England)*
Siti Khayriyyah Mohd Hanafiah, *Universiti Sains Malaysia (Malaysia)*
Quek Choon Lau, *Ngee Ann Polytechnic (Singapore)*
Maria del Carmen Montero-Calasanz, *Newcastle University (England)*

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ASM Recommended Curriculum Guidelines for Undergraduate Microbiology

The American Society for Microbiology (ASM) endorses a concept-based curriculum for undergraduate microbiology, emphasizing skills and concepts that have lasting importance beyond the classroom and laboratory. The ASM (in its *Curriculum Guidelines for Understanding Microbiology Education*) recommends deep understanding of 27 key concepts, 4 scientific thinking competencies, and 7 key skills. These guidelines follow scientific literacy reports and recommendations from the American Association for the Advancement of Science and the Howard Hughes Medical Institute by encouraging an active learning, student-based course. Consider these guiding statements as you progress through this book and master principles, problem solving, and laboratory skills in microbiology.

ASM Guideline Concepts and Statements

Evolution: Chapters 1, 9, 10–14, 20, 30

- Cells, organelles (e.g., mitochondria and chloroplasts), and all major metabolic pathways evolved from early prokaryotic cells.
- Mutations and horizontal gene transfer, with the immense variety of microenvironments, have selected for a huge diversity of microorganisms.
- Traditional concept of species is not readily applicable to microbes due to asexual reproduction and the frequent occurrence of horizontal gene transfer.
- Evolutionary relatedness of organisms is best reflected in phylogenetic trees.
- Human impact on the environment influences the evolution of microorganisms (e.g., emerging diseases and the selection of antibiotic resistance).

Cell Structure and Function: Chapters 1, 2, 5, 8, 11, 18

- Structure and function of microorganisms have been revealed by the use of microscopy (including bright-field, phase contrast, fluorescence, super-resolution, and electron).
- Bacteria have unique cell structures that can be targets for antibiotics, immunity, and phage infection.
- *Bacteria* and *Archaea* have specialized structures (e.g., flagella, endospores, and pili) that often confer critical capabilities.
- While microscopic eukaryotes (for example, fungi, protozoa, and algae) carry out some of the same processes as bacteria, many of the cellular properties are fundamentally different.
- Replication cycles of viruses (lytic and lysogenic) differ among viruses and are determined by their unique genomes.

Metabolic Pathways: Chapters 1, 3, 4, 7, 8, 12, 14

- *Bacteria and Archaea* exhibit extensive, and often unique, metabolic diversity (e.g., nitrogen fixation, methane production, anoxygenic photosynthesis).
- Interactions of microorganisms among themselves and with their environment are determined by their metabolic abilities (e.g., quorum sensing, oxygen consumption, nitrogen transformations).
- Survival and growth of any microorganism in a given environment depends on its metabolic characteristics.
- Growth of microorganisms can be controlled by physical, chemical, mechanical, or biological means.

Information Flow and Genetics: Chapters 1, 5–13

- Genetic variations can impact microbial functions (e.g., in biofilm formation, pathogenicity, and drug resistance).
- Although the central dogma is universal in all cells, the processes of replication, transcription, and translation differ in *Bacteria*, *Archaea*, and eukaryotes.
- Regulation of gene expression is influenced by external and internal molecular cues and/or signals.
- Synthesis of viral genetic material and proteins is dependent on host cells.
- Cell genomes can be manipulated to alter cell function.

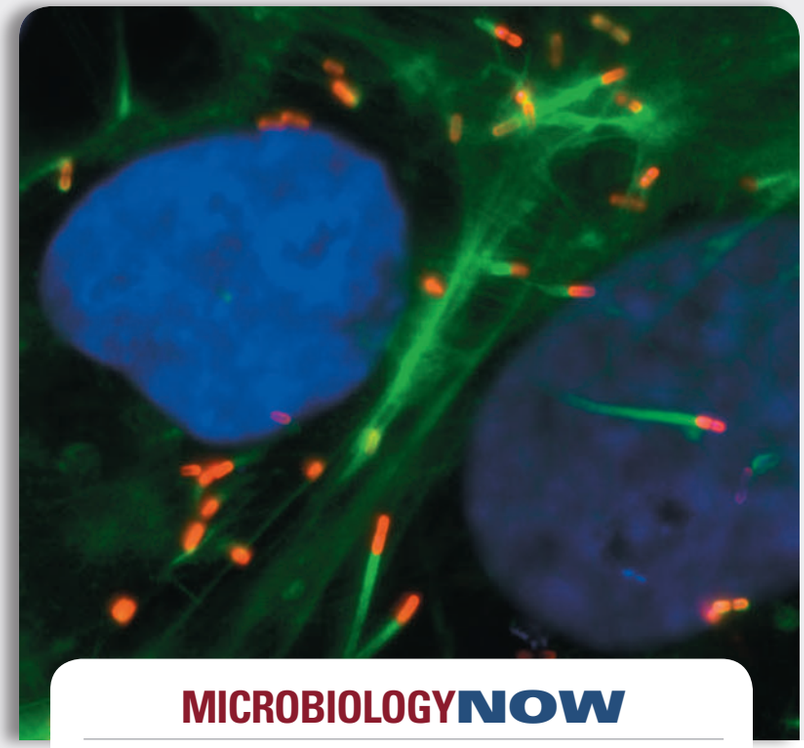
Microbial Systems: Chapters 1, 15–34

- Microorganisms are ubiquitous and live in diverse and dynamic ecosystems.
- Many bacteria in nature live in biofilm communities.
- Microorganisms and their environment interact with and modify each other.
- Microorganisms, cellular and viral, can interact with both human and nonhuman hosts in beneficial, neutral, or detrimental ways.

Impact of Microorganisms: Chapters 1, 6–8, 12, 19–34

- Microbes are essential for life as we know it and the processes that support life (e.g., in biogeochemical cycles and plant and/or animal microbiota).
- Microorganisms provide essential models that give us fundamental knowledge about life processes.
- Humans utilize and harness microorganisms and their products.
- Because the true diversity of microbial life is largely unknown, its effects and potential benefits have not been fully explored.

The Microbial World 1



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MICROBIOLOGY**NOW**

Microbiology in Motion

The microbial world is strange and fierce. It is teeming with life, ancient, diverse, and constantly changing. Microorganisms are Earth's life support system, and from our first breath they influence nearly every moment of our lives. Microbes are in our water and our food, and we carry them on us and in us. Indeed, microbes abound in any natural environment that will support life, including many environments too hostile for higher life forms.

While the microbial world is invisible, we can explore it through the science of microbiology. Microbiology evolves at a breathtaking pace. Even the microscope continues to evolve, providing an ever more detailed picture of the microbial world. The image above was made with a fluorescence microscope that uses lasers, guided by a computer, to map the three-dimensional structure of cells. The image shows neighboring human cells with their nuclei stained blue and actin filaments stained green. These cells are infected with the foodborne bacterial pathogen *Listeria monocytogenes*, stained red.

Listeria are soil organisms that sometimes find their way into our food. In soils they infect other microbes such as amoebae. Our cells are similar in many ways to those of microscopic organisms, and so *Listeria* finds itself well adapted to live within us. This bacterium has the unique ability to hijack cellular systems, causing actin to polymerize and propel the cell like a rocket within the host cytoplasm. The force of this propulsion causes *Listeria* to penetrate adjacent cells (image, lower left), spreading the infection. *Listeria* can also invade host vacuoles (not shown), where it hides and survives. This persistent state can prolong infection and promote resistance to antibiotic therapy. Research on *Listeria* has provided new insights on the biology of this pathogen and an ever-changing view of a microbial world in motion.

Source: Kortebi, M., et al. 2017. *Listeria monocytogenes* switches from dissemination to persistence by adopting a vacuolar lifestyle in epithelial cells. *PLoS Pathog. 13*: e1006734.

This chapter launches our journey into the microbial world. Here we will begin to discover what the science of microbiology is all about and what microorganisms are, what they do, and how they can be studied. We also place microbiology in historical context, as a process of scientific discovery driven by simple (yet powerful) experiments and insightful minds.

I • Exploring the Microbial World

The microbial world consists of microscopic organisms that have defined structures, unique evolutionary histories, and are of enormous importance to the biosphere.

1.1 Microorganisms, Tiny Titans of the Earth

Microorganisms (also called *microbes*) are life forms too small to be seen by the unaided human eye. These microscopic organisms are diverse in form and function, and they inhabit every environment on Earth that supports life. Many microbes are undifferentiated single-celled organisms, but some can form complex structures, and some are even multicellular. Microorganisms typically live in complex **microbial communities** (Figure 1.1), and their activities are regulated by interactions with each other, with their environments, and with other organisms. The science of microbiology is all about microorganisms, who they are, how they work, and what they do.

Microorganisms were teeming on the land and in the seas for billions of years before the appearance of plants and animals, and their diversity is staggering. Microorganisms represent a major fraction of Earth's biomass, and their activities are essential to sustaining life. Indeed, the very oxygen (O_2) we breathe is the result of microbial activities. Plants and animals are immersed in a world of microbes, and their evolution and survival are heavily influenced by microbial activities, by microbial symbioses, and by *pathogens*—those microbes that cause disease. Microorganisms are woven into the fabric of human life as well (Figure 1.2), from infectious diseases, to the food we eat, the water we drink, the fertility of our soils, the health of our animals, and even the fuel we put in automobiles. Microbiology is the study of the dominant form of life on Earth, and the effect that microbes have on our planet and all of the living things that call it home.

Microbiologists have many tools for studying microorganisms. Microbiology was born of the microscope, and microscopy is foundational to microbiology. Microbiologists have developed an array of methods for visualizing microorganisms, and these microscopic techniques are essential to microbiology. The cultivation of microorganisms is also foundational to microbiology. A microbial **culture** is a collection of cells that have been grown in or on a nutrient medium. A **medium** (plural, media) is a liquid or solid nutrient mixture that contains all of the nutrients required for a microorganism to grow. In microbiology, we use the word **growth** to refer to the increase in cell number as a result of cell division. A single microbial cell placed on a solid nutrient medium can grow and divide into millions or even billions of cells that form a visible **colony** (Figure 1.3). The formation of visible colonies makes it easier to see and grow microorganisms. Comprehension of the microbial basis of disease and microbial biochemical diversity has relied on the ability to grow microorganisms in the laboratory.

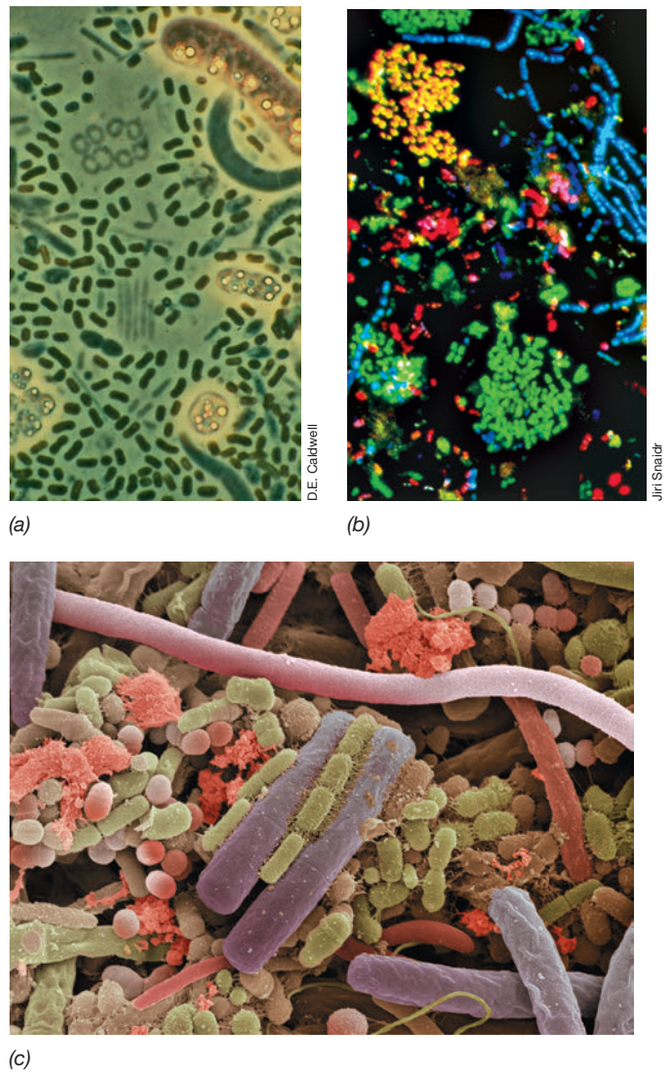


Figure 1.1 Microbial communities. *(a)* A bacterial community that developed in the depths of a small Michigan lake, including cells of various phototrophic bacteria. The bacteria were visualized using phase-contrast microscopy. *(b)* A bacterial community in a sewage sludge sample. The sample was stained with a series of dyes, each of which stained a specific bacterial group. From *Journal of Bacteriology 178*: 3496–3500, Fig. 2b. © 1996 American Society for Microbiology. *(c)* Colorized scanning electron micrograph of a microbial community scraped from a human tongue.

The ability to grow microorganisms rapidly under controlled conditions makes them highly useful for experiments that probe the fundamental processes of life. Most discoveries relating to the molecular and biochemical basis of life have been made using microorganisms. The study of molecules and their interactions is essential to defining the workings of microbial cells, and the tools of molecular biology and biochemistry are foundational to microbiology. Molecular biology has also provided a variety of tools to study microorganisms without need for their cultivation in the laboratory. These molecular tools have greatly expanded our knowledge of microbial ecology and diversity. Finally, the tools of genomics and molecular genetics are also cornerstones of modern

Figure 1.2 Microbial applications. Microorganisms have major impacts on the world in which we live. In the chapters that follow we will learn how microorganisms impact our health, the foods we eat, the water we drink, and even the air we breathe. We will learn how microbes can be used to produce valuable products and the many ways in which microorganisms touch our lives.

microbiology and allow microbiologists to study the genetic basis of life, how genes evolve, and how they regulate the activities of cells.

In the next section, we explore the basic elements of microbial cell structure and summarize the major physiological activities that take place in all cells, regardless of their structure.

Check Your Understanding

- In what ways are microorganisms important to humans?
- Why are microbial cells useful for understanding the basis of life?
- What is a microbial colony and how is one formed?

1.2 Structure and Activities of Microbial Cells

Microbial cells are living compartments that interact with their environment and with other cells in dynamic ways. We purposely exclude viruses in most of this discussion because although they resemble cells in many ways, viruses are not cells but instead a special category of microorganism. We consider the structure, diversity, and activities of viruses in Section 1.4 and in Chapters 5 and 11.

Elements of Microbial Structure

All cells have much in common and contain many of the same components (Figure 1.4). All cells have a permeability barrier called the **cytoplasmic membrane** that separates the inside of the cell, the **cytoplasm**, from the outside. The cytoplasm is an aqueous mixture of **macromolecules** (for example proteins, lipids, nucleic acids, and polysaccharides), small organic molecules (mostly the precursors of macromolecules), various inorganic ions, and ribosomes. All cells also contain **ribosomes**, which are the structures responsible for protein synthesis. Some cells have a **cell wall** that lends structural strength to a cell. The cell wall is a relatively permeable structure located outside the cytoplasmic membrane and is a much stronger layer than the membrane itself. Cell walls are typically found in plant cells and most microorganisms but are not found in animal cells.

There are two fundamental cell types that differ categorically in cellular organization: those having **prokaryotic** cell structure, and those having **eukaryotic** cell structure (Figure 1.4). Cells having eukaryotic cell structure are found in a group of organisms called the *Eukarya*. This group includes plants and animals as well as diverse microbial eukaryotes such as algae, protozoa, and fungi. Eukaryotic cells contain an assortment of membrane-enclosed cytoplasmic structures called **organelles** (Figure 1.4*b*). These include, most prominently, the DNA-containing nucleus but also mitochondria and chloroplasts, organelles that specialize in supplying the cell with energy, and various other organelles.

Mastering Microbiology
Art Activity: Figure 1.3 Common elements of prokaryotic/eukaryotic cells

Prokaryotic cell structure is found within two different groups of organisms we know as *Bacteria* and *Archaea*. Prokaryotic cells have few internal structures, they lack a nucleus, and they typically lack organelles (Figure 1.4*a*). *Bacteria* and *Archaea* appeared long before the evolution of eukaryotes (Section 1.5). While all *Archaea* and

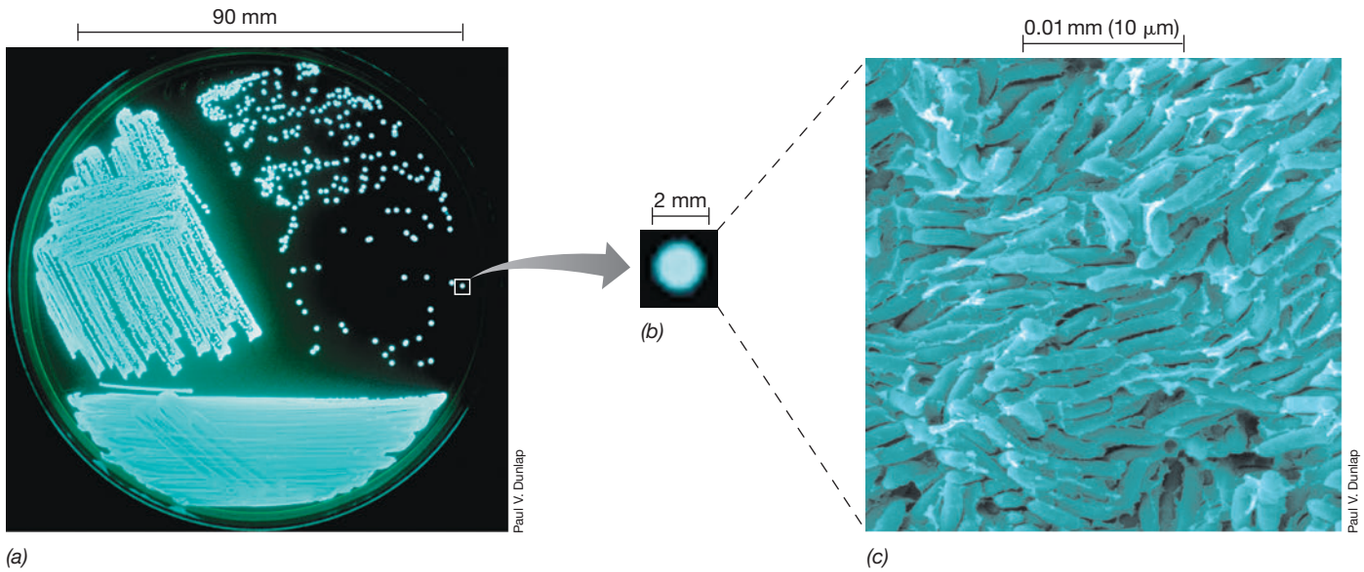


Figure 1.3 Microbial cells. *(a)* Bioluminescent (light-emitting) colonies of the bacterium *Photobacterium* grown in laboratory culture on a Petri plate. *(b)* A single colony can contain more than 10 million (10^7) individual cells. *(c)* Colorized scanning electron micrograph of cells of *Photobacterium*.

Bacteria have prokaryotic cell structure, these two groups diverged very early in the history of life and as a result many of their molecular and genetic characteristics differ at a fundamental level. Indeed, we will see later that in many ways *Archaea* and *Eukarya* are more similar to each other than either is to *Bacteria*.

Genes, Genomes, Nucleus, and Nucleoid

In addition to a cytoplasmic membrane and ribosomes, all cells also possess a DNA **genome**. The genome is the full set of genes in a cell. A gene is a segment of DNA that encodes a protein or an RNA molecule. The genome is the living blueprint of an organism; the characteristics, activities, and very survival of a cell are governed by its genome.

The genomes of prokaryotic cells and eukaryotic cells are organized into structures called **chromosomes**. In eukaryotic cells, DNA is present as several linear molecules (each one formed into its own chromosome) within the membrane-enclosed **nucleus**. By contrast, the genomes of *Bacteria* and *Archaea* are typically closed circular chromosomes (though some prokaryotic cells have linear chromosomes). The chromosome aggregates within the prokaryotic cell to form the **nucleoid**, a mass that is visible in the electron microscope (Figure 1.4*a*) but which is *not* enclosed by a membrane. Most prokaryotic cells have only a single chromosome, but many also contain one or more small circles of DNA distinct from that of the chromosome, called **plasmids** (Figure 1.4*a*). Plasmids typically contain genes that are not essential but often confer some special property on the cell (such as a unique metabolism, or antibiotic resistance). The genomes of *Bacteria* and *Archaea* are typically small and compact, and most contain between 500 and 10,000 genes encoded by 0.5 to 10 million base pairs of DNA. Eukaryotic cells typically have much larger and much less streamlined genomes than prokaryotic cells. A human cell, for example, contains approximately 3 billion base pairs, which encode about 20,000–25,000 genes.

Mastering Microbiology
Art Activity: Figure 1.4 Basic properties of microbial cells

Activities of Microbial Cells

To be competitive in nature, a microorganism must survive and reproduce. **Figure 1.5** considers structure and some of the activities that are performed by cells to drive survival and reproduction. All cells show some form of **metabolism** through which nutrients are acquired from the environment and transformed into new cellular materials and waste products. During these transformations, energy is used to support synthesis of new structures. Production of these new structures culminates in the division of the cell to form two cells. Microbial growth results from successive rounds of cell division.

Genes contain information that is used by the cell to perform the work of metabolism. Genes are decoded to form proteins that regulate cellular processes. **Enzymes**, those proteins that have catalytic activity, carry out reactions that supply energy and perform biosynthesis within the cell. Enzymes and other proteins are synthesized during *gene expression* in the sequential processes of transcription and translation. **Transcription** is the process by which the information encoded in DNA sequences is copied into an RNA molecule, and **translation** is the process whereby the information in an RNA molecule is used by a ribosome to synthesize a protein (Chapter 6). Gene expression and enzyme activity in a microbial cell are coordinated and highly regulated to ensure that the cell remains optimally tuned to its surroundings. Ultimately, microbial growth requires replication of the genome through the process of **DNA replication**, followed by cell division. All cells carry out the processes of transcription, translation, and DNA replication.

Microorganisms have the ability to sense and respond to changes in their local environment. Many microbial cells are capable of **motility**, typically by self-propulsion (Figure 1.5). Motility allows cells to relocate in response to environmental conditions. Some microbial cells undergo **differentiation**, which may result

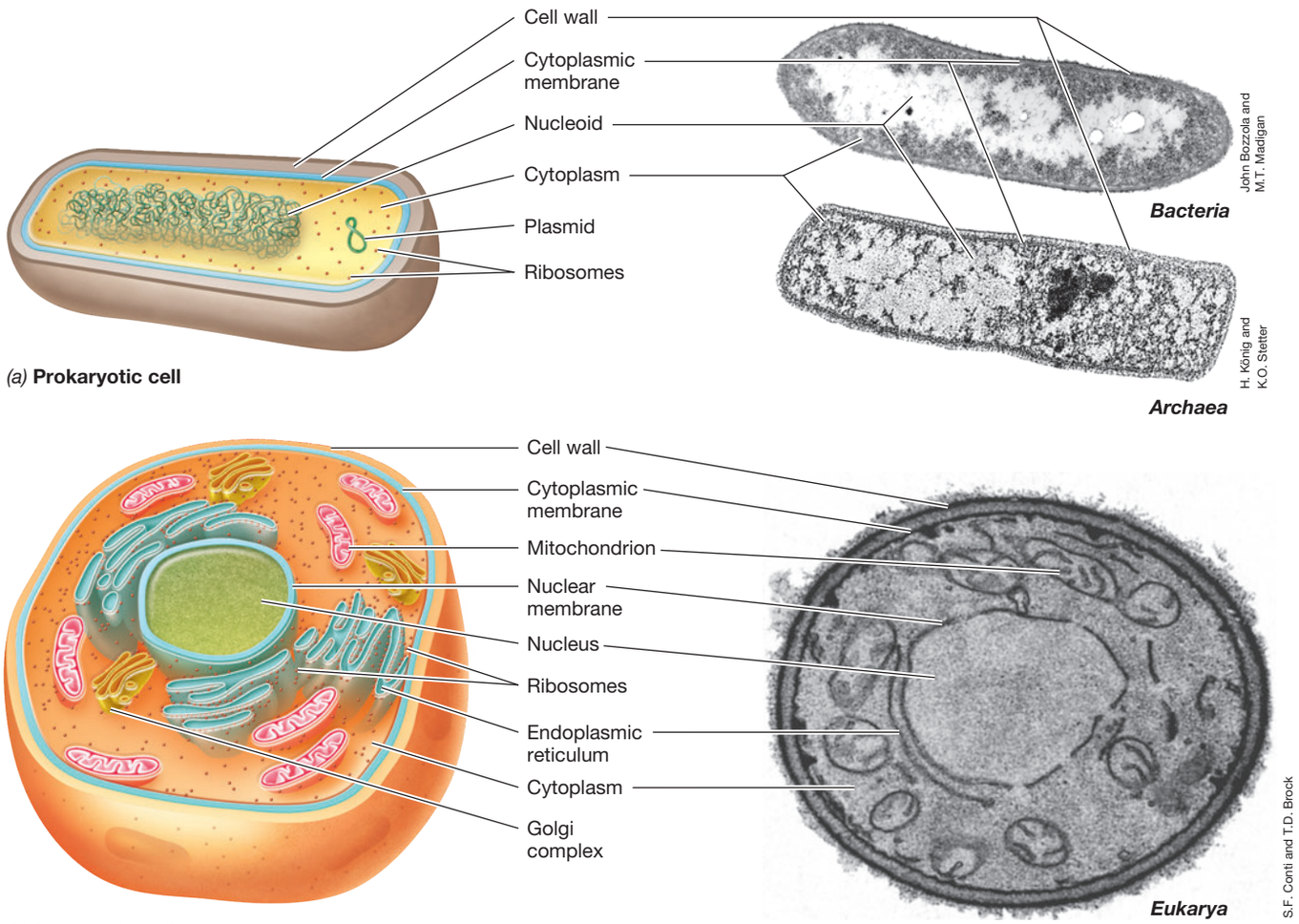


Figure 1.4 **Microbial cell structure.** *(a)* (Left) Diagram of a prokaryotic cell. (Right) Electron micrograph of *Heliobacterium modesticaldum* (*Bacteria*, cell is about 1 μm in diameter) and *Thermoproteus neutrophilus* (*Archaea*, cell is about 0.5 μm in diameter). *(b)* (Left) Diagram of a eukaryotic cell. (Right) Electron micrograph of a cell of *Saccharomyces cerevisiae* (*Eukarya*, cell is about 8 μm in diameter). In terms of relative scale, the bacterial cell in *a* is about the same size as the mitochondria of *Saccharomyces* in *b*.

in the formation of modified cells specialized for growth, dispersal, or survival. Cells respond to chemical signals in their environment, including those produced by other cells of either the same or different species, and these signals often trigger new cellular activities. Microbial cells thus exhibit **intercellular communication**; that is, they are "aware" of their neighbors and can respond accordingly. Many prokaryotic cells can also exchange genes with neighboring cells, regardless of their species, in the process of **horizontal gene transfer**.

Evolution (Figure 1.5) results when genes in a population of cells change in sequence and frequency over time, leading to descent with modification. The evolution of microorganisms can be very rapid relative to the evolution of plants and animals. For example, the indiscriminate use of antibiotics in human and veterinary medicine has selected for the proliferation of antibiotic resistance in pathogenic bacteria. The rapid pace of microbial evolution can be attributed in part to the ability of microorganisms to grow very quickly and to acquire new genes though the process of horizontal gene transfer. Not all of the processes depicted in Figure 1.5 occur in all cells. Metabolism, growth, and evolution, however, are universal and will be major areas of emphasis throughout this text.

We now move on to consider the diversity of cell shapes and sizes found in the microbial world.

Check Your Understanding

- What structures are universal to all type of cells?
- What processes are universal to all types of cells?
- What structures can be used to distinguish between prokaryotic cells and eukaryotic cells?

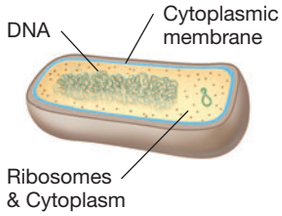
1.3 Cell Size and Morphology

Microscopic examination of microorganisms immediately reveals their **morphology**, which is defined by cell size and shape. A variety of cell shapes pervade the microbial world, and although microscopic by their very nature, microbial cells come in a variety of sizes.

Properties of *all* cells:

Structure

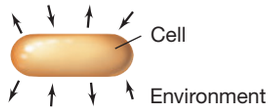
All cells have a cytoplasmic membrane, cytoplasm, a genome made of DNA, and ribosomes.



Metabolism

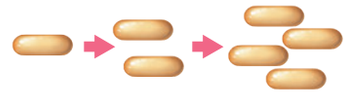
All cells use information encoded in DNA to make RNA and protein. All cells take up nutrients, transform them, conserve energy, and expel wastes.

1. **Catabolism** (transforming molecules to produce energy and building blocks)
2. **Anabolism** (synthesizing macromolecules)



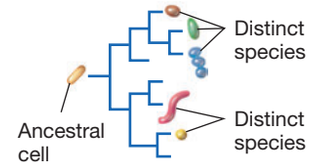
Growth

Information from DNA is converted into proteins, which do work. Proteins are used to convert nutrients from the environment into new cells.



Evolution

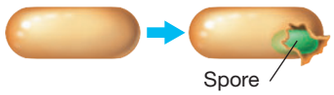
Chance mutations in DNA cause new cells to have new properties, thereby promoting evolution. Phylogenetic trees built from DNA sequences capture evolutionary relationships between species.



Properties of *some* cells:

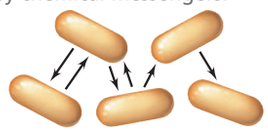
Differentiation

Some cells can form new cell structures such as a spore.



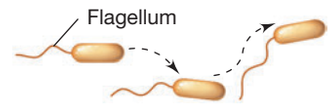
Communication

Cells interact with each other by chemical messengers.



Motility

Some cells are capable of self-propulsion.



Horizontal gene transfer

Cells can exchange genes by several mechanisms.

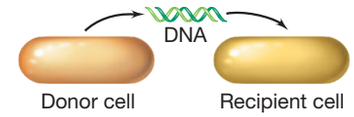


Figure 1.5 The properties of microbial cells. While cells are tremendously diverse in form and function, certain properties are shared by all cells.

Cell shape can be useful for distinguishing different microbial cells and often has ecological significance. Moreover, the very small size of most microbial cells has a profound effect on their ecology and dictates many aspects of their biology. We begin by considering cell size and then consider cell shape.

The Small World

A micrometer (μm or micron) is one-millionth of a meter in length. The unaided human eye has difficulty resolving objects that are less than 100 μm in diameter, but this is the scale of the microbial world. Most prokaryotic cells are small, ranging between 0.5 and 10 μm in length, but prokaryotic cells can vary widely in size. For example, the smallest prokaryotic cells are about 0.2 μm in diameter and the largest can be more than 600 μm long (**Table 1.1**). In contrast, most eukaryotic cells are larger on average than prokaryotic cells, being between 5 and 100 μm in length, but eukaryotic cells can vary widely in size too. For example, the smallest eukaryotic microorganism known is about 0.8 μm in diameter and the largest eukaryotic cells can be many centimeters in length (Section 1.4).

Cell size is influenced fundamentally by cell structure. Eukaryotic cells, owing to their complex intracellular structure and organelles (Figure 1.4), can actively transport molecules and macromolecules within the cytoplasm. Prokaryotic cells, in contrast, rely on diffusion for transport through the cytoplasm and this limits their size. While diffusion is very fast at small distances, the rate of diffusion increases as the square of the distance traveled. Hence, the metabolic rate in a prokaryotic cell varies inversely with the square of its size. This relationship means that, as cell size increases, it becomes advantageous to have cellular structures that facilitate transport and compartmentalize cellular activities as seen in eukaryotic cells. In contrast, since diffusion is rapid at small spatial scales, high metabolic rates can be maintained in small prokaryotic cells without a need for complex cellular structures.

It is possible, though unusual, for prokaryotic cells to be visible to the human eye; the largest are more than 600 μm (0.6 mm) long. To achieve this size, these bacteria must have traits that allow them to overcome diffusional limitation. The bacterium *Epulopiscium fishelsoni* (**Figure 1.6*a***; Figure 1.9), which is found in the gut of the surgeonfish, can be more than 75 μm wide and 600 μm long (Table 1.1). One of the traits that allows this bacterium to get so large is that it can have more than 10,000 copies of its genome distributed throughout its cytoplasm, thereby preventing diffusional limitation between the genome and any region of the cytoplasm. Cells of the largest known bacterium, the sulfur-oxidizing chemolithotroph *Thiomargarita* (Figure 1.6*b*, Table 1.1), are even larger than those of *Epulopiscium*, about 750 μm in diameter. *Thiomargarita* achieves this enormous size by having a large vacuole that fills the center of the cell. Hence, the cytoplasm of *Thiomargarita* occurs as a thin layer squeezed between the cytoplasmic membrane and this central vacuole. In this way, the cytoplasm is never more than 1 μm from the membrane. In addition, *Thiomargarita*, like *Epulopiscium*, also has many copies of its genome, which are distributed throughout its cytoplasm.

TABLE 1.1 Cell size and volume of some cells of *Bacteria*, from the largest to the smallest

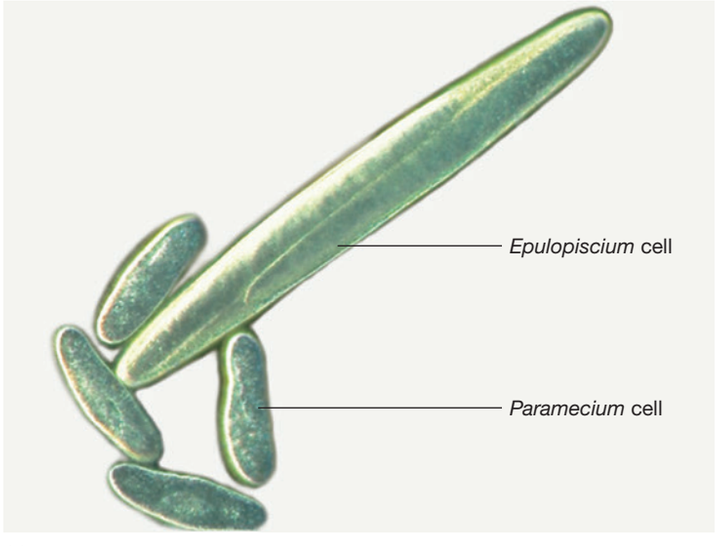
Organism	Characteristics	Morphology	Size[a] (μm^3)	Cell volume (μm^3)	Volumes compared to *E. coli*
Thiomargarita namibiensis	Sulfur chemolithotroph	Cocci in chains	750	200,000,000	100,000,000×
Epulopiscium fishelsoni[a]	Chemoorganotroph	Rods with tapered ends	80 × 600	3,000,000	1,500,000×
Beggiatoa species[a]	Sulfur chemolithotroph	Filaments	50 × 160	1,000,000	500,000×
Achromatium oxaliferum	Sulfur chemolithotroph	Cocci	35 × 95	80,000	40,000×
Lyngbya majuscula	Cyanobacterium	Filaments	8 × 80	40,000	20,000×
Thiovulum majus	Sulfur chemolithotroph	Cocci	18	3,000	1,500×
Staphylothermus marinus[a]	Hyperthermophile	Cocci in irregular clusters	15	1,800	900×
Magnetobacterium bavaricum	Magnetotactic bacterium	Rods	2 × 10	30	15×
Escherichia coli	Chemoorganotroph	Rods	1 × 2	2	1×
Pelagibacter ubique[a]	Marine chemoorganotroph	Rods	0.2 × 0.5	0.014	0.007×
Ultra-small bacteria[a]	Uncultured, from groundwater	Variable	<0.2	0.009	0.0045×
Mycoplasma pneumoniae	Pathogenic bacterium	Pleomorphic[b]	0.2	0.005	0.0025×

[a]Where only one number is given, this is the diameter of spherical cells. The values given are for the largest cell size observed in each species. For example, for *T. namibiensis*, an average cell is only about 200 μm in diameter. But on occasion, giant cells of 750 μm are observed. Likewise, an average cell of *S. marinus* is about 1 μm in diameter. The species of *Beggiatoa* here is unclear, and *E. fishelsoni*, *M. bavaricum*, and *P. ubique* are not formally recognized names in taxonomy. For more on ultra-small bacteria, see Explore the Microbial World "Tiny Cells."
[b]*Mycoplasma* is a bacterium that lacks a cell wall and can thus take on many shapes (*pleomorphic* means "many shapes").
Source: Data obtained from Schulz, H.N., and B.B. Jørgensen. 2001. *Annu. Rev. Microbiol. 55*: 105–137, and Luef, B., et al. 2015. *Nat. Commun.* doi:10.1038/ncomms7372.

At the opposite end of the spectrum from these large prokaryotic cells are very small prokaryotic cells. Exactly how small a cell can be is not precisely known. However, cells 0.2 μm in diameter exist (see Explore the Microbial World, "Tiny Cells"), and the lower limit is probably only a bit smaller than this. Ultimately, the lower limit to cell size is likely a function of the amount of space needed to house the essential biochemical components—proteins, nucleic acids, ribosomes and so on (Section 1.2)—that all cells need to survive and reproduce.

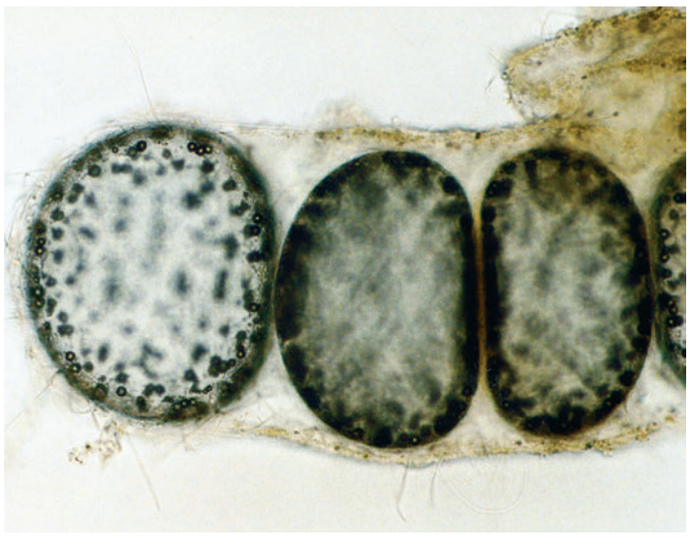
Surface-to-Volume Ratios, Growth Rates, and Evolution

For a cell, there are advantages to being small. Small cells have more surface area relative to cell volume and thus have a higher *surface-to-volume ratio* than larger cells. To understand this principle, consider a spherical cell. The volume of a sphere is a function of the cube of its radius ($V = \frac{4}{3}\pi r^3$), whereas its surface area is a function of the square of the radius ($S = 4\pi r^2$). Therefore, the S/V ratio of a coccus is $3/r$ (**Figure 1.7**). As cell size *increases*, its S/V ratio *decreases*.



Esther R. Angert, Harvard University

(a)



Heide Schulz-Vogt

(b)

Figure 1.6 Two very large *Bacteria*. *(a) Epulopiscium fishelsoni.* The rod-shaped cell is about 600 μm (0.6 mm) long and 75 μm wide and is shown with four cells of the protist *Paramecium* (a microbial eukaryote), each of which is about 150 μm long. *(b) Thiomargarita namibiensis*, a large sulfur chemolithotroph and currently the largest known of all prokaryotic cells. Cell widths vary from 400 to 750 μm.

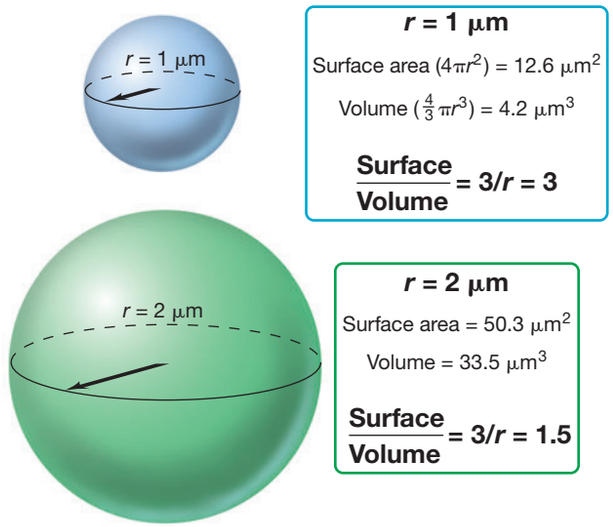


Figure 1.7 Surface area and volume relationships in cells. As a cell increases in size, its *S*/*V* ratio decreases.

To illustrate this, consider the *S*/*V* ratio for some of the cells of different sizes listed in Table 1.1: *Pelagibacter ubique*, 22; *Escherichia coli*, 4.5; and *E. fishelsoni* (Figure 1.6*a*), 0.05. The *S*/*V* of a rod-shaped organism can be estimated as if it were a cylinder; hence, the *S*/*V* of the cell will *decrease* as its radius *increases*.

The *S*/*V* ratio of a cell controls many of its properties, including how fast it grows (its *growth rate*) and shape. Cellular growth rate depends in part on the rate at which cells exchange nutrients and waste products with their environment. As cell size decreases, the *S*/*V* ratio of the cell increases, and this means that small cells can exchange nutrients and wastes more rapidly (per unit cell volume) than can large cells. As a result, free-living cells that are smaller tend to be more efficient than those that are larger, and any given mass of nutrients will support the synthesis of more small cells than large cells. We will see that cell morphology is also often predicated on the effect of cell shape on *S*/*V* ratio. For example, cell shapes that increase the overall membrane area of the cell, such as those having long thin appendages or invaginations, allow bacteria to increase their *S*/*V* ratio for a given mass of cytoplasm. We will see that prokaryotic cell morphology is remarkably diverse and different cell shapes can convey different benefits upon the cell.

Major Morphologies of Prokaryotic Cells

Common morphologies of prokaryotic cells are shown in **Figure 1.8**. A cell that is spherical or ovoid in morphology is called a *coccus* (plural, cocci). A cylindrically shaped cell is called a *rod* or a *bacillus* (plural, bacilli). A spiral-shaped cell is called a *spirillum* (plural, spirilla). A cell that is slightly curved and comma-shaped is called a *vibrio*. A *spirochete* is a special kind of organism (▶ Section 15.17) that has a spiral shape but which differs from spirilla because the cells of spirochetes are flexible, whereas cells of spirilla are rigid. Some bacteria are irregular in shape. Appendages, such as stalks and hyphae, are used by some cells for attachment or to increase surface area. In addition, asymmetrical cell division such as budding can result in irregular and asymmetrical cell shapes.

Cell division has a major impact on morphology because cells that remain attached to each other can form distinctive shapes. For instance, some cocci occur in pairs (diplococci), some form long chains (streptococci), others occur in three-dimensional cubes (tetrads or sarcinae), and still others occur in grapelike clusters (staphylococci). Filamentous bacteria are long, thin, rod-shaped bacteria that divide terminally and then form long filaments composed of many cells attached end to end.

The cell morphologies described here are representative but certainly not exhaustive; many variations of these morphologies are known. For example, there can be fat rods, thin rods, short rods, and long rods, rods that occur as single cells, as pairs of cells, or rods that

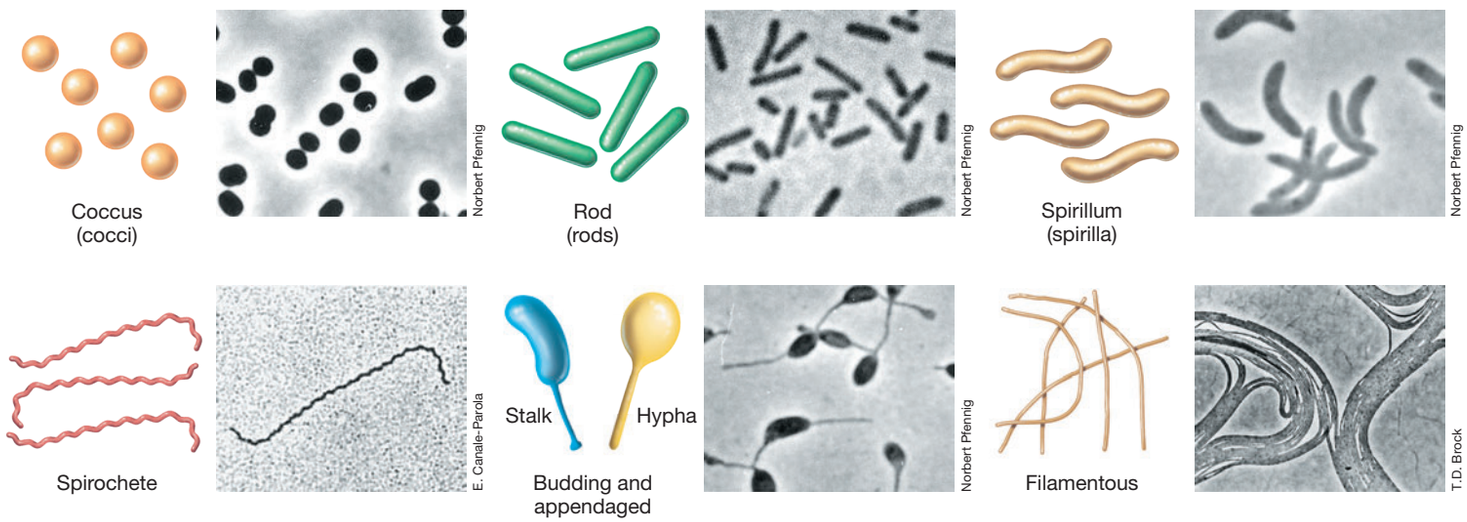


Figure 1.8 Cell morphologies. Beside each drawing is a phase-contrast photomicrograph of cells showing that morphology. Coccus (cell diameter in photomicrograph, 1.5 μm); rod (1 μm); spirillum (1 μm); spirochete (0.25 μm); budding (1.2 μm); filamentous (0.8 μm). All photomicrographs are of species of *Bacteria*. Not all of these morphologies are known among the *Archaea*, but cocci, rods, and spirilla are common.

Tiny Cells

Viruses are very small microbes and range in diameter from as small as 20 nm to almost 750 nm. Although no cells exist that are as small as most viruses, the recent discovery of ultra-small bacterial cells[1,2] has pushed the lower limits of cell size to what microbiologists feel must be very close to the minimal value. And, because microbiologists today can deduce amazing amounts of information about cells in nature without culturing them, the lack of laboratory cultures of these tiny cells has been only a minor impediment to understanding their biology in detail.

Microbiologists collected groundwater, which travels through Earth's deep subsurface, from a Colorado (USA) aquifer (**Figure 1**) and passed it through a membrane filter whose pores were only 0.2 μm in diameter. The liquid that passed through the filter was then subjected to microbiological analyses. Surprisingly, since filters with 0.2-μm pores have been used for decades to remove bacterial cells from solutions to generate "sterile solutions," prokaryotic cells were present in the groundwater filtrate. In fact, a diverse array of *Bacteria* were present in the filtrate, revealing that the groundwater was inhabited by a microbial community of tiny cells[1] that microbiologists have come to call ultramicrobacteria.

Figure 1 **Sampling the anoxic groundwater aquifer that parallels the Colorado River near Rifle, Colorado.**

Electron cryotomography, a microscopic technique in which a specimen is examined at extremely cold temperatures without fixation (chemical treatment that can alter a cell's morphology, see Section 1.10), showed the groundwater ultramicrobacteria to consist primarily of oval-shaped cells about 0.2 μm in diameter (**Figure 2**). The volume of these cells was calculated to be about 1/200 that of a cell of the bacterium *Escherichia coli* (see Table 1.1) such that more than 200 of the small cells could fit into one *E. coli* cell! Each of the tiny cells contained about 50 ribosomes, which is also about 1/100 of the number present in a slowly growing (100-min generation time) cell of *E. coli*. The very small size of the groundwater ultramicrobacteria gives them an enormous surface-to-volume ratio, and it is hypothesized that this advantage benefits them in extracting resources from their nutrient-deficient habitat.

Despite the fact that the tiny groundwater bacteria have yet to be cultured in the laboratory, much is already known about them because their small genomes—less than 1 megabase (Mb) in size—were obtained and analyzed.[2] From a phylogenetic perspective, the different species detected were distantly related to major phyla of *Bacteria* known from environmental analyses of diverse environments but which have thus far defied laboratory culture. Further analyses showed that genes encoding the enzymes for several core metabolic pathways widely distributed among microorganisms were absent from the genomes of the groundwater ultramicrobacteria. This suggests a metabolically minimalist lifestyle for these tiny cells and a survival strategy of cross-feeding essential nutrients with neighboring species in their microbial community.

A strategy of obtaining nutrients from other organisms is one widely used in the microbial world. As we will see later in this book, many disease-causing (pathogenic or parasitic) bacteria have very small genomes that are missing many key genes otherwise necessary for a free-living lifestyle. However, the pathogenic or parasitic way of life of these microbes lets them "get away" with a minimal genomic complement because any essential molecules they are unable to biosynthesize are supplied by the host.

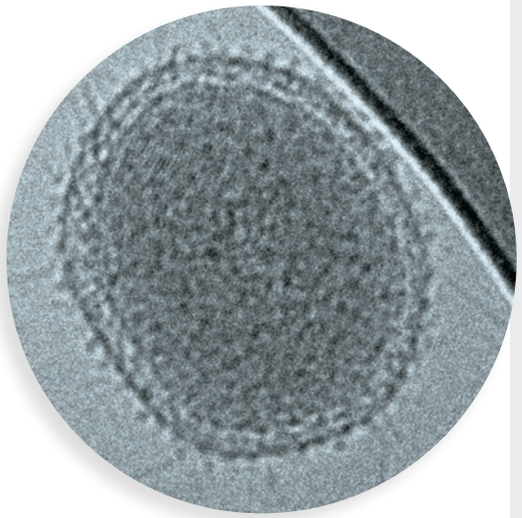


Figure 2 **A tiny bacterial cell from anoxic groundwater that passed through a filter with 0.2-μm pores.** The cell is not quite 0.2 μm in diameter.

Although we do not yet know exactly how small a microbial cell can be, microbiologists are closing in on this number from environmental analyses such as the Colorado groundwater study. From the same samples that yielded ultra-small *Bacteria* in this study, ultra-small *Archaea* were also detected and found to contain small and highly reduced genomes.[2]

It is thus likely that a large diversity of very small prokaryotic cells occurs in nature, and from the continued study of these tiny cells, more precise values for both the lower limits to cell size and the minimal genomic requirements for life should emerge. Moreover, theoretical considerations of cell size have shown that DNA and proteins dominate the volume of very small cells and that the theoretical lower limit to cell size agrees closely with the smallest bacteria observed in nature thus far.[3]

[1]Luef, B., et al. 2015. *Nat. Commun.* doi:10.1038/ncomms7372.
[2]Castelle, C.J., et al. 2015. *Curr. Biol. 25:* 1–12.
[3]Kempes, C.P., et al. 2016. *ISME J. 10:* 2145–2157.

form into filaments. As we will see, there are even square bacteria, hexagon-shaped bacteria, and star-shaped bacteria! Cell morphologies thus form a continuum, with some shapes, such as rods and cocci, being very common, whereas others, such as spiral, budding, and filamentous shapes, are less common.

Check Your Understanding

- What properties of the cell change as it gets smaller?
- Why is it that eukaryotic cells are typically larger than prokaryotic cells?
- What traits have allowed the bacteria *Epulopiscium* and *Thiomargarita* to have such large cells?

1.4 An Introduction to Microbial Life

As we have seen, microorganisms vary dramatically in size, shape, and structure. In this section we will learn more about different evolutionary (phylogenetic) lineages of cells. All cells fall into one of three major groups: *Bacteria*, *Archaea*, or *Eukarya*. These three major cell lineages are called **domains**, and all known cellular organisms belong to one of these three domains. In addition, while much of our focus in this chapter is on cellular forms of life, not all microbes form cells. In this section, we will also consider viruses, which are a group of microorganisms that lack a cellular structure. All known microorganisms can be classified into one of these four groups.

Bacteria

Bacteria have a prokaryotic cell structure (Figure 1.4*a*). *Bacteria* are often thought of as undifferentiated single cells with a length that ranges from 0.5 to 10 μm. While bacteria that fit this description are common, the *Bacteria* are actually tremendously diverse in appearance, size, and function (**Figure 1.9**). Although most bacteria are unicellular, some bacteria can differentiate to form multiple cell types and others are even multicellular (for example, *Magnetoglobus*, Figure 1.9).

Among the *Bacteria*, 30 major phylogenetic lineages (called *phyla*) have at least one species that has been grown in culture, though many more phyla exist which remain largely uncharacterized. Some of these phyla contain thousands of described species while others contain only a few. More than 90% of cultivated bacteria belong to one of only four phyla: *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. The analyses of environmental DNA sequences provide evidence for the existence of at least 80 bacterial phyla (Section 1.15).

Archaea

Like *Bacteria*, *Archaea* also have a prokaryotic cell structure (Figure 1.4*a*). The domain *Archaea* consists of five described phyla: *Euryarchaeota*, *Crenarchaeota*, *Thaumarchaeota*, *Nanoarchaeota*, and *Korarchaeota*. *Archaea* have historically been associated with extreme environments; the first isolates came from hot, salty, or acidic sites. But not all *Archaea* are extremophiles. *Archaea* are indeed common in

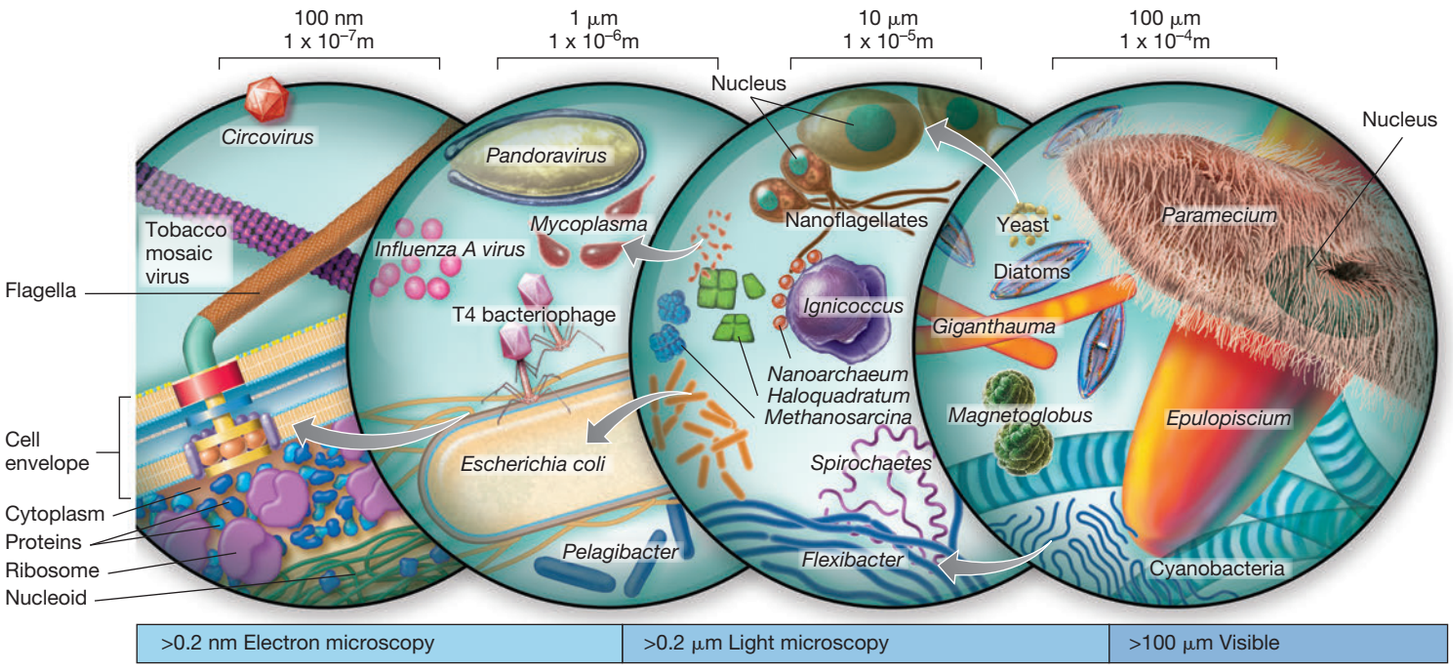


Figure 1.9 Microorganisms vary greatly in size and shape. The smallest known microbe is the circovirus (20 nm) and the largest shown here is the bacterium *Epulopiscium* (700 μm), which represents a 35,000-fold difference in length! Certain protozoa can be even larger than *Epulopiscium* (>2 mm long) and are visible to the unaided eye. Included in the figure are *Eukarya*: *Paramecium* (300 μm × 85 μm), diatoms (*Navicula*, 50 μm × 12 μm), yeast (*Saccharomyces*, 5 μm), and nanoflagellates (*Cafeteria*, 2 μm); *Bacteria*: *Epulopiscium* (700 μm × 80 μm), cyanobacteria (*Oscillatoria*, 10-μm-diameter multicellular filaments), *Magnetoglobus* (multicellular aggregate, 20 μm diameter), *Spirochaetes* (2–10 μm × 0.25 μm), *Flexibacter* (5–100 μm × 0.5 μm filaments), *Escherichia coli* (2 μm × 0.5 μm), *Pelagibacter* (0.4 μm × 0.15 μm), and *Mycoplasma* (0.2 μm); *Archaea*: *Giganthauma* (10-μm-diameter multicellular filament), *Ignicoccus* (6 μm), *Nanoarchaeum* (0.4 μm), *Haloquadratum* (2 μm), *Methanosarcina* (2 μm per cell in packet); and viruses: *Pandoravirus* (1 μm × 0.4 μm), T4 bacteriophage (200 nm × 90 nm), *Influenza A virus* (100 nm), *Tobacco mosaic virus* (300 nm × 20 nm), *Circovirus* (20 nm).

the most extreme environments that support life, such as those associated with volcanic systems, and species of *Archaea* hold many of the records that define the chemical and physical limits of life as we know them. However, in addition to these, *Archaea* are found widely in nature in nonextreme environments. For example, methane-producing *Archaea* (methanogens) are common in wetlands and in the guts of animals (including humans) and have a major impact on the greenhouse gas composition of our atmosphere. In addition, species of *Thaumarchaeota* inhabit soils and oceans worldwide and are important contributors to the global nitrogen cycle.

Archaea are also notable in that this domain lacks any known disease-causing (pathogenic or parasitic) species of plants or animals. Most described species of *Archaea* fall within the phyla *Crenarchaeota* and *Euryarchaeota* while only a handful of species have been described for the *Nanoarchaeota*, *Korarchaeota*, and *Thaumarchaeota*. Analysis of environmental DNA sequences indicate more than 12 archaeal phyla likely exist. We discuss *Archaea* in detail in Chapter 17.

Eukarya

Plants, animals, and fungi are the most well-known groups of *Eukarya*. These groups are phylogenetically relatively young compared with *Bacteria* and *Archaea*, originating during an evolutionary burst called the *Cambrian explosion*, which began about 600 million years ago. The first eukaryotes, however, were unicellular microbes. Microbial eukaryotes, which include diverse algae and protozoa, may have first appeared as early as 2 billion years ago, well before the origin of plants, animals, and fungi (Section 1.5). The major lineages of *Eukarya* are traditionally called *kingdoms* instead of phyla. There are at least six kingdoms of *Eukarya*, and this diverse domain contains microorganisms as well as the plants and animals.

Microbial eukaryotes vary dramatically in size, shape, and physiology (Figure 1.9). Among the smallest are the nanoflagellates, which are microbial predators that can be as small as 2 μm long. In addition, *Ostreococcus*, a genus of green algae that contains species whose cells are only 0.8 μm in diameter, are smaller than many bacteria. The largest single-celled organisms are eukaryotes, but they are hardly microbial. Xenophyophores are amoeba-like, single-celled organisms that live exclusively in the deep oceans and can be up to 10 *centimeters* in length. In addition, plasmodial slime molds consisting of a single cytoplasmic compartment can be up to 30 cm in diameter. In Chapter 18 we consider microbial eukaryotes in detail.

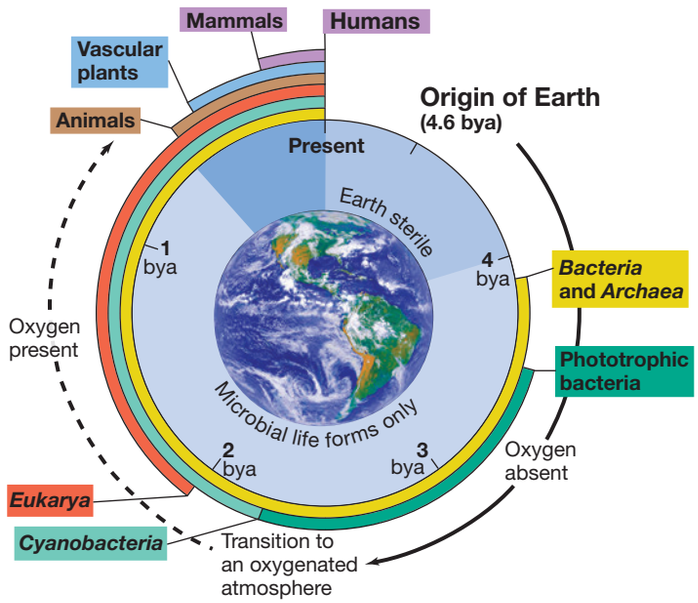
Viruses

Viruses are not found on the tree of life, and for a variety of reasons, it can be argued that they are not truly alive. Although viruses can replicate—a hallmark of cells—viruses are obligate parasites that can only replicate within the cytoplasm of a host cell. Viruses are not cells, and they lack the cytoplasmic membrane, cytoplasm, and ribosomes found in all forms of cellular life. Viruses do not carry out metabolic processes; instead, they take over the metabolic systems of infected cells and turn them into vessels for producing more viruses. Unlike cells, which all have genomes composed of double-stranded DNA, viruses have genomes composed of DNA *or* RNA that can be either double- or single-stranded. Viral genomes are often quite small, with the smallest having only three genes. The small size of most viral genomes means that no genes are conserved among all viruses, or between all viruses and all cells.

Although they are not cells, viruses are as diverse as the cells they infect, and different viruses are known to infect cells from all three domains of life. Viruses are often classified on the basis of their structure, genome composition, and host specificity. Viruses that infect bacteria are called *bacteriophages* (or *phages*, for short). Bacteriophages have been used as model systems to explore many aspects of viral biology. While most viruses are considerably smaller than bacterial cells (Figure 1.9), there are also unusually large viruses such as the Pandoraviruses, which can be more than 1 micrometer long and have a genome that contains as many as 2500 genes, larger than that of many bacteria! We will learn much more about viruses in Chapters 5 and 11.

Check Your Understanding

- How are viruses different from *Bacteria*, *Archaea*, and *Eukarya*?
- What four bacterial phyla contain the largest number of well-characterized species?
- What phylum of *Archaea* is common worldwide in soils and in the oceans?



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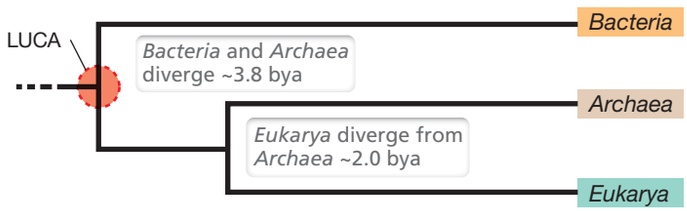


(b)

Figure 1.10 A summary of life on Earth through time and origin of the cellular domains. *(a)* At its origin, Earth was sterile and anoxic. Cellular life, in the form of *Bacteria* and *Archaea*, was present on Earth by 3.8 billion years ago (bya). The evolution of phototrophic bacteria called *Cyanobacteria* caused Earth's atmosphere to become oxygenated over time. While the first evidence for oxygen in Earth's atmosphere appears 2.4 bya, current levels of atmospheric O_2 were not achieved until 500–800 million years ago. *(b)* The three domains of cellular organisms are *Bacteria*, *Archaea*, and *Eukarya*. *Bacteria* and *Archaea* appeared first and *Eukarya* evolved later, diverging from the *Archaea*. LUCA, last universal common ancestor.

1.5 Microorganisms and the Biosphere

Microbes are the oldest form of life on Earth, and they have evolved to perform critical functions that sustain the biosphere. In this section we will learn how microbes have changed our planet and how they continue to do so.

A Brief History of Life on Earth

Earth is about 4.6 billion years old, and microbial cells first appeared between 3.8 and 4.3 billion years ago (**Figure 1.10**). During the first 2 billion years of Earth's existence, its atmosphere was anoxic (O_2 was absent), and only nitrogen (N_2), carbon dioxide (CO_2), and a few other gases were present. Only microorganisms capable of anaerobic metabolism (that is, metabolisms that do not require O_2) could survive under these conditions.

The evolution of phototrophic microorganisms—organisms that harvest energy from sunlight—occurred within 1 billion years of the formation of Earth (Figure 1.10*a*). The first phototrophs were anoxygenic (non-oxygen-producing), such as the purple sulfur bacteria and green sulfur bacteria we know today (**Figure 1.11**). *Cyanobacteria*—oxygen-producing (oxygenic) phototrophs (Figure 1.11*f*)—evolved nearly a billion years later (Figure 1.10*a*) and began the slow process of oxygenating Earth's atmosphere. These early phototrophs lived in structures called *microbial mats*, which are still found on Earth today (Figure 1.11*a–c*). After the oxygenation of Earth's atmosphere, multicellular life forms eventually evolved, culminating in the plants and animals we know today. But plants and animals have only existed for about half a billion years. The timeline of life on Earth (Figure 1.10*a*) shows that *80% of life's history was exclusively microbial*, and thus in many ways, Earth can be considered a microbial planet.

As evolutionary events unfolded, three major lineages of microbial cells—the *Bacteria*, the *Archaea*, and the *Eukarya* (Figure 1.10*b*)—were distinguished. All cellular organisms share certain characteristics (Figure 1.5) and as a result, certain genes are found in all cells. For example, approximately 60 genes are universally present in cells of all three domains. Examination of these genes reveals that all three domains have descended from a common ancestor, the *last universal common ancestor* (LUCA, Figure 1.10*b*). Over enormous periods of time, microorganisms derived from these three domains have evolved to fill every habitable environment on Earth.

Microbial Abundance and Activity in the Biosphere

Microorganisms are present everywhere on Earth that will support life. They constitute a major fraction of global biomass and are key reservoirs of nutrients essential for life. There are an estimated 2×10^{30} microbial cells on Earth. To put this number in context, the universe in all its vast extent is estimated to contain merely 7×10^{22} stars. The total amount of carbon present in all microbial cells is a significant fraction of Earth's biomass (**Figure 1.12**). Moreover, the total amount of nitrogen and phosphorus (essential nutrients for life) within microbial cells is almost four times that in all plant and animal cells combined. Microbes also represent a major fraction of the total DNA in the biosphere (about 31%), and their genetic diversity far exceeds that of plants and animals.

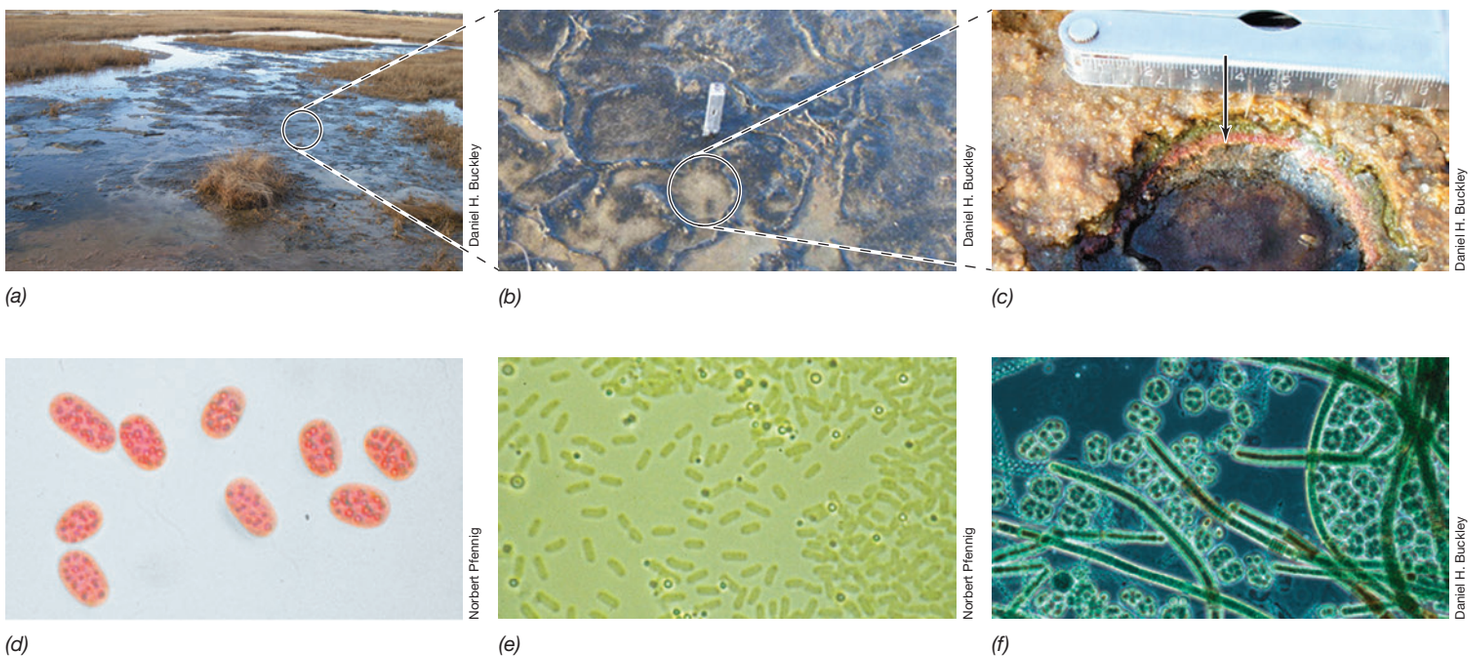


Figure 1.11 Phototrophic microorganisms. The earliest phototrophs lived in microbial mats. *(a)* Microbial mats in the Great Sippewissett Marsh, a salt marsh in Massachusetts, USA. *(b)* Mats develop a cohesive structure that forms at the sediment surface. *(c)* A slice through the mat shows colored layers that form due to the presence of photopigments. Cyanobacteria form the green layer nearest the surface, purple sulfur bacteria form the purple and yellow layers below, and green sulfur bacteria form the bottommost green layer. The scale on the knife is in cm. *(d)* Purple sulfur bacteria, *(e)* green sulfur bacteria, and *(f)* cyanobacteria imaged by bright-field and phase-contrast microscopy. Purple and green sulfur bacteria are anoxygenic phototrophs that appeared on Earth long before oxygenic phototrophs (that is, *Cyanobacteria*) evolved (see Figure 1.10*a*).

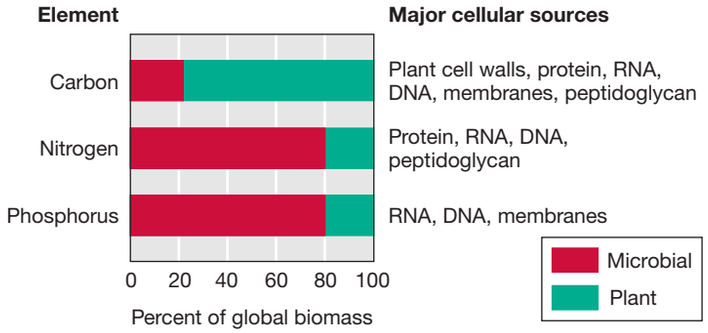


Figure 1.12 Contribution of microbial cells to global biomass. Microorganisms comprise a significant fraction of the carbon (C) and a majority of the nitrogen (N) and phosphorus (P) in the biomass of all organisms on Earth. C, N, and P are the macronutrients required in the greatest quantity by living organisms. Animal biomass is a minor fraction (<0.1%) of total global biomass and is not shown.

Microbes are even abundant in habitats that are much too harsh for other forms of life, such as volcanic hot springs, glaciers and ice-covered regions, high-salt environments, extremely acidic or alkaline habitats, and deep in the sea or deep in the earth at extremely high pressure. Such microorganisms are called **extremophiles** and their properties define the physiochemical limits to life as we know it (Table 1.2). We will revisit many of these organisms in later chapters and discover the special structural and biochemical properties that allow them to thrive under extreme conditions.

All ecosystems are influenced to one extent or another by microbial activities. The metabolic activities of microorganisms can change the habitats in which they live, both chemically and physically, and these changes can affect other organisms. For example, excess nutrients added to a habitat can cause aerobic (O_2-consuming) microorganisms to grow rapidly and consume O_2, rendering the habitat anoxic (O_2-free). Many human activities release nutrients into the coastal oceans, thereby stimulating excessive microbial growth, which can cause enormous anoxic zones in these waters. These "dead zones" cause massive mortality of fish and shellfish in coastal oceans worldwide, because most aquatic animals require O_2 and die if it is not available. Only by understanding microorganisms and microbiology can we predict and minimize the effects of human activity on the biosphere that sustains us.

Though diverse habitats are influenced strongly by microorganisms, their contributions are easy to overlook because of their small sizes. Within the human body, for example, more microbial cells can be present than human cells, and more than 200 microbial genes are present for every human gene. These microbes provide benefits and services that are essential to human health. In later chapters, we will return to a consideration of the ways in which microorganisms affect animals, plants, and the entire global ecosystem. This is the science of **microbial ecology**, perhaps the most exciting subdiscipline of microbiology today. We will see that microbes are important to myriad issues of global importance to humans including climate change, agricultural productivity, and even energy policy.

We focus now on the effects of microbes on humans and human activities.

Check Your Understanding

- How old is Earth and when did cells first appear on Earth?
- Name the three domains of life. Which of these contain eukaryotic life forms?
- Why were cyanobacteria so important in the evolution of life on Earth?

1.6 The Impact of Microorganisms on Human Society

Microbiologists have made great strides in discovering how microorganisms function, and application of this knowledge has greatly advanced human health and welfare. Besides understanding microorganisms as agents of disease, microbiology has made great

TABLE 1.2 Classes and examples of extremophiles[a]

Extreme	Descriptive term	Genus, species	Domain	Habitat	Minimum	Optimum	Maximum
Temperature							
High	Hyperthermophile	*Methanopyrus kandleri*	*Archaea*	Undersea hydrothermal vents	90°C	106°C	122°C[b]
Low	Psychrophile	*Psychromonas ingrahamii*	*Bacteria*	Sea ice	−12°C[c]	5°C	10°C
pH							
Low	Acidophile	*Picrophilus oshimae*	*Archaea*	Acidic hot springs	−0.06	0.7[d]	4
High	Alkaliphile	*Natronobacterium gregoryi*	*Archaea*	Soda lakes	8.5	10[e]	12
Pressure	Barophile (piezophile)	*Moritella yayanosii*	*Bacteria*	Deep ocean sediments	500 atm	700 atm[f]	>1000 atm
Salt (NaCl)	Halophile	*Halobacterium salinarum*	*Archaea*	Salterns	15%	25%	32% (saturation)

[a]The organisms listed are the current "record holders" for growth in laboratory culture at the extreme condition listed.
[b]Anaerobe showing growth at 122°C only under several atmospheres of pressure.
[c]The permafrost bacterium *Planococcus halocryophilus* can grow at −15°C and metabolize at −25°C. However, the organism grows optimally at 25°C and grows up to 37°C and thus is not a true psychrophile.
[d]*P. oshimae* is also a thermophile, growing optimally at 60°C.
[e]*N. gregoryi* is also an extreme halophile, growing optimally at 20% NaCl.
[f]*M. yayanosii* is also a psychrophile, growing optimally near 4°C.

advances in understanding the important roles microorganisms play in food and agriculture, and microbiologists have exploited microbial activities to produce valuable human products, generate energy, and clean up the environment.

Microorganisms as Agents of Disease

The statistics summarized in **Figure 1.13** show how microbiologists and clinical medicine have combined to conquer infectious diseases in the past 120 years. At the beginning of the twentieth century, more than half of all humans died from infectious diseases caused by bacterial and viral **pathogens**. Today, however, infectious diseases are largely preventable due to advances in our understanding of microbiology. Microbiology has fueled advances in medicine such as vaccination and antibiotic therapy, advances in engineering such as water and wastewater treatment, advances in food safety such as pasteurization, and a better understanding of how microorganisms are transmitted. Infectious diseases now cause fewer than 5% of all deaths in countries where these interventions, made possible by microbiology, are readily available. However, while infectious diseases are preventable, the World Health Organization has documented that they still account for more than a third of all deaths in countries where microbial interventions are less available, such as those having low-income economies. As we will see later in this chapter, the development of microbiology as a science can be traced to pioneering studies of infectious disease.

While pathogens and infectious disease remain a major threat to humanity, and combating these harmful organisms remains a major focus of microbiology, most microorganisms are not harmful to humans. In fact, most microorganisms are beneficial, and in many cases are even essential to human welfare and the functioning of the planet. We turn our attention to these microorganisms and microbial activities now.

Microorganisms, Agriculture, and Human Nutrition

Agriculture benefits from nutrient cycling performed by microorganisms, in particular, the cycling of nitrogen, sulfur, and carbon compounds. For example, legumes are a diverse family of plants that include major crop species such as soybeans, peas, and lentils, among others. Legumes live in close association with bacteria that form structures called *nodules* on their roots. In the nodules, these bacteria convert atmospheric nitrogen (N_2) into ammonia (NH_3) through the process of *nitrogen fixation*. NH_3 is the major nutrient found in fertilizer and is used as a nitrogen source for plant growth (**Figure 1.14**). In this way bacteria allow legumes to make their own fertilizer, thereby reducing the need for farmers to apply fertilizers produced industrially. When plants die they are decomposed by bacteria in the soil, and this process produces the nutrients that form the basis of soil fertility. Bacteria regulate nutrient cycles (Figure 1.14), in soils and throughout the biosphere, transforming and recycling the nutrients required by plants and animals.

Also of major agricultural importance are microorganisms that inhabit the rumen of ruminant animals, such as cattle and sheep. Ruminants, like most animals, lack enzymes for breaking down the polysaccharide cellulose, the major component of plant cell walls. The digestive tract of ruminants has a large specialized chamber called the *rumen* in which cellulose is digested. The rumen contains a dense and diverse community of microorganisms that digest and ferment cellulose. Without these symbiotic microorganisms, ruminants could not digest plant matter like grass and hay, most of which consists of cellulose. Ruminants ultimately get their nutrition by metabolizing the waste products of microbial fermentation and by digesting dead microbial cells. Many domesticated and wild herbivorous mammals—including deer, bison, camels, giraffes, and goats—are also ruminants.

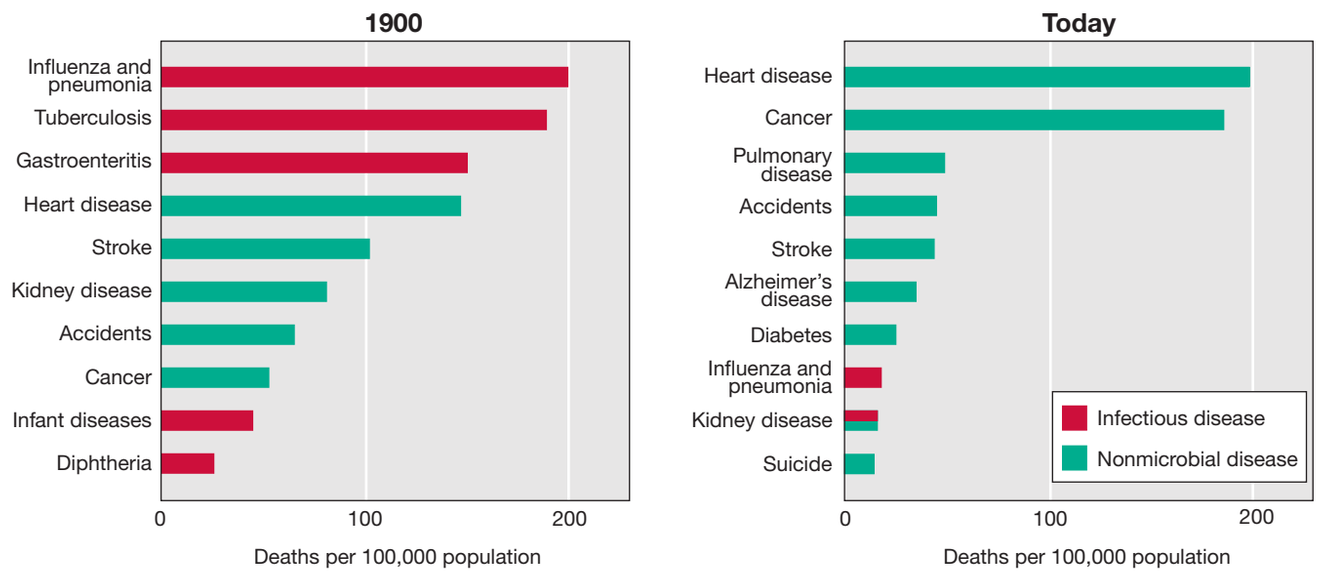


Figure 1.13 Death rates for the leading causes of death in the United States: 1900 and 2016. Infectious diseases were the leading causes of death in 1900, whereas today they account for relatively few deaths. Kidney diseases can be caused by microbial infections or systemic sources (diabetes, cancers, toxicities, metabolic diseases, etc.). Data are from the United States National Center for Health Statistics and the Centers for Disease Control and Prevention.

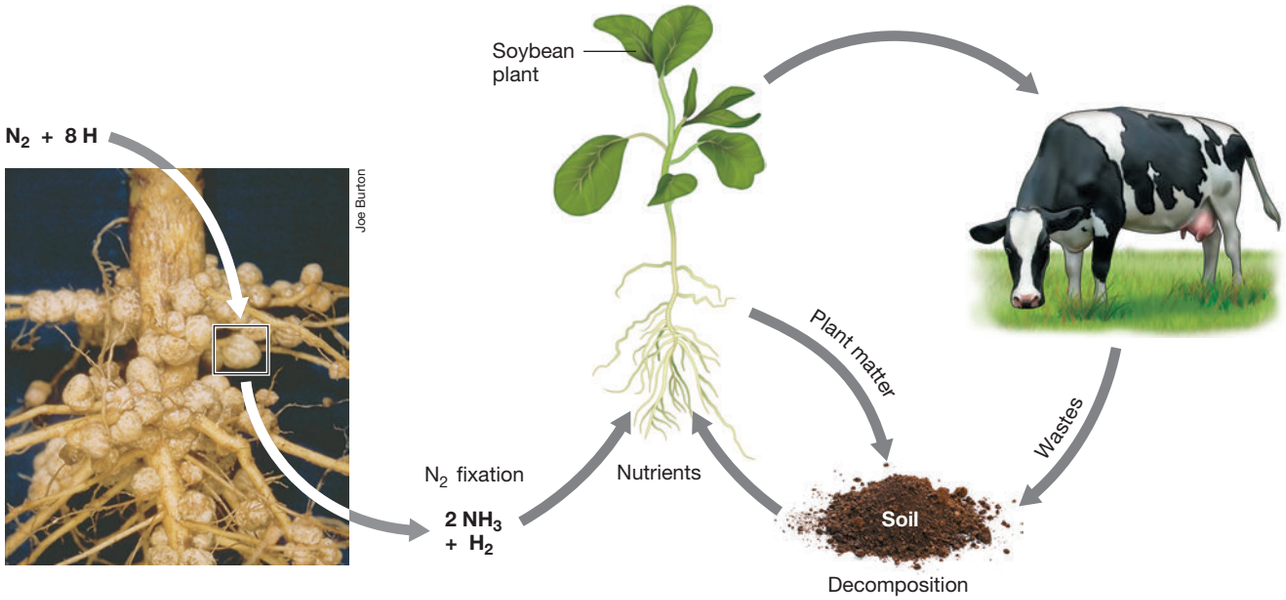


Figure 1.14 Microorganisms in modern agriculture. Root nodules on this soybean plant contain bacteria that fix atmospheric nitrogen (N_2) to form nitrogenous compounds used by the plant. Ruminant animals such as cows and sheep require rumen microbes to digest cellulose from plants. Plant matter and animal wastes are decomposed in soil to produce nutrients that are the basis of soil fertility and which are required for plant growth.

The human gastrointestinal (GI) tract lacks a rumen, but we too rely on microbial partners for our nutrition. Human enzymes lack the ability to break down complex carbohydrates (which can represent 10–30% of food energy) and so we rely on our **gut microbiome** for this purpose. The colon, or large intestine (**Figure 1.15**), follows the stomach and small intestine in the human digestive tract, and it contains about 10^{11} microbial cells per gram of colonic contents. Microbial cell numbers are low in the very acidic (pH 2) stomach (about 10^4 per gram) but increase to about 10^8 per gram near the end of the small intestine (pH 4–5) and then reach maximal numbers in the colon (pH 7) (Figure 1.15). The colon contains diverse microbial species that assist in the digestion of complex carbohydrates, and that synthesize vitamins and other nutrients essential to host nutrition. The gut microbiome develops from birth, but it can change over time with the human host. The composition of the gut microbiome has major effects on GI function and human health as we will see in Chapter 24.

Microorganisms and Food

Microbes are intimately associated with the foods we eat. Microbial growth in food can cause food spoilage and foodborne disease. The manner in which we harvest and store food (for example, canning, refrigeration, drying, salting, etc.), the ways in which we cook it, and even the spices we use, have all been fundamentally influenced by the goal of eliminating harmful organisms from our food. Microbial food safety and prevention of food spoilage is a major focus of the food industry and a major cause of economic loss every year.

While some microbes can cause foodborne disease and food spoilage, not all microorganisms in foods are harmful. Indeed, beneficial microbes have been used for thousands of years to improve food safety and to preserve foods (**Figure 1.16**). For example, cheeses, yogurt, and buttermilk are all produced by microbial fermentation of dairy products. Microbial production of lactic acid in these foods improves their shelf life and prevents the growth of foodborne pathogens. Lactic acid–producing bacteria are used to produce a variety of sour-tasting foods, including sauerkraut, kimchi, pickles, and even certain sausages. Even the production of chocolate and coffee rely on microbial fermentation. Moreover, the fermentative activities of yeast are essential for baking (by generating carbon dioxide—CO_2—to raise the dough), and for the production of alcoholic beverages (by generating alcohol). The products of microbial fermentation affect the flavor and taste of foods and can prevent spoilage as well as the growth of deleterious organisms.

Microorganisms and Industry

Microorganisms play important roles in all manner of human activity. The field of *industrial microbiology* is focused on the use of microorganisms as tools for major industries such as pharmaceuticals and brewing (**Figure 1.17**). For example, in large industrial settings, naturally occurring microorganisms are grown on a massive scale in bioreactors called *fermentors* to make large amounts of products, such as antibiotics, enzymes, alcohol, and certain other chemicals, at relatively low cost. By contrast, *biotechnology* employs genetically engineered microorganisms to synthesize products of high commercial value, such as insulin or other human proteins, usually on a small scale.

Microorganisms can also be used to produce *biofuels* (▶ Section 12.19 and Figure 12.33). For example, as previously discussed,

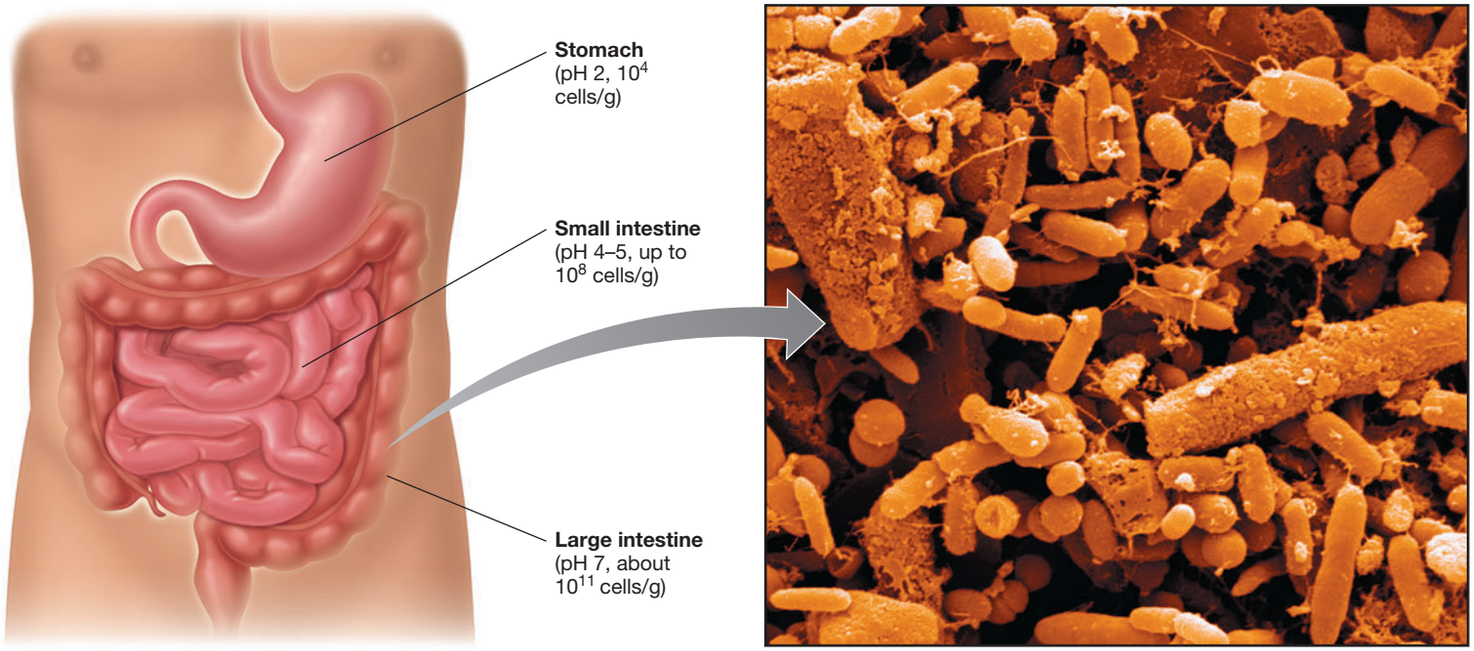


Figure 1.15 **The human gastrointestinal tract.** *(a)* Diagram of the human GI tract showing the major organs. *(b)* Scanning electron micrograph of microbial cells in the human colon (large intestine). Cell numbers in the colon can reach as high as 10^{11} per gram. As well as high *numbers* of cells, the microbial *diversity* in the colon is also quite high.

natural gas (methane, CH_4) is a product of the anaerobic metabolism of methanogenic *Archaea*. Ethyl alcohol (ethanol) is a major fuel supplement, which is produced by the microbial fermentation of glucose obtained from carbon-rich feedstocks such as sugarcane, corn, or rapidly growing grasses. Microorganisms can even convert waste materials, such as domestic refuse, animal wastes, and cellulose, into ethanol and methane. In producing these biofuels, humans are simply exploiting the metabolic features of particular microbes, but at the same time, are reducing the use of fossil fuels. As we will document in Chapter 21, CO_2 levels have been rising

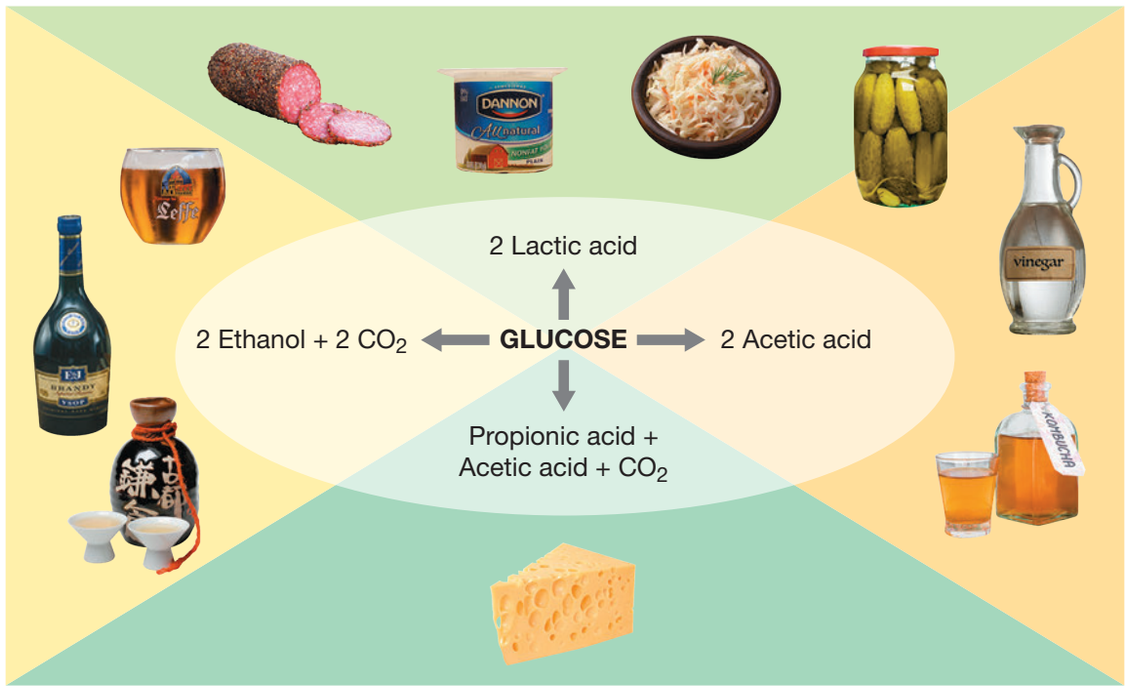


Figure 1.16 **Fermented foods.** Major fermentations in various fermented foods. It is the fermentation product (ethanol, or lactic, propionic, or acetic acids) that both preserves the food and renders in it a characteristic flavor.

Wastewater Treatment: Microbes are used to clean wastewater.

Bioremediation: Microbes are used to clean contaminated environments.

Biofilms: Microbes grow on surfaces and can foul pipes and pipelines.

Biotechnology: Microbes can be genetically modified to produce high-value products such as pharmaceuticals and enzymes.

Fermentation: Microbes are used at industrial scale to make chemicals, solvents, enzymes, and pharmaceuticals.

Biofuels: Microbes are used to convert biomass into ethanol and wastes into natural gas (methane).

Figure 1.17 Industrial microbiology. Microbes have major impacts on human industry. Microbes can be used to produce valuable products and biofuels and they can also be used to clean up our wastes. Microbial biofilms have major impacts on industry because biofilms can clog and corrode pipelines and holding tanks in factories, in ships, and in the oil industry.

rapidly on Earth in the industrial era, and the link between this "greenhouse gas" and Earth's rising temperatures is firm. Thus, as a sustainable fuel source, biofuels should help cool our planet and are one facet of the "green revolution" many countries support today.

Microorganisms are also used to clean up wastes. Wastewater treatment is essential to sanitation and human health. *Wastewater treatment* relies on microbes to treat water contaminated with human waste so that it can be reused or returned safely to the environment. Waterborne diseases such as cholera and typhoid (major killers before the blossoming of microbiology: see gastroenteritis, Figure 1.13) can proliferate in the absence of proper wastewater treatment. Microbes can also be used to clean up industrial pollution in a process called *bioremediation*. In bioremediation, microorganisms are used to transform spilled oil, solvents, pesticides, heavy metals, and other environmentally toxic pollutants into nontoxic forms. Bioremediation accelerates the cleanup process either by adding special microorganisms to a polluted environment or by adding nutrients that stimulate indigenous microorganisms to degrade the pollutants. In either case the goal is to accelerate disappearance of the pollutant.

Microbes can grow in almost any environment containing liquid water, including structures made by humans. For example, microbes often grow on submerged surfaces, forming *biofilms*. Biofilms that grow in pipes and drains can cause fouling and blockages in factory settings and pipelines, in sewers, and even in water distribution systems. In addition, biofilms that grow on ships' hulls can cause marked reductions in speed and efficiency. Biofilms can even grow in tanks that store oil and fuel, leading to spoilage of these products. We will learn that biofilms are also of great importance in medicine, as biofilms that form on implanted medical devices (▶ Section 4.9) can cause infections that are extremely difficult to treat.

As these examples show, the influence of microorganisms on humans is great and their activities are essential for the functioning of the planet. Or, as the famous French chemist and early microbiologist Louis Pasteur so aptly put it: "The role of the infinitely small in nature is infinitely large." Microscopes provide an essential portal through which microbiologists such as Pasteur gazed into the world of microbes. We therefore continue our introduction to the microbial world with an overview of microscopy.

Check Your Understanding

- How do microbes contribute to the nutrition of animals such as humans and cows?
- Describe several ways in which microorganisms are important in the food and agricultural industries.
- What is wastewater treatment and why is it important?

II • Microscopy and the Origins of Microbiology

The microscope first revealed the microbial world, and the several different types of microscopes available today remain among the microbiologist's foremost tools.

Historically, the science of microbiology has taken its greatest leaps forward as new tools are developed and old tools improve. The microscope is the microbiologist's oldest and most fundamental tool for studying the microbial world. Indeed, microbiology did not exist before the invention of the microscope. Many forms of microscopy are available, and some are extremely powerful. Throughout this text you will see images of microorganisms that were taken through the microscope using a variety of different techniques. So let's take a moment to explore how microscopy can be used to visualize microbial cells, starting at the very beginning with the invention of the microscope.

1.7 Light Microscopy and the Discovery of Microorganisms

Although the existence of creatures too small to be seen with the naked eye had been suspected for centuries, their discovery had to await invention of the microscope. The English mathematician and natural historian Robert Hooke (1635–1703) was an excellent microscopist. In his famous book *Micrographia* (1665), the first book devoted to microscopic observations, Hooke illustrated many microscopic images including the fruiting structures of molds (**Figure 1.18**). This was the first known description of microorganisms.

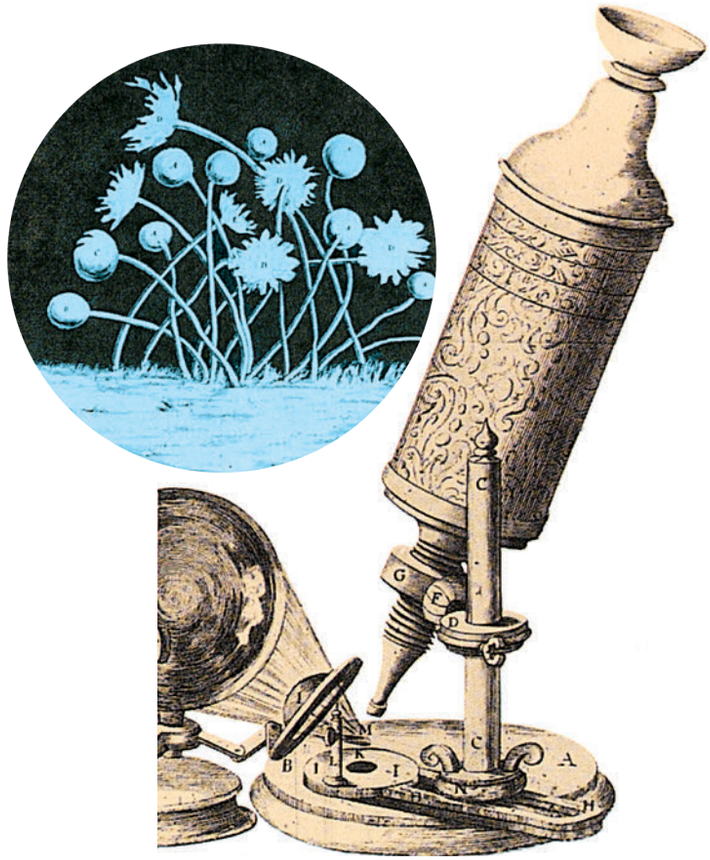


Figure 1.18 Robert Hooke and early microscopy. A drawing of the microscope used by Robert Hooke in 1664. The lens was fitted at the end of an adjustable bellows (G) and light focused on the specimen by a separate lens (1). Inset: Hooke's drawing of a bluish mold he found degrading a leather surface; the round structures contain spores of the mold.

The first person to see bacteria, the smallest microbial cells, was the Dutch draper and amateur microscopist Antoni van Leeuwenhoek (1632–1723). Van Leeuwenhoek constructed extremely simple microscopes containing a single lens to examine various natural substances for microorganisms (**Figure 1.19**). These microscopes were crude by today's standards, but by careful manipulation and focusing, van Leeuwenhoek was able to see bacteria. He discovered bacteria in 1676 while studying pepper–water infusions and reported his observations in a series of letters to the prestigious Royal Society of London, which published them in English translation in 1684. Drawings of some of van Leeuwenhoek's "wee animalcules," as he referred to them, are shown in Figure 1.19*b*, and a photo taken through a van Leeuwenhoek microscope is shown in Figure 1.19*c*.

Van Leeuwenhoek's microscope was a *light* microscope, and his design used a simple lens that could magnify an image

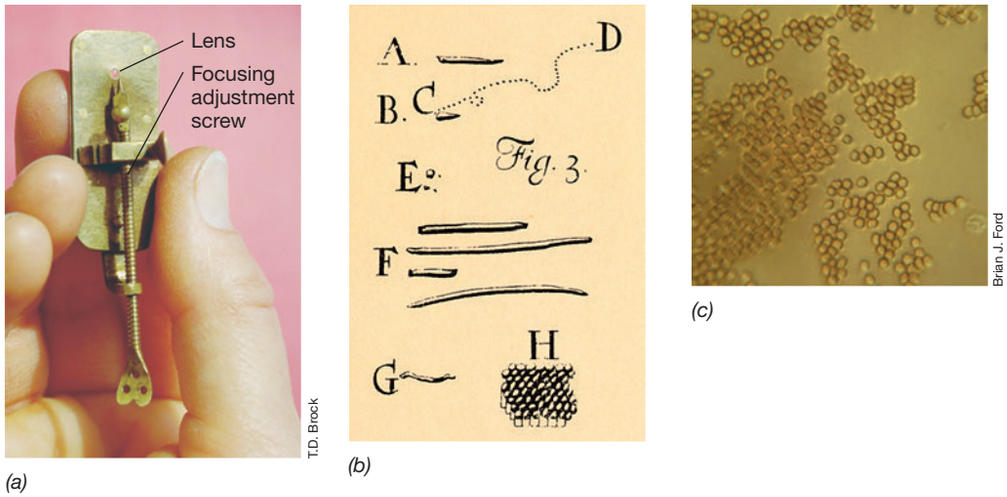


Figure 1.19 The van Leeuwenhoek microscope. *(a)* A replica of Antoni van Leeuwenhoek's microscope. *(b)* Van Leeuwenhoek's drawings of bacteria, published in 1684. Even from these simple drawings we can recognize several shapes of common bacteria: A, C, F, and G, rods; E, cocci; H, packets of cocci. *(c)* Photomicrograph of a human blood smear taken through a van Leeuwenhoek microscope. Red blood cells are clearly apparent.

at least 266 times. In a light microscope the sample is illuminated with visible light. **Magnification** describes the capacity of a microscope to enlarge an image. All microscopes employ lenses that provide magnification. Magnification, however, is not the limiting factor in our ability to see small objects. It is *resolution* that governs our ability to see the very small. **Resolution** is the ability to distinguish two adjacent objects as distinct and separate. The limit of resolution for a light microscope is about 0.2 μm (μm is the abbreviation for micrometer, 10^{-6} m). What this means is that two objects that are closer together than 0.2 μm cannot be resolved as distinct and separate.

Microscopy has improved remarkably since the days of van Leeuwenhoek. Several types of light microscopy are now available, including *bright-field*, *phase-contrast*, *differential interference contrast*, *dark-field*, and *fluorescence*. With the modern compound light microscope, light is focused on the specimen by the condenser (**Figure 1.20**) and this light passes through the sample and is collected by the lenses. The modern compound light microscope contains two types of lenses, *objective* and *ocular*, that function in combination to magnify the image. Microscopes used in microbiology have ocular lenses that magnify 10–30× and objective lenses that magnify 10–100× (Figure 1.20*b*). The total magnification of a compound light microscope is the *product* of the magnification of its objective and ocular lenses (Figure 1.20*b*). Magnification of 1000× is required to resolve objects 0.2 μm in diameter, which is the limit of resolution for most light microscopes (increasing magnification beyond 1000× provides little improvement in the resolution of a light microscope).

In addition to magnification, the limit of resolution for a light microscope is a function of the wavelength of light used and the light-gathering ability of the objective lens, a property known as its *numerical aperture*. There is a correlation between the magnification of a lens and its numerical aperture; lenses with higher magnification typically have higher numerical apertures. The diameter of the smallest object resolvable by any lens is equal to 0.5λ/numerical aperture, where λ is the wavelength of light used. With objectives that have a very high numerical aperture (such as the 100× objective), an optical-grade oil is placed between the microscope slide and the objective. Lenses on which oil is used are called *oil-immersion* lenses. Immersion oil increases the light-gathering ability of a lens, that is, it increases the amount of light that is collected and viewed by the lens.

Mastering Microbiology
Microbiology Animation: Light Microscopy

In light microscopy, specimens are visualized because of differences in **contrast** that exist between them and their surroundings (**Figure 1.21**). In bright-field microscopy, contrast results when cells absorb or scatter light differently from their surroundings. Bacterial cells typically lack contrast, that is, their optical properties are similar to the surrounding liquid, and hence they are difficult to see well with the bright-field microscope. Pigmented microorganisms are an exception because the color of the organism adds contrast, thus improving visualization by bright-field optics (Figure 1.21). For cells lacking pigments there are several ways to boost contrast, and we consider these methods in the next section.

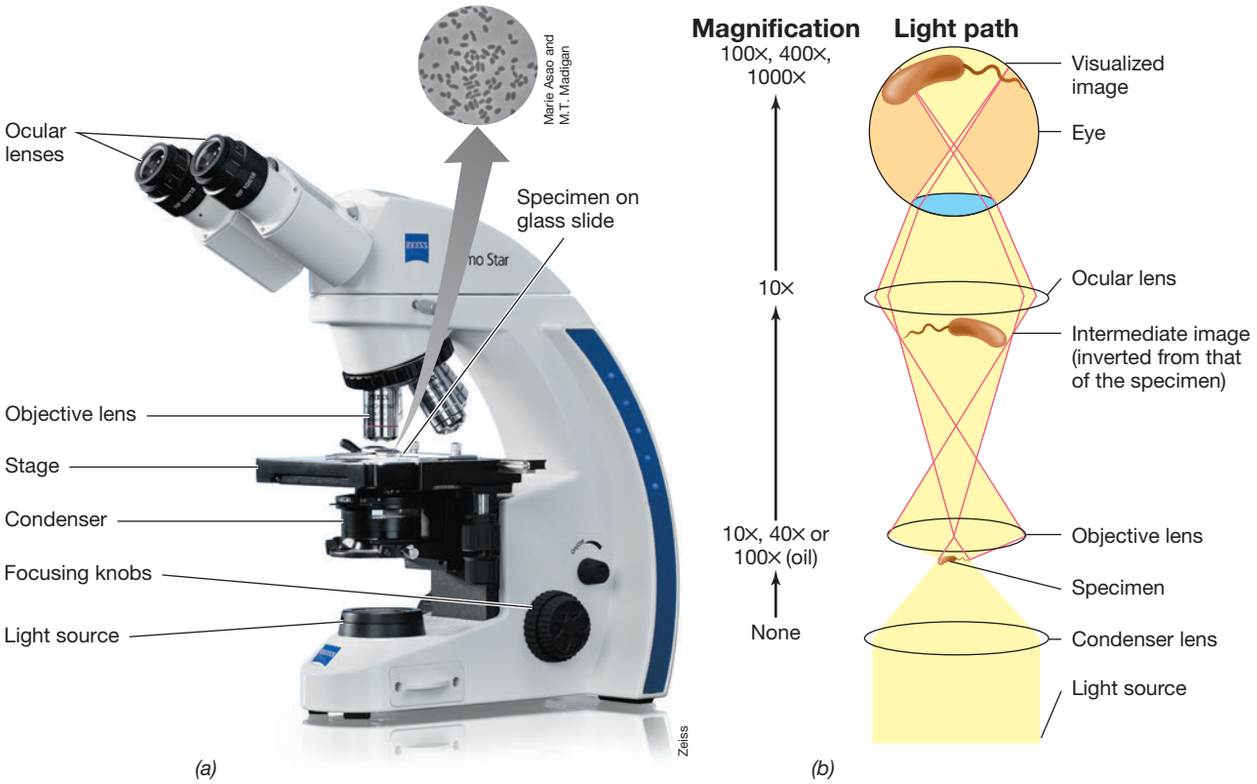
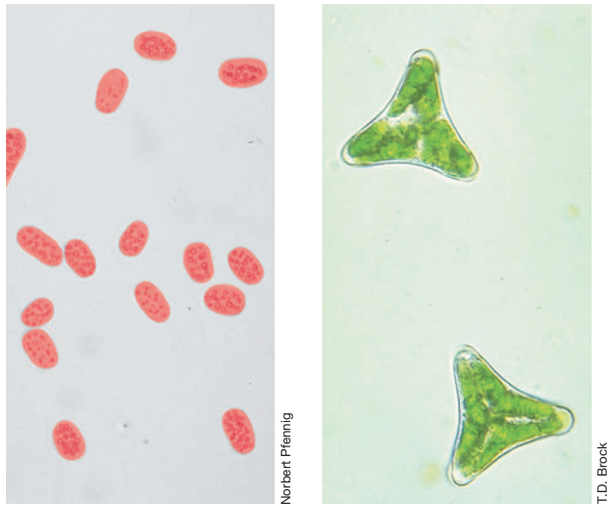


Figure 1.20 Microscopy. *(a)* A compound light microscope (inset photomicrograph of unstained cells taken through a phase-contrast light microscope). *(b)* Path of light through a compound light microscope. Figure 1.24 compares cells visualized by bright field with those visualized by phase contrast.



(a) (b)

Figure 1.21 Bright-field photomicrographs of pigmented microorganisms. *(a)* Purple phototrophic bacteria (*Bacteria*). The bacterial cells are about 5 μm wide. *(b)* A green alga (eukaryote). The green structures are chloroplasts. The algal cells are about 15 μm wide. Purple bacteria are anoxygenic phototrophs, whereas algae are oxygenic phototrophs. Both groups contain photosynthetic pigments, but only oxygenic phototrophs produce O_2 (Section 1.5 and Figure 1.10*a*).

Check Your Understanding

- Define the terms magnification and resolution.
- What is the limit of resolution for a bright-field microscope? What defines this limit?

1.8 Improving Contrast in Light Microscopy

Contrast is necessary in light microscopy to distinguish microorganisms from their surroundings. Cells can be stained to improve contrast, and staining is commonly used to visualize bacteria with bright-field microscopy. In addition to staining, other methods of light microscopy have been developed to improve contrast with or without staining, and we consider all of these methods here.

Staining: Increasing Contrast for Bright-Field Microscopy

Dyes can be used to stain cells and increase their contrast so that they can be more easily seen in the bright-field microscope. Each class of dye has an affinity for specific cellular materials. Many dyes used in microbiology are positively charged, and for this reason, they are called *basic dyes*. Examples of basic dyes include methylene blue, crystal violet, and safranin. Basic dyes bind strongly to negatively charged cell components, such as nucleic acids and acidic polysaccharides. These dyes also stain the surfaces of cells because cell surfaces tend to be negatively charged. These properties make basic dyes useful general-purpose stains that nonspecifically stain most bacterial cells.

To perform a *simple* stain, one begins with dried preparations of cells (Figure 1.22). A clean glass slide containing a dried suspension of cells is flooded for a minute or two with a dilute solution of a basic dye, rinsed several times in water, and blotted dry. Because bacterial cells are so small, it is common to observe dried, stained preparations of those cells with a high-power (oil-immersion) lens.

Differential Stains: The Gram Stain

Stains that render different kinds of cells different colors are called *differential* stains. An important differential-staining procedure used in microbiology is the **Gram stain** (Figure 1.23). On the basis of their reaction in the Gram stain, bacteria can be divided into two major groups: **gram-positive** and **gram-negative**. After Gram staining, gram-positive bacteria appear purple-violet and gram-negative bacteria appear pink (Figure 1.23*b*). The color difference in the Gram stain arises because of differences in the cell wall structure of gram-positive and gram-negative cells (▶ Section 2.3). Staining with a basic dye such as crystal violet renders cells purple in color. Cells are then treated with ethanol, which decolorizes gram-negative cells but

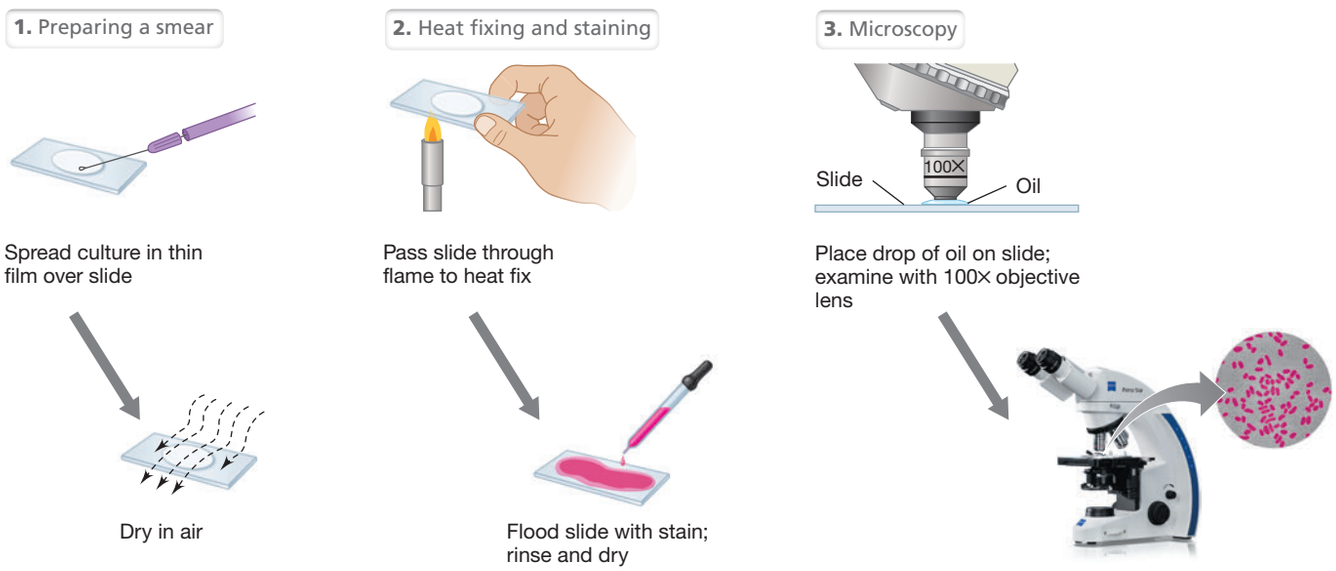


Figure 1.22 Staining cells for microscopic observation. Stains improve the contrast between cells and their background. Step 3 lower right: Same cells as shown in Figure 1.20*a* inset but stained with a basic dye.

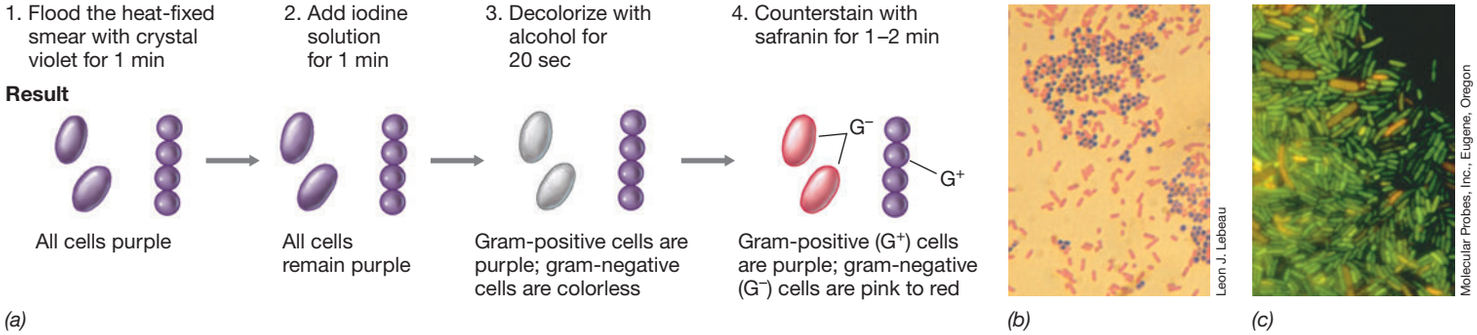


Figure 1.23 **The Gram stain.** *(a)* Steps in the procedure. *(b)* Microscopic observation of gram-positive (purple) and gram-negative (pink) bacteria. The organisms are *Staphylococcus aureus* and *Escherichia coli*, respectively. *(c)* Cells of *Pseudomonas aeruginosa* (gram-negative, green) and *Bacillus cereus* (gram-positive, orange) stained with a one-step fluorescent staining method. This method allows for differentiating gram-positive from gram-negative bacteria in a single staining step.

not gram-positive cells. Finally, cells are counterstained with a different-colored stain, typically the red stain safranin. As a result, gram-positive and gram-negative cells can be distinguished microscopically by their different colors (Figure 1.23*b*).

The Gram stain is the most common staining procedure used in microbiology, and it is often performed to begin the characterization of a new bacterium. If a fluorescence microscope is available, the Gram stain can be reduced to a one-step procedure; gram-positive and gram-negative cells fluoresce different colors when treated with a special chemical (Figure 1.23*c*).

Phase-Contrast and Dark-Field Microscopy

Although staining is widely used in light microscopy, staining often kills cells and can distort their features. Two forms of light microscopy improve image contrast of unstained (and thus live) cells. These are phase-contrast microscopy and dark-field microscopy (**Figure 1.24**). The phase-contrast microscope in particular is widely used in teaching and research for the observation of living preparations.

Phase-contrast microscopy is based on the principle that cells differ in refractive index (that is, the ability of a material to alter the speed of light) from their surroundings. Light passing through a cell thus differs in phase from light passing through the surrounding liquid. This subtle difference is amplified by a device in the objective lens of the phase-contrast microscope called the *phase ring*, resulting in a dark image on a light background (Figure 1.24*b*; see also inset to Figure 1.20*a*). The ring consists of a phase plate that amplifies the variation in phase to produce the higher-contrast image.

In the dark-field microscope, light does not pass through the specimen. Instead, light is directed from the sides of the specimen and only light that is scattered when it hits the specimen reaches the lens. Thus, the specimen appears light on a dark background (Figure 1.24*c*). Dark-field microscopy often has better resolution than light microscopy, and some objects can be resolved by dark-field that cannot be resolved by bright-field or even by phase-contrast microscopes. Dark-field microscopy is a particularly good way to observe microbial motility, as bundles of flagella (the structures responsible for swimming motility) are often resolvable with this technique.

Fluorescence Microscopy

The fluorescence microscope visualizes specimens that *fluoresce* (emit light). In fluorescence microscopy, cells are made to fluoresce by illuminating them from above with light of a single color. Filters are used so that only fluorescent light is seen, and thus cells appear to glow in a black background (**Figure 1.25**).

Cells fluoresce either because they contain naturally fluorescent substances such as chlorophyll (autofluorescence, Figure 1.25*b*, *d*)

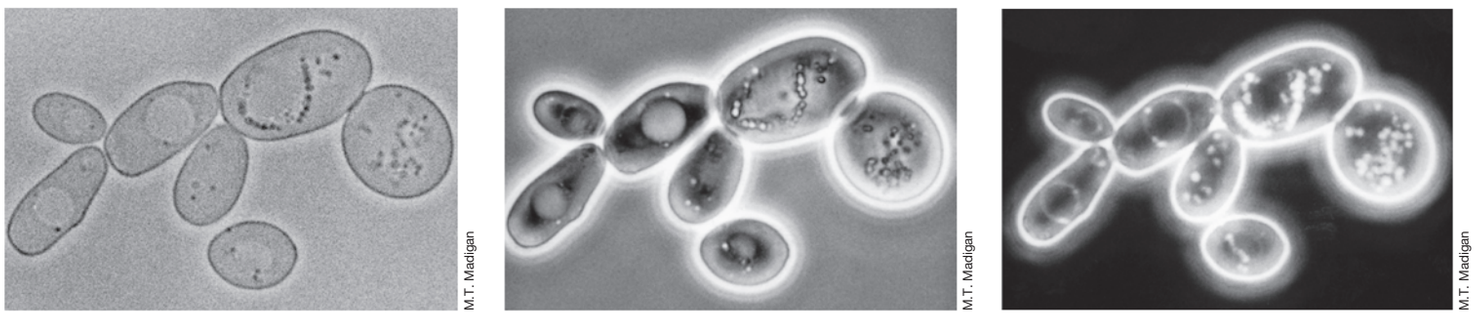


Figure 1.24 **Cells visualized by different types of light microscopy.** The same field of cells of the yeast *Saccharomyces cerevisiae* visualized by *(a)* bright-field microscopy, *(b)* phase-contrast microscopy, and *(c)* dark-field microscopy. Cells average 8–10 μm wide.

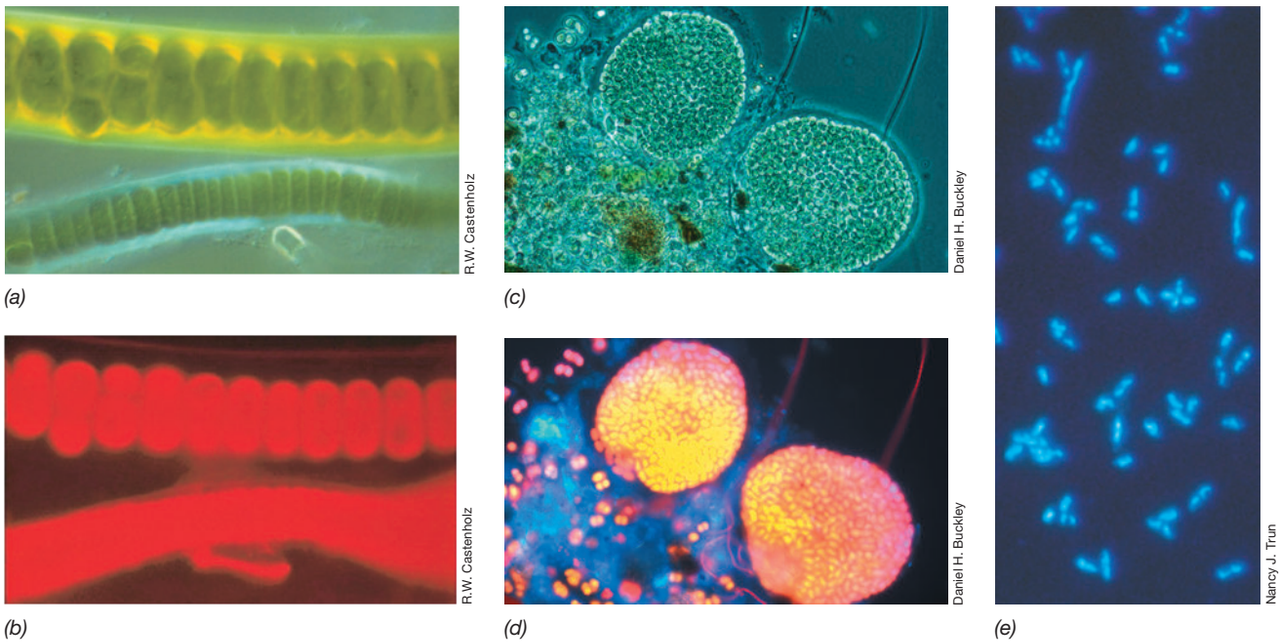


Figure 1.25 **Fluorescence microscopy.** *(a, b, c, d)* Cyanobacteria. The same cells are observed in *a* and *b*, and likewise in *c* and *d*. The top photos are taken with phase-contrast microscopy and the bottom photos with fluorescence microscopy. The cells fluoresce because they contain chlorophyll *a* and other pigments. The image in *b* was generated using a filter specific for the fluorescence of chlorophyll *a*, while the image in *d* was generated using a permissive filter that shows fluorescence from a range of pigments that occur naturally in cyanobacteria. *(e)* Fluorescence photomicrograph of cells of *Escherichia coli* made fluorescent by staining with the fluorescent dye DAPI, which binds to DNA.

or because they have been stained with a fluorescent dye (Figure 1.25*e*). DAPI (4′,6-*dia*midino-2-*p*heny*l*indole) is a widely used fluorescent dye that stains cells bright blue because it complexes with the cell's DNA (Figure 1.25*e*). DAPI can be used to visualize cells in their natural habitats, such as soil, water, food, or a clinical specimen. Fluorescence microscopy using DAPI is widely used in clinical diagnostic microbiology and also in microbial ecology for enumerating bacteria in a natural environment or in a cell suspension (Figure 1.25*e*).

Check Your Understanding

- What color will a gram-negative cell be after Gram staining by the conventional method?
- What major advantage does phase-contrast microscopy have over staining?
- How can cells be made to fluoresce?

1.9 Imaging Cells in Three Dimensions

Thus far we have only considered forms of microscopy in which the rendered images are two-dimensional. Two methods of light microscopy can render a more three-dimensional image, and in this section we explore these forms of microscopy.

Differential Interference Contrast Microscopy

Differential interference contrast (DIC) microscopy is a form of light microscopy that employs a polarizer in the condenser to produce polarized light (light in a single plane). The polarized light then passes through a prism that generates two distinct beams. These beams pass through the specimen and enter the objective lens, where they are recombined into one. Because the two beams pass through substances that differ in refractive index, the combined beams are not totally in phase but instead interfere with each other. This optical effect provides a three-dimensional perspective, which enhances subtle differences in cell structure.

Using DIC microscopy, cellular structures such as the nucleus of eukaryotic cells (**Figure 1.26**), or various types of inclusions present in some bacterial cells, appear more three-dimensional than in

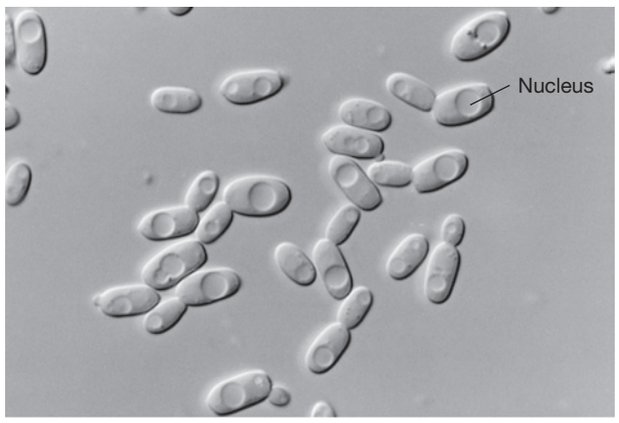


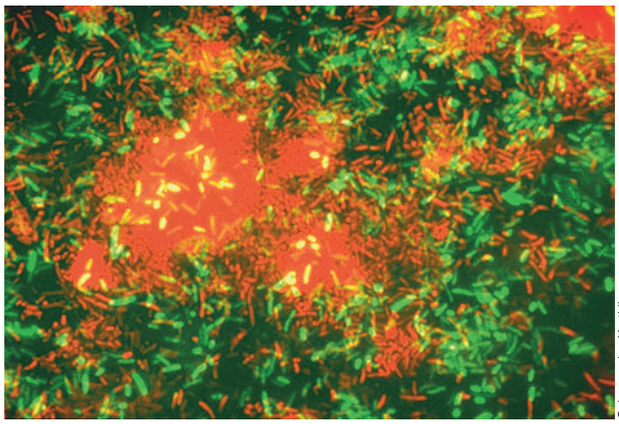
Figure 1.26 **Differential interference contrast microscopy.** The yeast cells are about 8 μm wide. Note the clearly visible nucleus and compare to the bright-field image of yeast cells in Figure 1.24*a*.

other forms of light microscopy. DIC microscopy is typically used on unstained cells as it can reveal internal cell structures that are nearly invisible by bright-field microscopy without the need for staining (compare Figure 1.24*a* with Figure 1.26).

Confocal Scanning Laser Microscopy

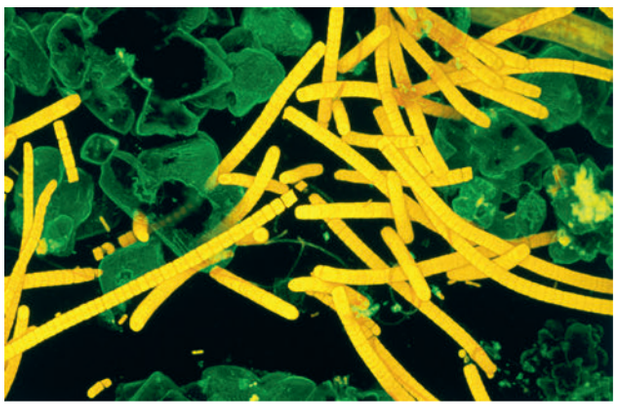
A confocal scanning laser microscope (CSLM) is a computer-controlled microscope that couples a laser to a fluorescence microscope. The laser generates a high-contrast, three-dimensional image and allows the viewer to access several planes of focus in the specimen (**Figure 1.27**). The laser beam is precisely adjusted such that only a particular layer within a specimen is in perfect focus at one time. Cells struck by the laser fluoresce to generate the image as in fluorescence microscopy (Section 1.8).

Cells in CSLM preparations can also be stained with fluorescent dyes to make them more distinct (Figure 1.27*a*). The laser then scans up and down through the layers of the sample, generating an image for each layer. A computer assembles the images to compose the many layers into a single high-resolution, three-dimensional image. Thus, for a relatively thick specimen (such as a bacterial biofilm, Figure 1.27*a*), not only can cells on the surface of the biofilm be observed, as with conventional light microscopy, but cells in the various layers are also observed by adjusting the plane of focus of the laser beam. CSLM is particularly useful when thick specimens need to be examined.



Subramanian Karthikeyan

(a)



Gernot Arp and Christian Boeker, Carl Zeiss, Jena

(b)

Figure 1.27 Confocal scanning laser microscopy. *(a)* Confocal image of a microbial biofilm community. The green, rod-shaped cells are *Pseudomonas aeruginosa* experimentally introduced into the biofilm. Cells of different colors are present at different depths in the biofilm. *(b)* Confocal image of a filamentous cyanobacterium growing in a soda lake. Cells are about 5 μm wide.

Check Your Understanding

- What structure in eukaryotic cells is more easily seen in DIC than in bright-field microscopy? (*Hint:* Compare Figures 1.24*a* and 1.26).
- Why is CSLM able to show different layers in a thick preparation while bright-field microscopy cannot?

1.10 Probing Cell Structure: Electron Microscopy

Electron microscopes use electrons instead of visible light (photons) to image cells and cell structures. In the electron microscope, electromagnets function as lenses, and the whole system operates in a vacuum (**Figure 1.28**). Electron microscopes are fitted with cameras to allow a photograph, called an *electron micrograph*, to be taken. Two types of electron microscopy are in routine use in microbiology: transmission and scanning.

Transmission Electron Microscopy

The *transmission electron microscope* (TEM) is used to examine cells and cell structure at very high magnification and resolution. The resolving power of a TEM is much greater than that of the light

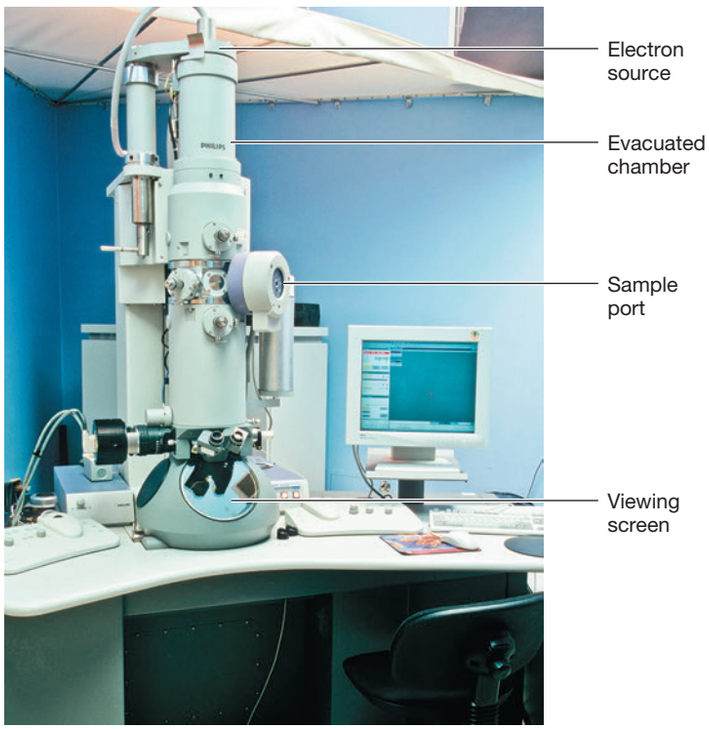
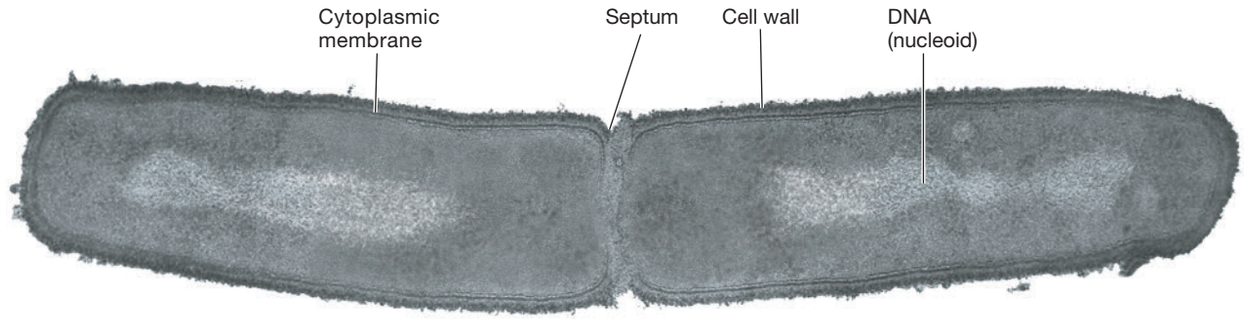
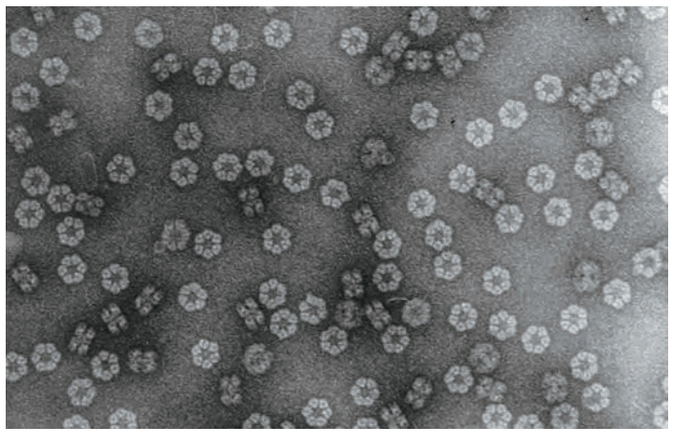


Figure 1.28 The electron microscope. This instrument encompasses both transmission and scanning electron microscope functions.



(a)



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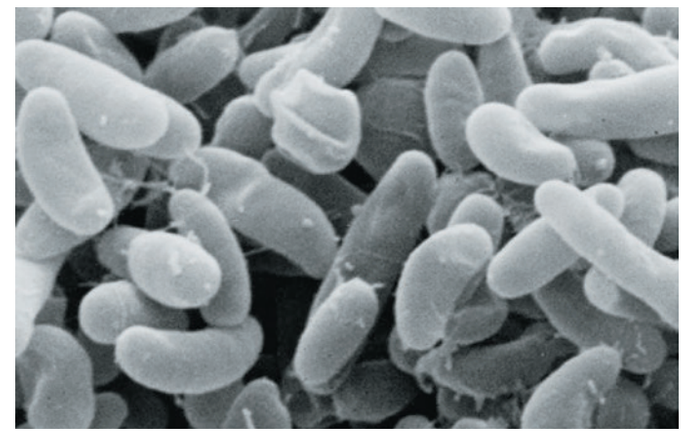


(c)

Figure 1.29 **Electron micrographs.** *(a)* Micrograph of a thin section of a dividing bacterial cell, taken by transmission electron microscopy (TEM). The cell is about 0.8 μm wide. *(b)* TEM of negatively stained molecules of hemoglobin. Each hexagonal-shaped molecule is about 25 nanometers (nm) in diameter and consists of two doughnut-shaped rings, a total of 15 nm thick. *(c)* Scanning electron micrograph (SEM) of bacterial cells. A single cell is about 0.75 μm wide.

microscope, even allowing one to view structures at the molecular level (**Figure 1.29**). This is because the wavelength of electrons is much shorter than the wavelength of visible light, and, as we have learned, wavelength affects resolution (Section 1.7). For example, whereas the resolving power of a light microscope is about 0.2 *micrometer*, the resolving power of a TEM is about 0.2 *nanometer*, a thousandfold improvement. With such powerful resolution, objects as small as individual protein and nucleic acid molecules can be visualized by transmission electron microscopy (Figure 1.29*b*).

Unlike photons, electrons are very poor at penetrating; even a single cell is too thick to penetrate with an electron beam. Consequently, to view the internal structure of a cell, *thin sections* of the cell are needed, and the sections must be stabilized and stained with various chemicals to make them visible. A single bacterial cell, for instance, is cut into extremely thin (20- to 60-nm) slices, which are then examined individually by TEM (Figure 1.29*a*). To obtain sufficient contrast, the sections are treated with stains such as osmic acid, or permanganate, uranium, lanthanum, or lead salts. Because these substances are composed of atoms of high atomic weight, they scatter electrons well and thus improve contrast. If only the *external* features of an organism are to be observed, thin sections are unnecessary. Intact cells or cell components can be observed directly in the TEM by a technique called *negative staining* (Figure 1.29*b*).

Electron cryotomography (cryoET) is an imaging technique in which TEM is used to obtain three-dimensional images. In cryoET, samples are prepared by rapid freezing to very low temperatures so that they are immobilized in noncrystalline vitreous ice. They are then imaged at very low temperatures ($<-150°C$), thereby preserving cell structures in their native states. Finally, samples are tilted as they are being imaged and this series of tilted images is assembled computationally to generate a three-dimensional image of the interior of the cell with a resolution of 4 nm.

Scanning Electron Microscopy

For optimal three-dimensional imaging of cells, a *scanning electron microscope* (SEM) is used. In scanning electron microscopy, the specimen is coated with a thin film of a heavy metal, typically gold. An electron beam then scans back and forth across the specimen. Electrons scattered from the metal coating are collected and projected on a monitor to produce an image (Figure 1.29*c*). In the SEM, even fairly large specimens can be observed, and the depth of field (the portion of the image that remains in sharp focus) is extremely good. A wide range of magnifications can be obtained with the SEM, from as low as 15× up to about 100,000×, but only the *surface* of an object is typically visualized.

Electron micrographs taken by either TEM or SEM are black-and-white images. Although the original image contains the maximum amount of scientific information that is available, color is often added to scanning electron micrographs by manipulating them in a computer. However, such false color does not improve resolution of a micrograph. In this text, false color will be used sparingly in electron micrographs so as to present the micrographs in their original scientific context.

Check Your Understanding

- What is an electron micrograph? Why do electron micrographs have greater resolution than light micrographs?
- What type of electron microscope would be used to view a cluster of cells? What type would be used to observe internal cell structure?

III • Microbial Cultivation Expands the Horizon of Microbiology

Microbes can be cultured in the laboratory, and microbial cultures have played a major role in unraveling the metabolic diversity and medical importance of the microbial world.

Following the discovery of microorganisms through microscopic methods, advances in microbial cultivation fueled major discoveries in microbiology. Important advances included the development of **aseptic technique**, a collection of practices that allow for the preparation and maintenance of **sterile** (that is, without the presence of living organisms) nutrient media and solutions (Chapter 4). Aseptic technique is essential for the isolation and maintenance of pure cultures of bacteria. **Pure cultures** are those that contain cells from only a single type of microorganism and are of great value for the study of microorganisms. Finally, **enrichment culture** techniques, which allow for the isolation from nature of microbes having particular metabolic characteristics, facilitate the discovery of diverse microorganisms.

Advances in microbial cultivation are directly responsible for success in fighting infectious disease, the discovery of microbial diversity, and the use of microbes as model systems to discover the fundamental properties of all living cells. Important advances in microbial cultivation occurred in the nineteenth century as microbiologists sought to answer two major questions of that time: (1) Does spontaneous generation occur? (2) What is the nature of infectious disease? Answers to these seminal questions emerged from the work of two giants in the field of microbiology: the French chemist Louis Pasteur and the German physician Robert Koch. We begin with the work of Pasteur.

1.11 Pasteur and Spontaneous Generation

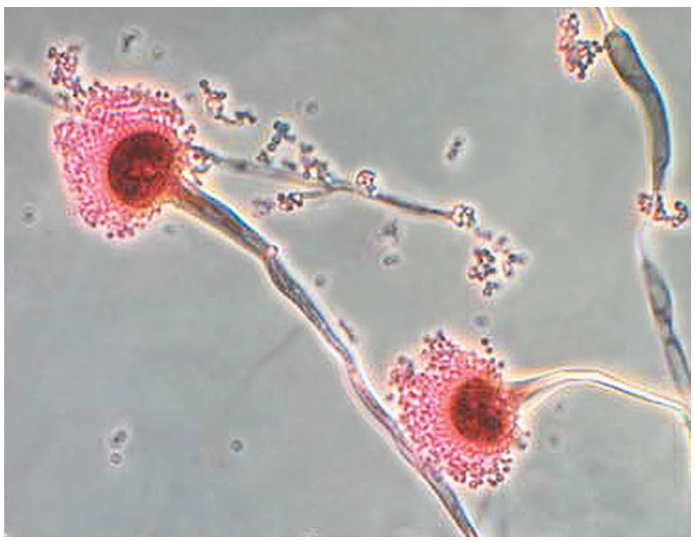
Pasteur was a chemist by training and was one of the first to recognize that many of what were thought to be strictly chemical reactions were actually catalyzed by microorganisms. Pasteur studied the chemistry of crystal formation and he used microscopes to examine crystal structure. His training in chemistry and microscopy prepared him to make a series of foundational discoveries to further the science of microbiology.

The Microbial Basis of Fermentation

Early in his career, Pasteur studied crystals formed during the production of alcohol. Through careful microscopic observation of tartaric acid crystals formed in wine, he observed two types of crystals that had mirror-image structures. He separated these by hand and observed that each type of crystal bent a beam of polarized light in a different direction. In this way he discovered that chemically identical substances can have *optical isomers*, which have different molecular structures that can influence their properties. Pasteur went on to discover that microorganisms could discriminate between optical isomers. For example, cultures of the mold *Aspergillus* (Figure 1.30) metabolized exclusively D-tartrate but not its optical isomer, L-tartrate. The fact that a living organism could discriminate between optical isomers led Pasteur to strongly suspect that many reactions previously thought to be abiotic were actually catalyzed by microbes.

While a professor of chemistry, Pasteur encountered a local businessman who produced alcohol industrially from beet juice. The businessman was losing money because many of his vats produced, instead of alcohol, a product that smelled like sour milk, which Pasteur determined to be lactic acid. In the mid-nineteenth century the production of alcohol was thought to be solely a chemical process. Pasteur studied the broth with his microscope, but instead of crystals he observed cells. Pasteur observed that the vats that produced alcohol were full of yeast, but the sour vats were full of rod-shaped bacteria. He hypothesized that these were living organisms whose growth produced either alcohol or lactic acid.

Pasteur needed to grow these organisms to prove his hypothesis. He prepared a hot-water extract of yeast cells, deducing that this would contain all of the nutrients that yeast need to grow. He then used a porcelain filter to remove all cells from this yeast extract



CDC/PHIL

Figure 1.30 Louis Pasteur and his observation of isomeric discrimination by *Aspergillus*. Light micrograph of cells of the mold *Aspergillus*.

nutrient medium, rendering it sterile. If he introduced living yeast back into this sterile yeast extract medium, he could observe their growth and show the production of alcohol, but if he instead introduced the small rods, he then observed lactic acid formation. Heating of these cultures eliminated growth *and* the production of either alcohol or lactic acid. In this way he proved that fermentation is carried out by microorganisms and that different microorganisms perform different fermentation reactions.

During his work on fermentation, Pasteur observed that other organisms would often grow in his yeast extract medium. He deduced that these organisms were being introduced from the air. Pasteur's work on fermentation had prepared him to conduct a series of classic experiments on spontaneous generation, experiments that are forever linked to his name and which helped establish microbiology as a modern science.

Spontaneous Generation

The concept of **spontaneous generation** existed for thousands of years and its basic tenet can be easily grasped. If food or some other perishable material is allowed to stand for some time, it putrefies. When examined microscopically, the putrefied material is teeming with microorganisms. From where do these organisms arise? Prior to Pasteur it was common belief that life arose spontaneously from nonliving materials, that is, by *spontaneous generation*.

Mastering Microbiology
Art Activity: Figure 1.26 Pasteur's swan-necked flask experiments

Pasteur became a powerful opponent of spontaneous generation. He predicted that microorganisms in putrefying materials were descendants of cells that entered from the air or cells that had initially been present on the decaying materials. Pasteur reasoned that if food were treated in such a way as to destroy all living organisms present—that is, if it were rendered sterile—and if it were kept sterile, it would not putrefy.

Pasteur used heat to kill contaminating microorganisms, and he found that extensive heating of a nutrient solution followed by sealing kept it from putrefying. Proponents of spontaneous generation criticized these experiments by declaring that "fresh air" was necessary for the phenomenon to occur. In 1864 Pasteur countered this objection simply and brilliantly by constructing a swan-necked flask, now called a *Pasteur flask* (**Figure 1.31**). In such a flask, nutrient solutions could be heated to boiling and sterilized. After the flask cooled, air could reenter, but the bend in the neck prevented particulate matter (including microorganisms) from entering the nutrient solution and initiating putrefaction. Nutrient solutions in such flasks remained sterile indefinitely. Microbial growth was observed only after particulate matter from the neck of the flask was allowed to enter the liquid in the flask (Figure 1.31*c*). This experiment settled the spontaneous generation controversy forever.

Pasteur's work on spontaneous generation demonstrated the importance of sterilization and led to the development of effective sterilization procedures that were eventually standardized and applied widely in microbiology, medicine, and industry. For example, the British physician Joseph Lister (1827–1912) deduced from Pasteur's discoveries that surgical infections were caused by microorganisms. He implemented a range of techniques designed to kill microorganisms and to prevent microbial infection of surgical patients. Lister is credited with the introduction of aseptic techniques for surgeries (1867), and his methods were adopted worldwide; these greatly reduced postoperative infections and greatly improved the survival rate of surgical patients. The food industry

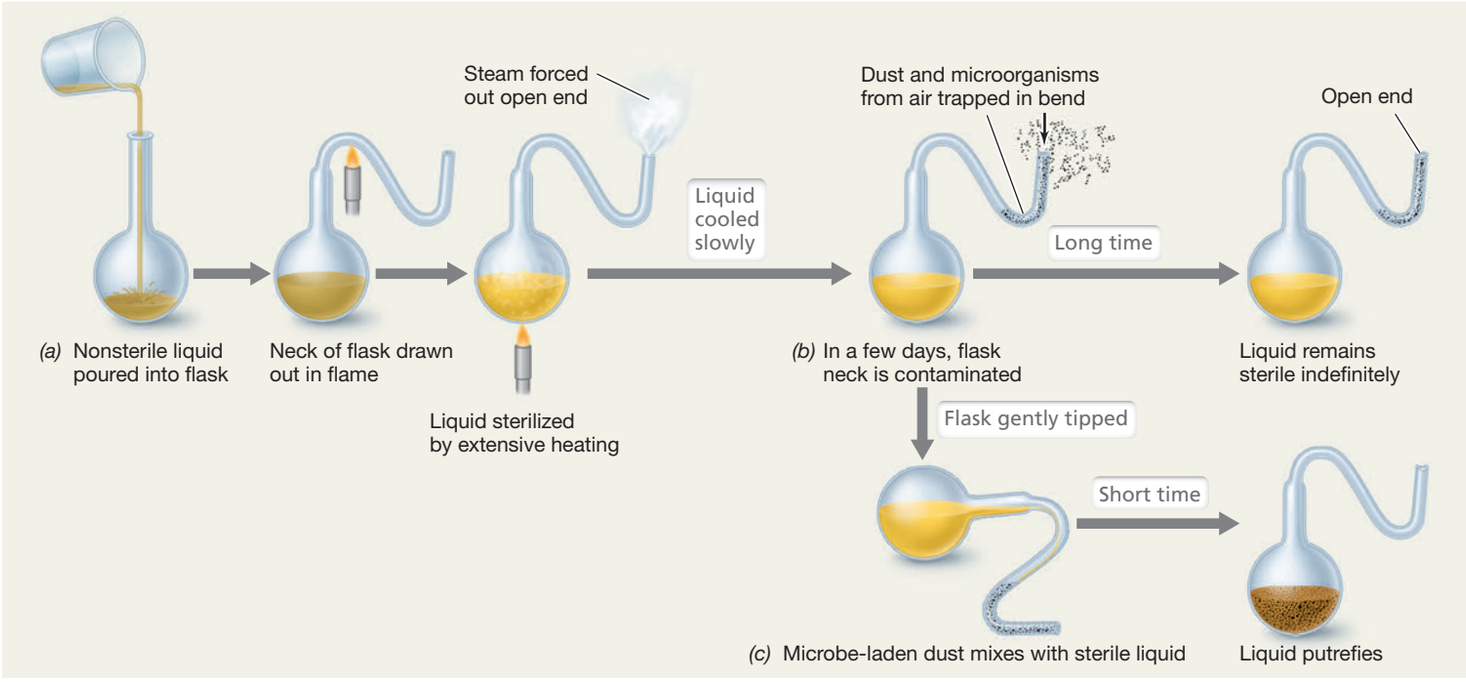


Figure 1.31 The defeat of spontaneous generation: Pasteur's swan-necked flask experiment. In *(c)* the liquid putrefies because microorganisms enter with the dust. The bend in the flask allowed air to enter (a key objection to Pasteur's sealed flasks) but prevented microorganisms from entering.

also benefited from the work of Pasteur, as his principles were quickly adapted for the preservation of milk and many other foods by heat treatment, which we now call *pasteurization*.

Other Major Accomplishments of Pasteur

Pasteur went on to many other triumphs in microbiology and medicine. Some highlights include his development of vaccines for the diseases anthrax, fowl cholera, and rabies. Pasteur's work on rabies was his most famous success, culminating in July 1885 with the first administration of a rabies vaccine to a human, a young French boy named Joseph Meister who had been bitten by a rabid dog. In those days, a bite from a rabid animal was invariably fatal. News spread quickly of the success of Meister's vaccination, and of one administered shortly thereafter to a young shepherd boy, Jean-Baptiste Jupille (**Figure 1.32*a***). Within a year several thousand people bitten by rabid animals had traveled to Paris to be treated with Pasteur's rabies vaccine.

Pasteur's fame was legendary and led the French government to establish the Pasteur Institute in Paris in 1888 (Figure 1.32*b*).

(a)

M.T. Madigan

(b)

Figure 1.32 Louis Pasteur and some symbols of his contributions to microbiology. *(a)* A French 5-franc note honoring Pasteur. The shepherd boy Jean-Baptiste Jupille is shown killing a rabid dog that had attacked children. Pasteur's rabies vaccine saved Jupille's life. In France, the franc preceded the euro as a currency. *(b)* Part of the Pasteur Institute, Paris, France. Today this structure, built for Pasteur by the French government, houses a museum that displays some of the original swan-necked flasks used in his experiments and a chapel containing Pasteur's crypt.

Originally established as a clinical center for the treatment of rabies and other contagious diseases, the Pasteur Institute today is a major biomedical research center focused on antiserum and vaccine research and production. The medical and veterinary breakthroughs of Pasteur not only were highly significant in their own right but helped solidify the concept of the germ theory of disease, whose principles were being developed at about the same time by a second giant of this era, Robert Koch.

Check Your Understanding

- Define the term sterile. What two methods did Pasteur use to make solutions sterile?
- How did Pasteur's experiments using swan-necked flasks defeat the theory of spontaneous generation?
- Besides ending the controversy over spontaneous generation, what other accomplishments do we credit to Pasteur?

1.12 Koch, Infectious Diseases, and Pure Cultures

Proof that some microorganisms can cause disease provided the greatest impetus for the development of microbiology as an independent biological science. As early as the sixteenth century it was suspected that some agent of disease could be transmitted from a diseased person to a healthy person. After microorganisms were discovered, a number of individuals proposed that they caused infectious diseases, but skepticism prevailed, and definitive proof was lacking. As early as 1847, the Hungarian physician Ignaz Semmelweis promoted sanitary methods including hand washing as a method for preventing infections. His methods are credited with saving many lives, but he could not prove why these methods worked and his advice was met with scorn by most of the medical community. The work of Pasteur and Lister provided strong evidence that microbes were the cause of infectious disease, but it was not until the work of the German physician Robert Koch (1843–1910) that the *germ theory* of infectious disease had direct experimental support.

The Germ Theory of Disease and Koch's Postulates

In his early work Koch studied anthrax, a disease of cattle and occasionally of humans. Anthrax is caused by the bacterium *Bacillus anthracis.* By careful microscopy and staining, Koch established that the bacteria were always present in the blood of an animal that was succumbing to the disease. However, Koch reasoned that the mere *association* of the bacterium with the disease was not actual proof of *cause and effect*, and he seized the opportunity to study cause and effect experimentally using anthrax and laboratory animals. The results of this study formed the standard by which infectious diseases have been studied ever since.

Koch used mice as experimental animals. Using appropriate controls, Koch demonstrated that when a small drop of blood from a mouse with anthrax was injected into a healthy mouse, the latter quickly developed anthrax. He took blood from this second animal, injected it into another, and again observed the characteristic disease symptoms. However, Koch carried this experiment a critically important step further. He discovered that the anthrax bacteria could be

Mastering Microbiology
Art Activity: Figure 1.29 Koch's Postulates for proving cause and effect in infectious diseases

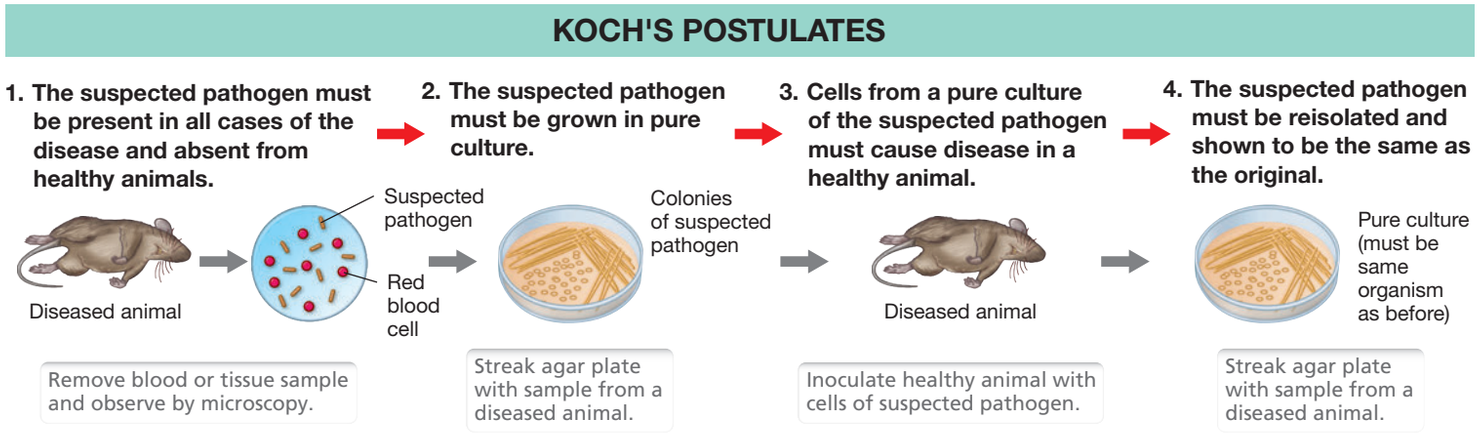


Figure 1.33 Koch's postulates for proving cause and effect in infectious diseases. Note that following isolation of a pure culture of the suspected pathogen, the cultured organism must both initiate the disease and be recovered from the diseased animal. Establishing the correct conditions for growing the pathogen is essential.

grown in a nutrient medium *outside the host* and that even after many transfers in laboratory culture, the bacteria still caused the disease when inoculated into a healthy animal.

On the basis of these experiments and others on the causative agent of tuberculosis, Koch formulated a set of rigorous criteria, now known as **Koch's postulates**, for definitively linking cause and effect in an infectious disease. Koch's postulates, summarized in **Figure 1.33**, stressed the importance of *laboratory culture* of the putative infectious agent followed by introduction of the suspected agent into virgin animals and recovery of the pathogen from diseased or dead animals. With these postulates as a guide, Koch, his students, and those that followed them discovered the causative agents of most of the important infectious diseases of humans and domestic animals. These discoveries also led to the development of successful treatments for the prevention and cure of many of these diseases, greatly improving the scientific basis of clinical medicine and human health and welfare (Figure 1.13).

Koch, Pure Cultures, and Microbial Taxonomy

The second of Koch's postulates states that the suspected pathogen must be isolated and grown away from other microorganisms in laboratory culture (Figure 1.33); in microbiology we say that such a culture is *pure*. To accomplish this important goal, Koch and his associates developed several simple but ingenious methods of obtaining and growing bacteria in pure culture, and many of these methods are still used today.

Koch started by using natural surfaces such as a potato slice to obtain pure cultures, but he quickly developed more reliable and reproducible growth media employing liquid nutrient solutions solidified with gelatin, and later with agar, an algal polysaccharide with excellent properties for this purpose. Along with his associate Walther Hesse, Koch observed that when a solid surface was incubated in air, masses of microbial cells called colonies developed, each having a characteristic shape and color (**Figure 1.34**). He inferred that each colony had arisen from a single bacterial cell that had grown to yield the mass of cells (see also Figure 1.3). Koch reasoned that each colony harbored a pure culture (a population of identical cells), and he quickly realized that solid media provided an easy way to obtain pure cultures. Richard Petri, another associate of Koch, developed the transparent double-sided "Petri dish" in 1887, and this quickly became the standard tool for obtaining pure cultures.

Koch was keenly aware of the implications his pure culture methods had for classifying microorganisms. He observed that colonies that differed in color and size (Figure 1.34) bred true and that cells from different colonies typically differed in size and shape and often in their nutrient requirements as well. Koch realized that these differences were analogous to the criteria taxonomists had established for the classification of larger organisms, such as plant and animal species, and he suggested that the different types of bacteria should be considered as "species, varieties, forms, or other suitable designation." Such insightful thinking was important for the rapid acceptance of microbiology as a new biological science, rooted as biology was in classification during Koch's era.

Koch and Tuberculosis

Koch's crowning scientific accomplishment was his discovery of the causative agent of tuberculosis. At the time Koch began this work (1881), one-seventh of all reported human deaths were caused by tuberculosis (Figure 1.13). There was a strong suspicion that

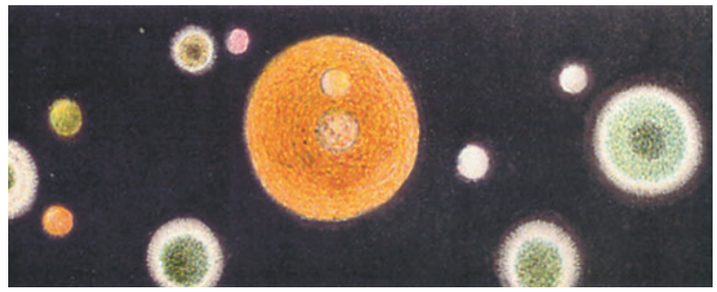


Figure 1.34 A hand-colored photograph taken by Walther Hesse of colonies formed on agar. The colonies include those of molds and bacteria obtained during Hesse's studies of the microbial content of air in Berlin, Germany, in 1882. From Hesse, W. 1884. "Ueber quantitative Bestimmung der in der Luft enthaltenen Mikroorganismen." *Mittheilungen aus dem Kaiserlichen Gesundheitsamte. 2*: 182–207.

tuberculosis was a contagious disease, but the suspected agent had never been seen, either in diseased tissues or in culture. Following his successful studies of anthrax, Koch set out to demonstrate the cause of tuberculosis, and to this end he brought together all of the methods he had so carefully developed in his previous studies with anthrax: microscopy, staining, pure culture isolation, and an animal model system (Figure 1.33).

The bacterium that causes tuberculosis, *Mycobacterium tuberculosis*, is very difficult to stain because *M. tuberculosis* cells contain large amounts of a waxlike lipid in their cell walls. Nevertheless, Koch devised a staining procedure for *M. tuberculosis* cells in lung tissue samples. Using this method, he observed the blue, rod-shaped cells of *M. tuberculosis* in tubercular tissues but not in healthy tissues (**Figure 1.35**). Obtaining cultures of *M. tuberculosis* was not easy, but eventually Koch succeeded in growing colonies of this organism on a solidified medium containing blood serum. Under the best of conditions, *M. tuberculosis* grows slowly in culture, but Koch's persistence and patience eventually led to pure cultures of this organism from human and animal sources.

From this point Koch used his postulates (Figure 1.33) to obtain definitive proof that the organism he had isolated was the cause of the disease tuberculosis. Guinea pigs can be readily infected with *M. tuberculosis* and eventually succumb to systemic tuberculosis. Koch showed that tuberculous guinea pigs contained masses of *M. tuberculosis* cells in their lungs and that pure cultures obtained from such animals transmitted the disease to healthy animals. In this way, Koch successfully satisfied all four of his postulates, and the cause of tuberculosis was understood. Koch announced his discovery of the cause of tuberculosis in 1882, and for this accomplishment he was awarded the 1905 Nobel Prize for Physiology or Medicine. Koch had many other triumphs in the growing field of infectious diseases, including the discovery of the causative agent of cholera (the bacterium *Vibrio cholerae*) and the development of methods to diagnose infection with *M. tuberculosis* (the tuberculin skin test).

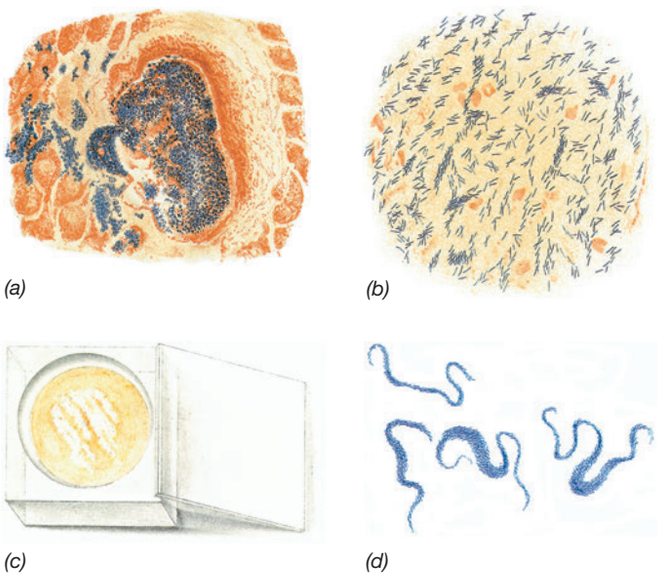


Figure 1.35 **Robert Koch's drawings of *Mycobacterium tuberculosis*.** *(a)* Section through infected lung tissue showing cells of *M. tuberculosis* (blue). *(b) M. tuberculosis* cells in a sputum sample from a tubercular patient. *(c)* Growth of *M. tuberculosis* on a glass plate of coagulated blood serum stored inside a glass box to prevent contamination. *(d) M. tuberculosis* cells taken from the plate in *c* and observed microscopically; cells appear as long, cordlike forms (▶ Section 16.11 and Figures 16.32 and 16.33). Original drawings from Koch, R. 1884. "Die Aetiologie der Tuberkulose." *Mittheilungen aus dem Kaiserlichen Gesundheitsamte 2*: 1–88.

Check Your Understanding

- How do Koch's postulates ensure that cause and effect of a given disease are clearly differentiated?
- What advantages do solid media offer for the isolation of microorganisms?
- What is a pure culture?

1.13 Discovery of Microbial Diversity

As microbiology entered the twentieth century, its initial focus on basic principles, methods, and medical aspects broadened to include studies of the microbial diversity of soil and water and the metabolic processes that microorganisms carried out in these habitats. Major contributors of this era included the Dutchman Martinus Beijerinck and the Russian Sergei Winogradsky.

Sergei Winogradsky and Chemolithotrophy

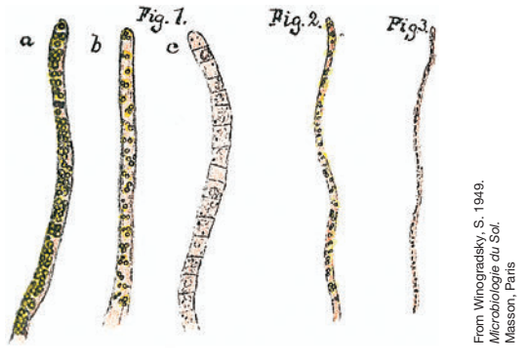
Sergei Winogradsky (1856–1953) was interested in the bacterial diversity of soils and waters and was highly successful in isolating several notable bacteria from natural samples. Winogradsky was particularly interested in bacteria that cycle nitrogen and sulfur compounds, such as the nitrifying bacteria and the sulfur bacteria (Chapters 14 and 15). He studied *Beggiatoa*, which are large sulfur-oxidizing bacteria found in marine sediments. *Beggiatoa* are morphologically distinctive and readily identified under the microscope (**Figure 1.36**), but Winogradsky found that they would not grow on the rich nutrient media used by Koch and Pasteur. In order to grow these *Beggiatoa*, Winogradsky designed a medium that chemically imitated the environment in which *Beggiatoa* lived. He showed that *Beggiatoa* are able to grow in the absence of organic nutrients, and that their growth requires only inorganic substances (compounds lacking carbon–carbon bonds). In this way, Winogradsky was the first to define **chemolithotrophy**, which is any metabolic process in which energy for growth is produced using only *inorganic* chemical compounds. Winogradsky also revealed that these chemolithotrophic bacteria obtain their carbon from CO_2, much like plants, though they get their energy from chemical reactions rather than from light. Winogradsky further showed that these organisms, which he called *lithotrophs* (meaning, literally, "stone eaters"), are widespread in nature.

Martinus Beijerinck, the Enrichment Culture Technique, and Nitrogen Fixation

Martinus Beijerinck (1851–1931) was a professor at the Delft Polytechnic School in Holland and a contemporary of Winogradsky. Beijerinck's greatest contribution to the field of microbiology was his clear formulation of the *enrichment culture technique*. Enrichment cultures were used by both Beijerinck and Winogradsky to discover many unique forms of metabolism that we now know to be essential to nutrient cycling in nature.

From *Microbiologie du Sol*, used with permission

(a)



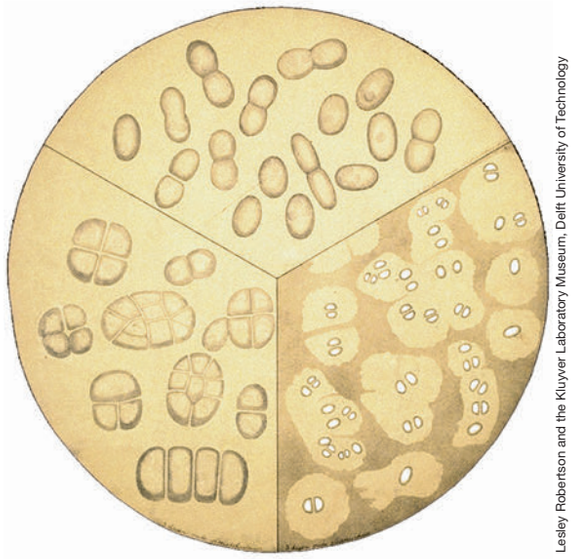
From Winogradsky, S. 1949. *Microbiologie du Sol.* Masson, Paris

(b)

Figure 1.36 Sulfur bacteria. The original drawings were made by Sergei Winogradsky in the late 1880s and then copied and hand-colored by his wife Hèléne. *(a)* Purple sulfur phototrophic bacteria. Figures 3 and 4 show cells of *Chromatium okenii* (compare with photomicrographs of *C. okenii* in Figures 1.1*a* and 1.11*d*, and 1.21*a*). *(b) Beggiatoa*, a sulfur chemolithotroph (compare with ▶ Figure 15.32*a*).

The growth media used by Pasteur and Koch (for example, yeast extract media) were rich in a wide variety of nutrients. These media support the growth of many microorganisms and are often suitable for growing pathogenic bacteria, but as Winogradsky discovered, most bacteria found in nature do not grow well on such media. Enrichment culture techniques are used to selectively encourage the growth of *specific* microorganisms. To do this, enrichment cultures employ culture media and selective incubation conditions that are deliberately designed to favor only certain types of microbes.

For example, Winogradsky used enrichment culture to isolate the first nitrogen-fixing bacterium. He devised a liquid growth medium that lacked a source of nitrogen, inoculated this medium with soil, and incubated it in the presence of air. He knew that all living things require nitrogen, and so when he observed bacteria growing in the medium he knew that they must be getting their nitrogen from N_2 in the air. In this way he enriched for and ultimately isolated the anaerobic nitrogen-fixing bacterium *Clostridium pasteurianum*, becoming the first to demonstrate the process of nitrogen fixation. The growth of nitrogen-fixing bacteria was favored in the enrichment culture because only those bacteria that can use nitrogen from the air can grow on media that lack a nitrogen source such as ammonia or nitrate. Beijerinck used a similar technique shortly thereafter to isolate the first aerobic nitrogen-fixing bacterium, *Azotobacter* (**Figure 1.37**). Beijerinck also devised a medium to isolate rhizobia and proved that these bacteria cause root nodules to form on legumes and that they perform nitrogen fixation within the nodules (Figure 1.14).



Lesley Robertson and the Kluyver Laboratory Museum, Delft University of Technology

Figure 1.37 Martinus Beijerinck and *Azotobacter*. The first aerobic nitrogen-fixing bacterium, *Azotobacter chroococcum*, was isolated by M. Beijerinck in 1900. The image is a painting by M. Beijerinck's sister, Henriëtte Beijerinck, showing cells of *Azotobacter chroococcum*. Beijerinck used such paintings to illustrate his lectures. Compare these drawings of *A. chroococcum* cells with the photomicrograph of cells of *Azotobacter* in Figure 15.24.

Using the enrichment culture technique, Beijerinck and Winogradsky isolated the first pure cultures of many important soil and aquatic microorganisms. For example, Winogradsky isolated the first nitrifying bacteria using an enrichment medium that contained ammonium salts and CO_2 since these chemolithotrophic bacteria oxidize ammonium as an energy source and are autotrophic. Beijerinck was the first to isolate sulfur-cycling bacteria such as sulfate-reducing bacteria and sulfur-oxidizing bacteria, fermentative bacteria such as the lactic acid bacteria, and many other physiological types of bacteria as well as microbial eukaryotes such as green algae.

Beijerinck was also the first person to observe a virus. While studying "mosaic disease" of tobacco, Beijerinck used selective filters to show that the infectious agent in this disease was smaller than any bacterium and that it somehow became incorporated into cells of the living host plant. In this insightful work, Beijerinck described not only the first virus, but also the basic principles of virology, which we expand upon in Chapters 5 and 11.

Check Your Understanding

- What is meant by the term "enrichment culture"?
- What is meant by the term "chemolithotrophy"? In what way are chemolithotrophs like plants?

IV • Molecular Biology and the Unity and Diversity of Life

Molecular analyses of microbial cells have unveiled the biochemical principles that govern all life and the evolutionary position of microbes in the tree of life.

The development of aseptic technique and methods for the enrichment, isolation, and propagation of bacteria at the end of the nineteenth century gave rise to explosive growth in the pace of microbiological discovery. Moreover, microbiologists realized that the ability to grow bacteria rapidly and in controlled laboratory conditions made them excellent model systems for exploring the fundamental nature of life.

1.14 Molecular Basis of Life

Experiments with bacterial cultures in the twentieth century were critical in forming the foundations of molecular biology, molecular genetics, and biochemistry. Microbiologists came to realize that while microorganisms were incredibly diverse, all cells appeared to operate on similar basic principles. Thus, the search was on for the overarching molecular processes that define life itself.

Unity in Biochemistry

Albert Jan Kluyver (1888–1956) was Beijerinck's successor at what was then called the Delft Institute of Technology. Kluyver recognized that though microbial diversity was tremendous, all microorganisms used many of the same biochemical pathways and their metabolic processes were governed by similar thermodynamic constraints. Kluyver promoted the study of comparative biochemistry to identify the unifying features of all cells. He famously proclaimed, "From elephant to butyric acid bacterium—it is all the same!" This was later reformulated by Jacques Monod (1910–1976) into the expression, "What is true for *E. coli* is also true for the elephant," a statement that proclaimed the importance of working with bacteria to understand the fundamental principles that govern all living things.

The use of microbes as metabolic model systems led to the discovery that certain macromolecules and biochemical reactions are universal, and that to understand their function in one cell is to understand their function in all cells. We will learn about these fundamental macromolecules and biochemical processes in later chapters. These discoveries were of central importance to understanding microbial evolution, and none were more important than the discovery of DNA as the molecular basis of heredity, a discovery that is less than 80 years old.

Cracking the Code of Life

In the early twentieth century, it was clear that some molecule carried the hereditary information from parent to offspring, but the molecular basis of heredity remained a mystery. Most biologists thought that proteins carried this hereditary information. DNA had been discovered but it was thought to be merely a structural molecule, and too simple in its composition to encode cellular functions. The hunt for the molecular basis of heredity began in earnest with a key experiment by the British bacteriologist Frederick Griffith (1879–1941).

Griffith worked with a virulent strain of *Streptococcus pneumoniae*, a cause of bacterial pneumonia in both humans and mice. This strain, strain S, produced a polysaccharide coat (that is, a capsule, ▶ Section 2.6) that caused cells to form smooth colonies on agar media and conferred the ability to kill infected mice (**Figure 1.38*a***).

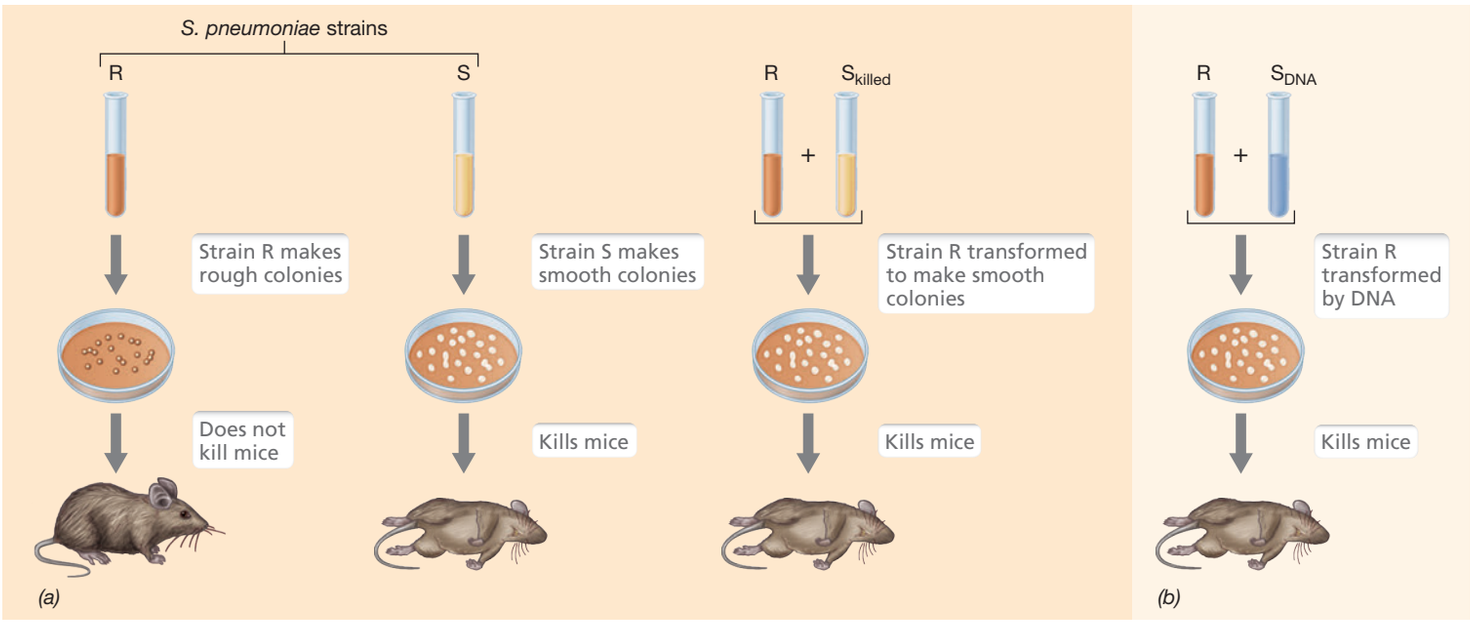


Figure 1.38 Early evidence that DNA is the molecular basis of heredity. *(a)* Griffith's experiment showed that bacteria can transfer genetic information. *Streptococcus pneumoniae* strain R makes rough colonies on agar media and does not kill mice, but strain S makes smooth colonies and does kill mice. Heat-killed cells of strain S do not cause disease, but if these killed cells are mixed with cells of strain R, then strain R is "transformed" to the S type and begins to make smooth colonies and kill mice. *(b)* The Avery–MacLeod–McCarty experiment showed that DNA contains genetic information. DNA isolated from strain S can transform strain R to cause disease, though the DNA itself does not cause disease. Degraded DNA (the *control* in the experiment) lacks the ability to transform strain R.

A related strain, strain R, lacked this polysaccharide and produced "rough" colonies that did not cause disease. However, Griffith observed that strain R could be *transformed* to type S, forming smooth colonies and causing disease, when it was mixed with the dead remains of cells of strain S (Figure 1.38*a*). He reasoned that some molecule that contained genetic information must have been transferred from strain R to strain S in this process, and this experiment showed that genetic transfer could be studied in bacteria.

Later, the Avery–MacLeod–McCarty experiment (1944), named for three scientists at the Rockefeller University, would show that this "transforming principle" was DNA. They treated the dead remains of cells of strain S with chemicals and enzymes that destroyed protein and left behind only DNA. They then repeated Griffith's experiment with the pure DNA of strain S and showed that this DNA was sufficient to cause transformation, causing strain R cells to become S-type cells and virulent (Figure 1.38*b*). They also demonstrated that transformation failed if the DNA from strain S was degraded. Collectively, these experiments proved that DNA is the genetic material of cells.

The discovery that DNA is the basis of heredity was followed by intense efforts to understand how this molecule stores genetic information. The structure of DNA was ultimately solved by James D. Watson (1928–) and Francis Crick (1916–2004) using X-ray diffraction images of DNA taken by their colleague Rosalind Franklin (1920–1958). They revealed that DNA is composed of a double helix that contains four nitrogenous bases: guanine, cytosine, adenine, and thymine, which form the genetic code (▶ Section 6.1). Later research would reveal how the genetic code is read from DNA and translated into protein, and these principles are covered in Chapter 6. Once again, however, this research to crack the code of life was enabled by a microbial model system, in this case, the bacterium *Escherichia coli*.

From DNA to Evolutionary Insight

Not long after the discovery that genetic information is encoded in the sequence of biological molecules, Emile Zuckerkandl (1922–2013) and Linus Pauling (1901–1994) proposed that molecular sequences could be used to reconstruct evolutionary relationships. They recognized that evolution, as described by Darwin, required variation in offspring and that these variations must be caused by changes in molecular sequences. They predicted that these sequence differences occur randomly in a clocklike fashion over time. This led to the conclusion that the evolutionary history of organisms is inscribed in the sequence of molecules such as DNA. Carl Woese seized upon these insights to pursue the ambitious goal of reconstructing the evolutionary history of all cells, and we explore the events that led up to this seminal discovery in evolutionary biology now.

Check Your Understanding

- Describe the experiments that proved DNA was the transforming principle described by Griffith.
- Why are microbes useful as model organisms to understand basic principles of biology?

1.15 Woese and the Tree of Life

Evolutionary relationships between microorganisms remained a mystery until it was discovered that certain molecular sequences maintain a record of evolutionary history. Here we will examine how the sequence of **ribosomal RNA (rRNA)** genes, present in all cells, revolutionized the understanding of microbial evolution and made it possible to construct the first universal tree of life. Ribosomal RNAs are components of the *ribosome*, the biosynthetic machine that all cells use to make proteins (Section 1.2).

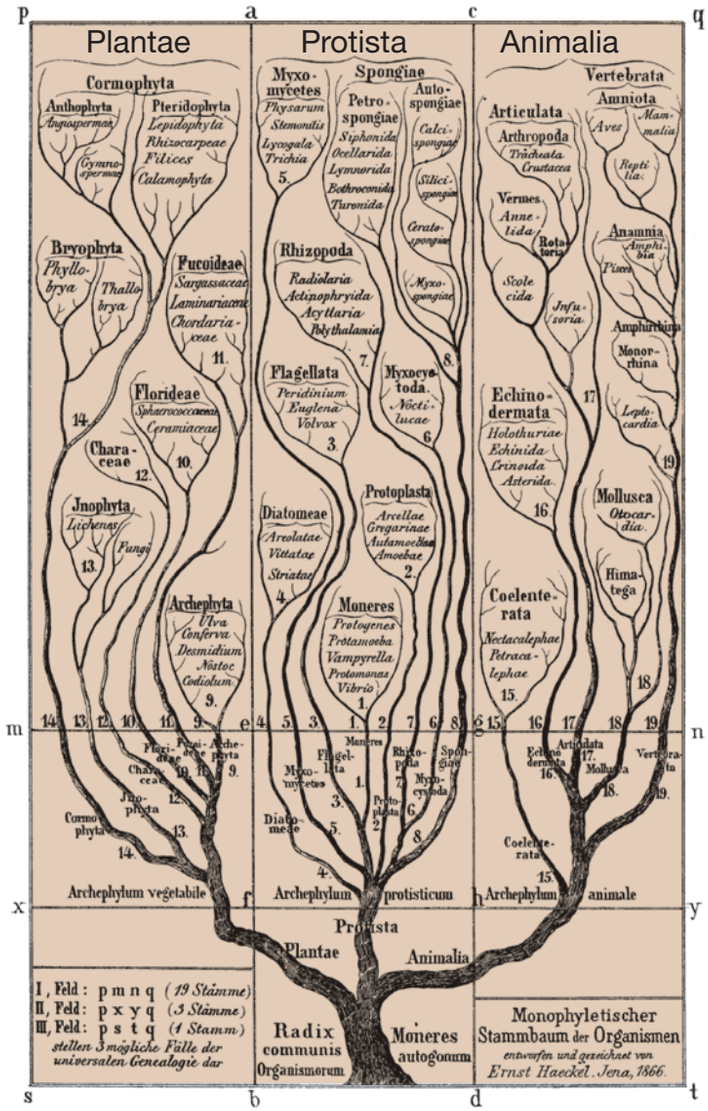
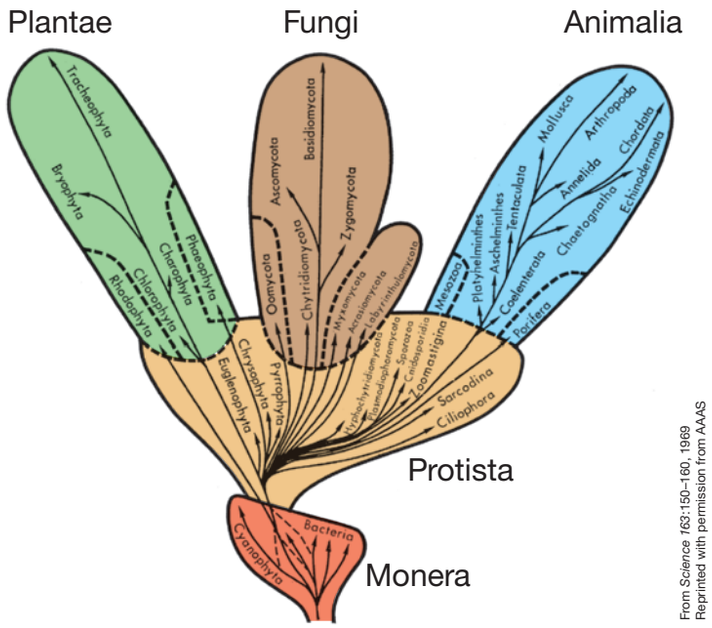


Figure 1.39 Early efforts to depict the universal tree of life. A conceptual tree of life proposed by Ernst Haeckel in *Generelle Morphologie der Organismen* (1866) shortly after Darwin published *On the Origin of Species* (1859). Haeckel proposed that four main groups of organisms (Plantae, Animalia, Protista, and Moneres) evolved from a common single-celled ancestor. The group that Haeckel proposed as Moneres included all microbes having prokaryotic cell structure, which we know today as *Bacteria* and *Archaea*.

Early Attempts to Portray the Evolutionary History of Life

For over a hundred years, following the 1859 publication of Charles Darwin's *On the Origin of Species*, evolutionary history was studied primarily with the tools of paleontology (through examining fossils) and comparative biology (through comparing the traits of living organisms). These approaches led to progress in understanding the evolution of plants and animals, but they were powerless to explain the evolution of microorganisms. The vast majority of microorganisms do not leave behind fossils, and their morphological and physiological traits provide few clues about their evolutionary history. Moreover, microorganisms do not share any morphological traits with plants and animals. Thus, it was impossible to create a robust evolutionary framework that included microorganisms.

The first attempt to depict the common evolutionary history of all living cells was published by Ernst Haeckel in 1866 (**Figure 1.39**). Haeckel correctly suggested that single-cell organisms, which he called *Monera* (labeled as Moneres near the root of the tree shown in Figure 1.39), were ancestral to other forms of life, but his scheme, which included plants, animals, and protists, did not attempt to resolve evolutionary relationships among microorganisms. The situation was little changed as late as 1969 when Robert Whittaker proposed a five-kingdom classification scheme (**Figure 1.40**). Whittaker's scheme distinguished the fungi as a distinct lineage, but it was still largely impossible to resolve evolutionary relationships among most microorganisms. Hence, our understanding of microbial evolution had made little progress since Haeckel's day.



From *Science* 163:150–160, 1969
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Figure 1.40 Later efforts to depict the universal tree of life. A conceptual tree of life published by Robert H. Whittaker (1969). Whittaker recognized the same groups as Haeckel (Figure 1.39), but he was the first to propose adding Fungi as a distinct kingdom. Compare this tree to Haeckel's tree and consider how little our knowledge of microbial evolution had changed between 1866 and 1969, prior to the development of nucleic acid sequencing.

Molecular Sequence Data Revolutionized Microbial Phylogeny

Everything changed after the structure of DNA was discovered and it was recognized that evolutionary history is recorded in DNA sequences. Carl Woese (1928–2012), a professor at the University of Illinois (USA), realized in the 1970s that the sequence of ribosomal RNA (rRNA) molecules and the genes that encode them could be used to infer evolutionary relationships between organisms. Woese recognized that genes encoding rRNAs were excellent candidates for phylogenetic analysis because they were (1) present in all cells, (2) functionally constant, (3) highly conserved (slowly changing) in their nucleic acid sequences, and (4) of adequate length to provide a deep view of evolutionary relationships.

Woese compared the sequences of rRNA molecules from many microorganisms. Among the first microbes he examined were methanogens. To his astonishment, he found that the rRNA sequences from methanogens were distinct from those of both *Bacteria* and *Eukarya*, the only two domains recognized at that time. He named this new group of prokaryotic cells the *Archaea* (originally *Archaebacteria*) and recognized them as the third domain of life alongside the *Bacteria* and the *Eukarya* (**Figure 1.41*b***). More importantly, Woese demonstrated that the analysis of rRNA gene sequences could be used to reveal evolutionary relationships between *all cells*, providing the first effective tool for the evolutionary classification of microorganisms.

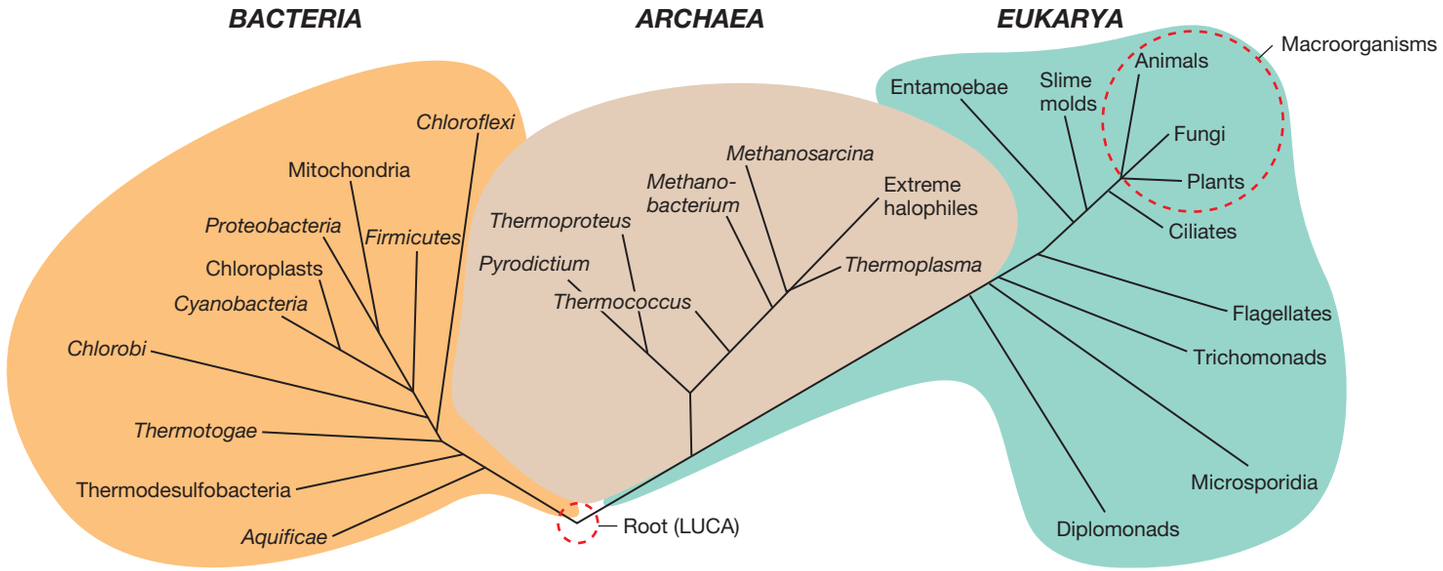
The Tree of Life Based on rRNA Gene Sequences

The universal tree of life based on rRNA gene sequences (Figure 1.41*b*) is a genealogy of all life on Earth. It is a true **phylogenetic tree**, a diagram that depicts the evolutionary history—the **phylogeny**—of all cells and clearly reveals the three domains. The root of the universal tree represents a point in time when all extant life on Earth shared a common ancestor, the last universal common ancestor, LUCA (Figures 1.10*b* and 1.41*b*). From the last universal common ancestor of all cells, evolution proceeded along two paths to form the domains *Bacteria* and *Archaea*. At some later time, the domain *Archaea* diverged to distinguish the *Eukarya* from the *Archaea* (Figures 1.10*b* and 1.41*b*). The three domains of cellular life are evolutionarily distinct and yet they share features indicative of their common descent from a universal cellular ancestor.

Revealing the Extent of Microbial Diversity

The tools Woese developed to build the tree of life were first used to determine the evolutionary history of microorganisms grown in pure culture (Figure 1.41*a*). However, Norman Pace (1942–), a professor at the University of Colorado (USA), realized that Woese's approach could be applied to rRNA molecules isolated *directly from the environment* as a way to probe the diversity of natural microbial communities without first cultivating their component organisms (Chapter 19). These *cultivation-independent* methods of rRNA gene analysis pioneered by Pace greatly improved our picture of microbial diversity (**Figure 1.42**). Despite many advances in the culturing of microorganisms from nature, many microbes have not yet been brought into laboratory culture. Hence, cultivation-independent methods provide us with valuable insights into the diversity and activities of microbes found in nature and oftentimes provide crucial hints for how they can be cultured. We also now know that although much progress has been made in describing microbial diversity, much of this diversity is yet to be explored and thus our work to describe the microbial world has only just begun.

(a)



(b)

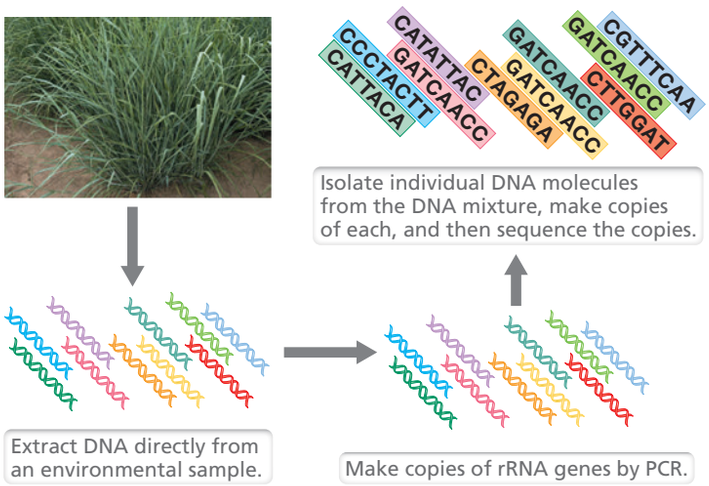
Figure 1.41 Evolutionary relationships and the phylogenetic tree of life. *(a)* The technology behind ribosomal RNA gene phylogenies. (1) DNA is extracted from cells. (2) Copies of the gene encoding rRNA are made by the polymerase chain reaction (PCR, a technique for making multiple copies of nucleic acid sequences, ▶ Section 12.1). (3) The gene is sequenced, and (4) the sequence is aligned and analyzed with sequences of the same gene from other organisms. A computer algorithm makes pairwise comparisons at each base and (5) generates a phylogenetic tree that depicts evolutionary relationships. In the example shown, the sequence differences are highlighted in yellow and are as follows: organism 1 versus organism 2, three differences; 1 versus 3, two differences; 2 versus 3, four differences. Thus organisms 1 and 3 are closer relatives than are 2 and 3 or 1 and 2. *(b)* The phylogenetic tree of life based on the analysis of rRNA gene sequences as proposed by Carl Woese (adapted from Woese, C.R., et al. *PNAS 87*: doi.org/10.1073/pnas.87.12.4576). The tree shows the three domains of organisms and a few representative groups in each domain. See detailed discussion of using molecular sequences as phylogenetic tools in Section 13.11.

Improvements in DNA sequencing technology have greatly improved our ability to study *Bacteria* and *Archaea* (Figure 1.42*b*). While the analysis of rRNA genes remains a cornerstone of microbial ecology, we now have the ability to sequence *entire microbial genomes* instead of just individual genes. Jo Handelsman (1959–), a professor at the University of Wisconsin (USA), was the first to propose *metagenomics*, a technique in which fragments of microbial genomes (or even entire genomes) can be recovered from a sample of environmental DNA. Metagenomics, the study of genomic information recovered directly from the environment, is currently providing us with profound insights on the evolution and diversity of life and new information on the metabolic potential of the microbial world.

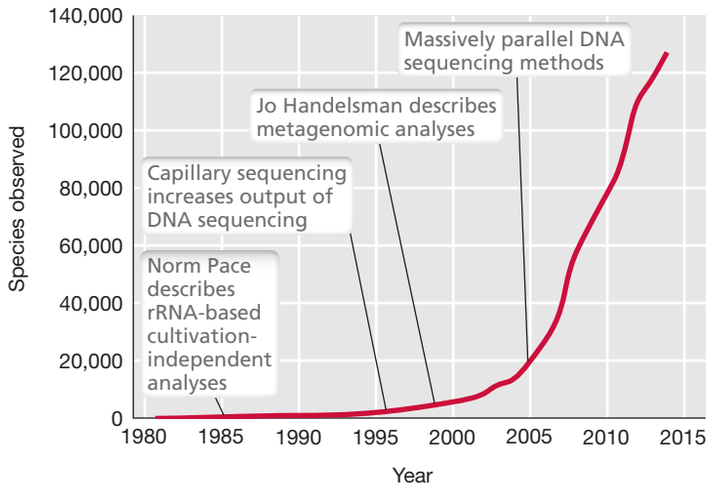
With an evolutionary framework of the microbial world in place, and with powerful new methods in hand to guide future research, advances in microbial diversity are happening quickly. Besides unveiling the previously hidden concept of three evolutionary domains of life, the contributions of Carl Woese and his associates have given microbiologists the tools they need to understand and explore the diversity of our microbial world. In the chapters that follow we will learn how microbiologists have unraveled many of the basic principles that govern living systems, how microorganisms control essential processes that sustain our biosphere, and how we can apply the lessons of microbiology to combat disease and improve our world.

Check Your Understanding

- What kinds of evidence support the three-domain concept of life?
- What is a phylogenetic tree?
- List three reasons why rRNA genes are suitable for phylogenetic analyses.



(a) **Cultivation-independent analysis of rRNA genes**



(b) **Revealing the extent of microbial diversity**

Figure 1.42 Analysis of environmental rRNA genes leads to discovery of new microbial species. *(a)* Norman Pace in 1985 described the first approach to sequence rRNA genes obtained directly from the environment without the need to grow microbes by cultivation in the laboratory. *(b)* Cultivation-independent analysis of microbial communities in environmental samples has revealed a tremendous diversity of microbial species. The application of cultivation-independent methods has been facilitated greatly by improvements in DNA sequencing technology over time. The graph shows the discovery over time of 16S rRNA gene sequences from natural samples that represent unique microbial species (data adapted from Schloss, P.D., et al. *mBio 6*: doi.org/10.1128/mBio.00201-16).

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CHAPTER REVIEW

I • Exploring the Microbial World

1.1 Microorganisms are single-celled microscopic organisms that are essential for the well-being and functioning of other life forms and the planet. The tools of microscopy, microbial cultivation, molecular biology, and genomics are cornerstones of modern microbiology.

Q What are bacterial colonies and how are they formed?

1.2 Prokaryotic and eukaryotic cells differ in cellular architecture, and an organism's characteristics are defined by its complement of genes—its genome. All cells have a cytoplasmic membrane, a cytoplasm, ribosomes, and a double-stranded DNA genome. All cells carry out activities including metabolism, growth, and evolution.

Q What cellular structures distinguish prokaryotic and eukaryotic cells? What are some differences between a cell wall and a cytoplasmic membrane? In what types of organisms would you expect to find these structures?

1.3 Prokaryotic cells, while typically smaller than eukaryotic cells, can range widely in size and morphology. The most common shapes of prokaryotic cells are rods, cocci, and spirilla, and while most bacteria are 0.5 to 10 μm in length, some can be more than 600 μm long.

Q How do diffusion and surface-to-volume (*S*/*V*) ratio influence the size and shape of prokaryotic cells?

1.4 *Bacteria*, *Archaea*, and *Eukarya* are the major phylogenetic lineages (domains) of cells. The greatest diversity of microorganisms is found in the *Bacteria*, while many extremophiles are found within the *Archaea*. Microbial eukaryotes can vary tremendously in size, with some species being smaller than bacteria. Viruses are acellular and because of this cannot be placed on the tree of life.

Q What features (or lack of features) can be used to distinguish between viruses, *Bacteria*, *Archaea*, and *Eukarya*?

1.5 Diverse microbial populations were widespread on Earth for billions of years before plants and animals appeared. Microbes are abundant in the biosphere, and their activities greatly affect the chemical and physical properties of their habitats.

Q Why can Earth, in many ways, be considered a microbial planet? Which event in Earth's history eventually lead to the evolution of multicellular life forms?

1.6 Microorganisms can be both beneficial and harmful to humans, although many more microorganisms are beneficial (or even essential) than are harmful. Agriculture, food, energy, and the environment are all affected in major ways by microorganisms.

Q The gut microbiome directly benefits humans by digesting complex carbohydrates and synthesizing vitamins and other nutrients. In what other ways do microorganisms benefit humans?

II • Microscopy and the Origins of Microbiology

1.7 Microscopes are essential for studying microorganisms. Bright-field microscopy, the most common form of microscopy, employs a microscope with a series of lenses to magnify and resolve the image. The limit of resolution for a light microscope is about 0.2 μm.

Q What is the difference between magnification and resolution? Can either increase without the other?

1.8 An inherent limitation of bright-field microscopy is the lack of contrast between cells and their surroundings. This problem can be overcome by the use of stains or by alternative forms of light microscopy, such as phase contrast or dark field.

Q What is the function of staining in light microscopy? What is the advantage of phase-contrast microscopy over bright-field microscopy?

1.9 Differential interference contrast (DIC) microscopy and confocal scanning laser microscopy allow enhanced three-dimensional imaging or imaging through thick specimens.

Q How is confocal scanning laser microscopy different from fluorescence microscopy? In what ways are they similar? How does differential interference contrast microscopy differ from bright-field microscopy?

1.10 Electron microscopes have far greater resolving power than do light microscopes, the limits of resolution being about 0.2 nm. The two major forms of electron microscopy are transmission, used primarily to observe internal cell structure, and scanning, used to examine the surface of specimens.

Q Why does an electron microscope have a higher resolution, or greater resolving power, than a light microscope?

III • Microbial Cultivation Expands the Horizon of Microbiology

1.11 Louis Pasteur devised ingenious experiments proving that living organisms cannot arise spontaneously from nonliving matter. Pasteur introduced many concepts and techniques central to the science of microbiology, including sterilization, and developed a number of key vaccines for humans and other animals.

Q Explain the principle behind the Pasteur flask in studies on spontaneous generation. Why were the results of this experiment inconsistent with the theory of spontaneous generation?

1.12 Robert Koch developed a set of criteria called Koch's postulates for linking cause and effect in infectious diseases. Koch also developed the first reliable and reproducible means for obtaining and maintaining microorganisms in pure culture.

Q What are Koch's postulates and how did they influence the development of microbiology? Why are Koch's postulates still relevant today?

1.13 Martinus Beijerinck and Sergei Winogradsky explored soil and water for microorganisms that carry out important natural processes, such as nutrient cycling and the biodegradation of particular substances. Out of their work came the enrichment culture technique and the concepts of chemolithotrophy and nitrogen fixation.

Q What were the major microbiological interests of Martinus Beijerinck and Sergei Winogradsky? It can be said that both men discovered nitrogen fixation. Explain.

IV • Molecular Biology and the Unity and Diversity of Life

1.14 All cells share certain characteristics, and microorganisms are used as model systems to explore the fundamental processes that define life. The discoveries of DNA as the molecular basis of heredity, and of its structure and function, paved the way for progress in molecular genetics, microbial phylogeny, and genomics.

Q Describe the experiments that proved DNA to be the molecule at the basis of heredity.

1.15 Carl Woese discovered that ribosomal RNA (rRNA) sequences can be used to determine the evolutionary history of microorganisms, and in so doing, he constructed a modern phylogenetic tree of life that revealed a new domain, the *Archaea*. Analysis of rRNA sequences from the environment has shown that microbial diversity is exceptional and that the majority of microorganisms have not yet been cultivated.

Q What insights led to the reconstruction of the tree of life? Which domain, *Archaea* or *Eukarya*, is more closely related to *Bacteria*? What evidence is there to justify your answer?

APPLICATION QUESTIONS

1. Pasteur's experiments on spontaneous generation contributed to the methodology of microbiology, understanding of the origin of life, and techniques for the preservation of food. Explain briefly how Pasteur's experiments affected each of these topics.
2. Describe the lines of proof Robert Koch used to definitively associate the bacterium *Mycobacterium tuberculosis* with the disease tuberculosis. How would his proof have been flawed if any of the tools he developed for studying bacterial diseases had not been available for his study of tuberculosis?
3. Imagine that all microorganisms suddenly disappeared from Earth. From what you have learned in this chapter, why do you think that animals would eventually disappear from Earth? Why would plants disappear? By contrast, if all higher organisms suddenly disappeared, what in Figure 1.10*a* tells you that a similar fate would not befall microorganisms?

CHAPTER GLOSSARY

Aseptic technique a series of steps taken to prevent contamination of laboratory cultures and media

Cell wall a rigid layer present outside the cytoplasmic membrane; it confers structural strength on the cell

Chemolithotrophy a form of metabolism in which energy is generated from the oxidation of inorganic compounds and carbon is obtained typically from CO_2

Chromosome a genetic element, usually circular in prokaryotic cells, carrying genes essential to cellular function

Colony a macroscopically visible population of cells growing on solid medium, arising from a single cell

Contrast the ability to resolve a cell or structure from its surroundings

Culture a collection of microbial cells grown using a nutrient medium

Cytoplasm the fluid portion of a cell, enclosed by the cytoplasmic membrane

Cytoplasmic membrane a semipermeable barrier that separates the cell interior (cytoplasm) from the environment

Differentiation modification of cellular components to form a new structure, such as a spore

Domain one of the three main evolutionary lineages of cells: the *Bacteria*, the *Archaea*, and the *Eukarya*

DNA replication the process by which information from DNA is copied into a new strand of DNA

Enrichment culture a culture that employs highly selective laboratory methods for obtaining microorganisms from natural samples

Enzyme a protein (or in some cases an RNA) catalyst that functions to speed up chemical reactions

Eukaryotic having a membrane-enclosed nucleus and various other membrane-enclosed organelles; cells of *Eukarya*

Evolution a change over time in gene sequence and frequency within a population of organisms, resulting in descent with modification

Extremophiles microorganisms that inhabit environments characterized by extremes of temperature, pH, pressure, or salinity

Genome the total complement of genes contained in a cell or virus

Gram-negative a bacterial cell with a cell wall containing small amounts of peptidoglycan and an outer membrane

Gram-positive a bacterial cell whose cell wall consists chiefly of peptidoglycan; it lacks the outer membrane of gram-negative cells

Gram stain a differential staining procedure that stains cells either purple (gram-positive cells) or pink (gram-negative cells)

Growth in microbiology, an increase in cell number with time

Gut microbiome the microbial communities present in the animal gastrointestinal tract

Horizontal gene transfer the unidirectional transfer of genes between cells through a process uncoupled from reproduction

Intercellular communication interactions between cells using chemical signals

Koch's postulates a set of criteria for proving that a given microorganism causes a given disease

Macromolecules a polymer of monomeric units, for example proteins, nucleic acids, polysaccharides, and lipids

Magnification the optical enlargement of an image

Medium (plural, media) in microbiology, the liquid or solid nutrient mixture(s) used to grow microorganisms

Metabolism all biochemical reactions in a cell

Microbial community two or more populations of cells that coexist and interact in a habitat

Microbial ecology the study of the interaction of microorganisms with each other and their environment

Microorganism an organism that is too small to be seen by the unaided human eye

Morphology the physical appearance of a cell as determined by cell shape, for example: rod, coccus, or spirillum

Motility the movement of cells by some form of self-propulsion

Nucleoid the aggregated mass of DNA that makes up the chromosome(s) of prokaryotic cells

Nucleus a membrane-enclosed structure in eukaryotic cells that contains the cell's DNA genome

Organelle a bilayer-membrane-enclosed structure such as the mitochondrion, found in eukaryotic cells

Pathogen a disease-causing microorganism

Phylogenetic tree a diagram that depicts the evolutionary history of an organism; consists of nodes and branches

Phylogeny the evolutionary history of organisms

Plasmid an extrachromosomal genetic element that is usually not essential to the cell

Prokaryotic lacking a membrane-enclosed nucleus and other organelles; cells of *Bacteria* or *Archaea*

Pure culture a culture containing a single kind of microorganism

Resolution the ability to distinguish two objects as distinct and separate when viewed under the microscope

Ribosomal RNA (rRNA) the types of RNA found in the ribosome; some participate actively in protein synthesis

Ribosome a cytoplasmic particle composed of ribosomal RNA and protein, whose function is to synthesize proteins

Spontaneous generation the hypothesis that living organisms can originate from nonliving matter

Sterile free of all living organisms (cells) and viruses

Transcription the synthesis of an RNA molecule complementary to one of the two strands of a double-stranded DNA molecule

Translation the synthesis of protein by a ribosome using the genetic information in a messenger RNA as a template

2 Microbial Cell Structure and Function

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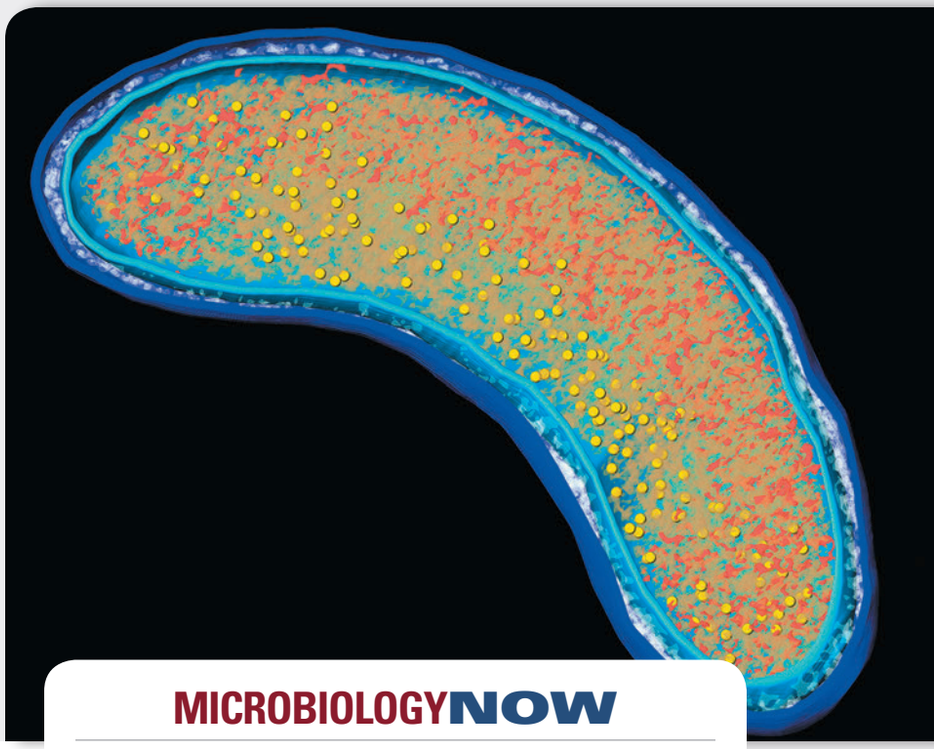


MICROBIOLOGYNOW

Exploring the Microbial Cell

New microscopic techniques are changing how we view microbial cells. Many cellular processes are performed by macromolecules and structures whose forms dictate their function. These structures are incredibly small and impossible to resolve with standard light microscopy. Cryogenic electron tomography (cryoET) images cells embedded within vitreous ice at ultra-low temperatures so they can be viewed in their native state without dehydration. During cryoET, samples are tilted in an electron microscope and images made at many different angles, much in the same way that a CT scan images a human body. These images are integrated using computer algorithms to generate complex, high-resolution, three-dimensional images that provide unprecedented structural detail. CryoET images have led to the discovery of new features within microbial cells and stunning new insights on well-known structures.

"*Candidatus* Pelagibacter ubique," one of the most abundant organisms on Earth, lives in microbial communities suspended in the upper waters of oceans worldwide. Cells of *Pelagibacter* are quite small (about 0.8 µm long), but their structures are readily visualized by cryoET (see image). Colors in the cryoET reconstruction indicate the outer membrane (blue), cytoplasmic membrane (cyan), peptidoglycan (white), cytoplasm (orange), nucleoid (red), and ribosomes (yellow). These new images reveal that the periplasm (the space between inner and outer membrane) in these cells is larger than expected and can occupy a remarkable 50–70% of cell volume in nongrowing cells. These images also reveal that the cytoplasm is divided into distinct regions that contain either nucleoid or ribosomes. Finally, mysterious structures such as membrane vesicles have been observed on the extracellular surface of the outer membrane and tethered to the cytoplasmic membrane (not shown). The function of these vesicles is unknown. New imaging techniques such as cryoET move microbiology forward and are revealing surprising complexity and diversity in the structures of prokaryotic cells.

Zhao, X., et al. 2017. Three-dimensional structure of the ultraoligotrophic marine bacterium "*Candidatus* Pelagibacter ubique." *Appl. Environ. Microbiol.* 83: e02807-16.

In this chapter, we consider the structure and function of microbial cells, both prokaryotic and eukaryotic. We will explore the components of microbial cells that control nutrient transport and cellular integrity but also several internal structures, which can be found in certain cells. We will also examine structures that give microbial cells the ability to move within their environment. We begin with two critical cell structures—the cytoplasmic membrane and cell wall—that collectively compose the cell envelope.

I • The Cell Envelope

The cytoplasm of the prokaryotic cell is surrounded by several layers that include a membrane, a cell wall, and other structures specific to the kind of cell. Collectively, these layers form the cell envelope and are essential for preserving the integrity and functioning of the cell.

The **cell envelope** consists of a series of layered structures that surround the cytoplasm and govern cellular interactions with the external environment. The cell envelope has many important functions: It governs transport of nutrients into the cell and wastes out of the cell, it is the site of energy conservation, it governs cell shape, it protects the cell from mechanical stress, and it can help the cell attach to surfaces and even protect the cell from attack.

The diversity of microorganisms is in part reflected in the composition of the cell envelope; but we will also learn that certain envelope structures are highly conserved and that knowledge of cell envelope structure can help us to identify and classify microorganisms. In the first few sections, we will learn about major components of cell envelope structure including the *cytoplasmic membrane*, *cell wall*, *outer membrane*, and *S-layers*. We start our tour by considering the cytoplasmic membrane, a structure found in all cells.

2.1 The Cytoplasmic Membrane

The **cytoplasmic membrane** surrounds the *cytoplasm*—the mixture of macromolecules and small molecules inside the cell—and separates it from the environment. The cytoplasmic membrane is physically rather weak but is an ideal structure for its major cellular function: selective permeability. In order for a cell to grow, nutrients must be transported inwards and waste products outwards. Both of these events occur across the cytoplasmic membrane. A variety of proteins located in the cytoplasmic membrane facilitate these reactions, and many other membrane proteins play important roles in energy metabolism.

Bacterial Cytoplasmic Membranes

The cytoplasmic membrane of all bacterial and eukaryal cells is a phospholipid bilayer containing embedded proteins. The cytoplasmic membrane is only 8–10 nanometers wide but can be resolved easily by transmission electron microscopy (**Figure 2.1*a***). Phospholipids are composed of both hydrophobic (water-repelling) and

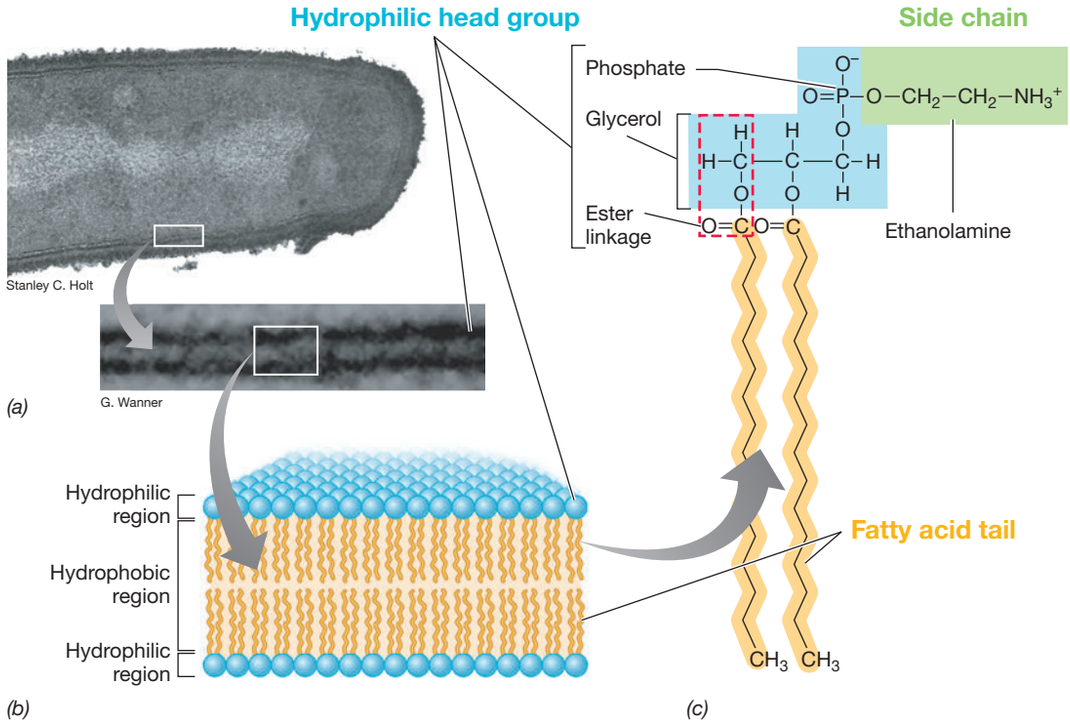


Figure 2.1 Phospholipid bilayer membrane. *(a)* Transmission electron micrograph of a cell with membrane region shown in detail. *(b)* General architecture of a bilayer membrane; phospholipids are composed of a hydrophilic head group (blue spheres) with fatty acid tails (yellow lines). The phospholipid head groups of the bilayer are visible in *a* as parallel dark lines, between which can be found a lighter region comprising the hydrophobic region of the membrane. *(c)* Structure of the phospholipid phosphatidylethanolamine. Each fatty acid side chain is connected to the head group by an ester bond (boxed with a red dashed line); ester linkages are a characteristic feature of lipids from *Bacteria* and *Eukarya* but not those of *Archaea*.

hydrophilic (water-attracting) components (Figure 2.1*b*). In *Bacteria* and *Eukarya*, the hydrophobic component consists of fatty acid "tails" and the hydrophilic component consists of a glycerophosphate (a glycerol molecule bound to a phosphate) and one of several other functional groups (such as sugars, ethanolamine, or choline) also bonded to the phosphate (Figure 2.1*c*). The membrane is comprised of two phospholipid layers in which the fatty acid tails associate together to form a hydrophobic region, leaving the hydrophilic "head groups" exposed to either the environment or the cytoplasm (Figure 2.1*b*). That is, the *outer* surface of the cytoplasmic membrane faces the environment while the *inner* surface faces the cytoplasm and interacts with the cytoplasmic milieu. This type of membrane structure is called a *lipid bilayer*, or a *unit membrane*.

Mastering Microbiology
Art Activity: Figure 2.5 Structure of the cytoplasmic membrane

A variety of proteins are attached to or integrated into the cytoplasmic membrane; membrane proteins typically have hydrophobic domains that span the membrane and hydrophilic domains that contact the environment or the cytoplasm (**Figure 2.2**). Proteins significantly embedded in the membrane are called *integral* membrane proteins. Many, though not all, integral membrane proteins extend completely across the membrane, and these are called *transmembrane* proteins. By contrast, *peripheral* membrane proteins are more loosely attached. Some peripheral membrane proteins are lipoproteins, proteins that contain a hydrophobic lipid tail that anchors the protein into the membrane. Other peripheral membrane proteins have residues that associate with the hydrophilic head groups of phospholipids, or they associate indirectly with membranes by binding to other proteins anchored in the membrane. Peripheral membrane proteins typically interact with integral membrane proteins in important cellular processes such as energy metabolism and transport.

Archaeal Cytoplasmic Membranes

The cytoplasmic membrane of *Archaea* is structurally similar to those of *Bacteria* and *Eukarya*, but the chemistry is somewhat different. In the lipids of *Bacteria* and *Eukarya* the hydrophobic *fatty acid* tails are bound to glycerol by *ester* linkages (Figure 2.1); in contrast, the lipids of *Archaea* have hydrophobic *isoprenoid* (rather than fatty acid) tails, which are bound to glycerol by *ether* bonds (**Figure 2.3**). The hydrophobic region of archaeal membranes is formed from repeating units of the five-carbon hydrocarbon *isoprene*, rather than from fatty acids (compare Figures 2.1 and 2.3).

The cytoplasmic membrane of *Archaea* is constructed from either phosphoglycerol diethers, which can have C_{20} side chains (called a *phytanyl* group), or diphosphoglycerol tetraethers (C_{40} side chains, called a *biphytanyl* group) (Figure 2.3). In the tetraether lipid structure, the ends of the inwardly pointing isoprenoid chains are covalently linked at their termini to form a *lipid monolayer* (Figure 2.3*c*) instead of a lipid bilayer (Figure 2.3*a*) membrane.

Archaeal lipids can have many different isoprenoid chains including some that contain ring structures. For example, *crenarchaeol*, a

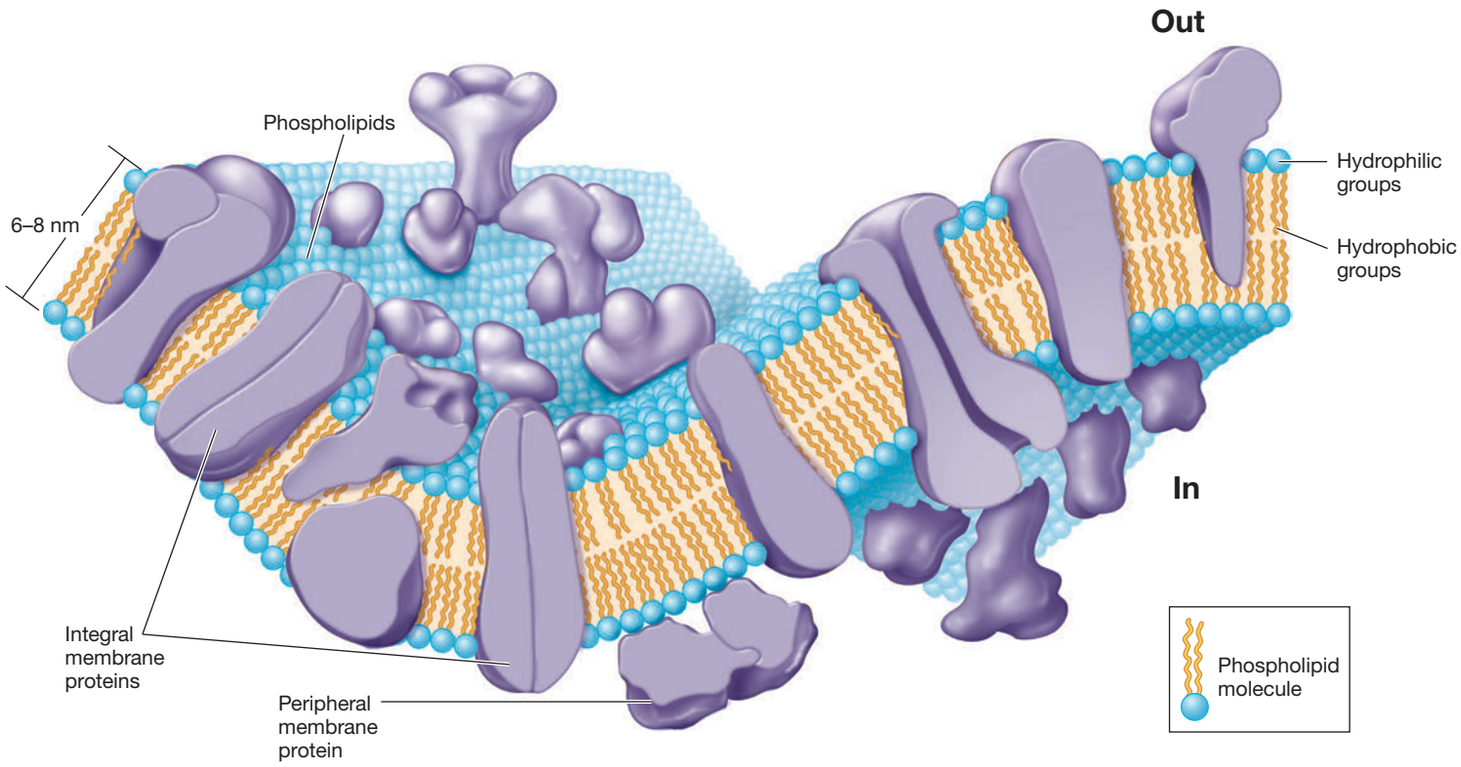


Figure 2.2 Structure of the cytoplasmic membrane. The inner surface (**In**) faces the cytoplasm and the outer surface (**Out**) faces the environment. Phospholipids compose the matrix of the cytoplasmic membrane with proteins embedded (integral) or surface associated (peripheral). The general design of the cytoplasmic membrane is similar in both prokaryotic and eukaryotic cells, although there can be differences in the chemistry between different species. Note that this membrane is shown in a relaxed shape to better illustrate its inner and outer surfaces; in a living cell, cytoplasmic turgor pressure would cause the membrane to have convex curvature.

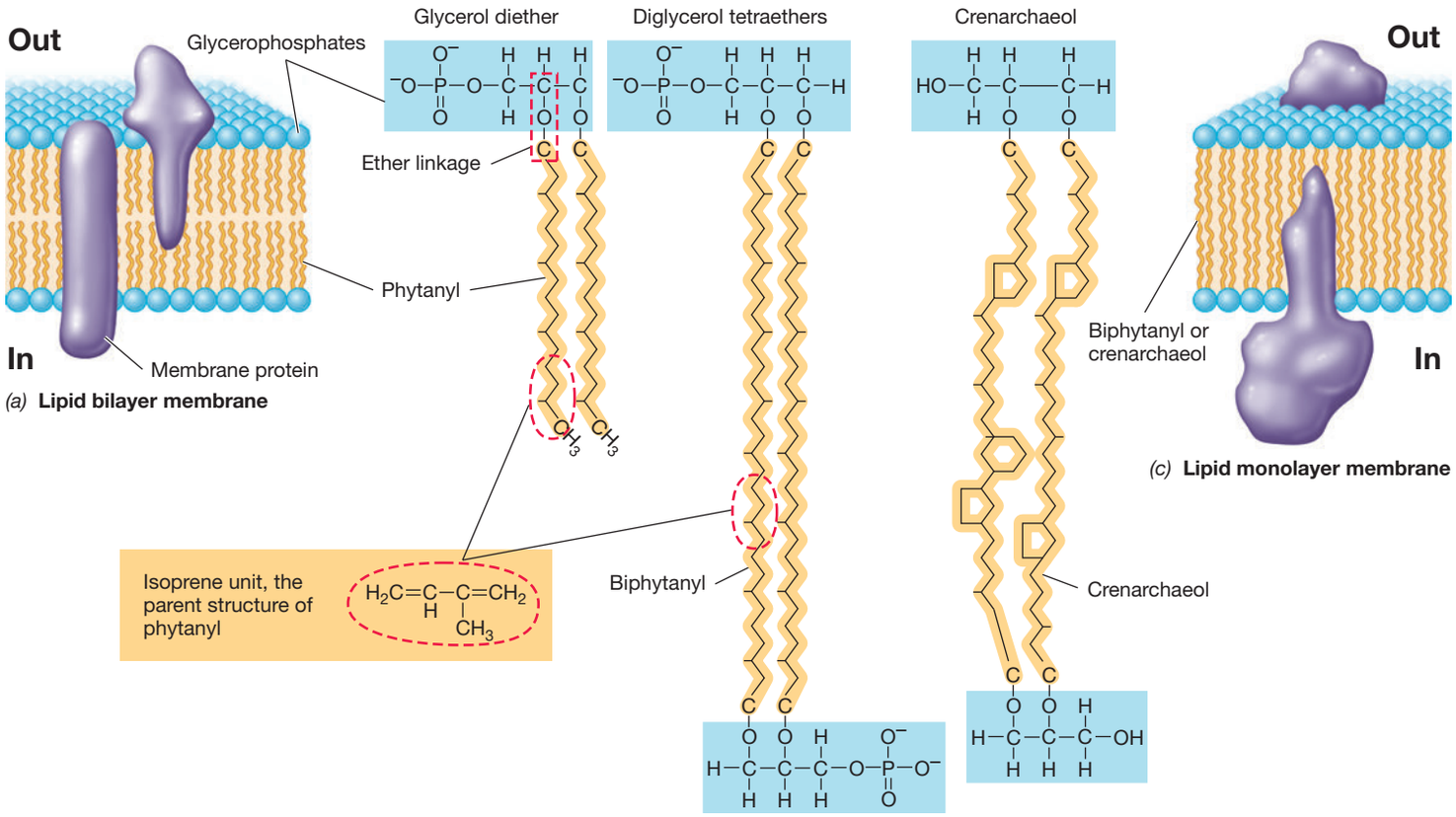


Figure 2.3 **Major lipids of *Archaea* and the architecture of archaeal membranes.** *(a, b) Archaea* can have lipid bilayers composed of phosphoglycerol diether lipids. The hydrophobic portions of archaeal lipids are comprised of isoprenoid chains synthesized from repeated units of isoprene (in dashed red ovals); this contrasts with the lipids of *Bacteria* and *Eukarya*, which have fatty acid tails (Figure 2.1). Note that these isoprenoids are bonded to glycerol by an ether linkage (in dashed red box). *(b, c)* Some *Archaea* can also have lipid monolayers composed of diphosphoglycerol tetraether lipids or other isoprenoid lipids such as crenarchaeol. The isoprenoid lipids in *b* are phytanyl (C_{20}), biphytanyl (C_{40}), and crenarchaeol. Isoprene lipids can often contain 5- and 6-carbon rings such as those present in crenarchaeol. The membrane structure in *Archaea* may form a lipid bilayer or a lipid monolayer (or a mix of both).

common membrane lipid in cells of *Thaumarchaeota* (a major phylum of *Archaea*, ▶ Section 17.5) contains four C_5 rings and one C_6 ring (Figure 2.3*b*). These rings affect the chemical properties of the lipids and thus influence membrane function. As in other organisms, the polar head groups in archaeal lipids can be sugars, ethanolamine, or a variety of other molecules.

Despite differences in chemistry between the cytoplasmic membranes of *Archaea* and organisms in the other phylogenetic domains, the fundamental construction of the archaeal cytoplasmic membrane—inner and outer hydrophilic surfaces and a hydrophobic interior—is the same as that of membranes in all cells. Obviously, evolution has selected this fundamental design as the best solution to the major functions of the cytoplasmic membrane, an issue we turn to now.

Cytoplasmic Membrane Function

The cytoplasmic membrane has at least *three* major functions (**Figure 2.4**). First, it is the cell's permeability barrier, preventing the passive leakage of solutes into or out of the cell. Second, the cytoplasmic membrane anchors several proteins that catalyze a suite of key cell functions. And third, the cytoplasmic membrane of *Bacteria* and *Archaea* plays a major role in energy conservation and consumption.

The cytoplasmic membrane is a barrier to the diffusion of most substances, especially polar or charged molecules. Because the cytoplasmic membrane is so impermeable, most substances that enter or leave the cell must be carried in or out by *transport proteins*. These are not simply ferrying proteins but instead function to *accumulate* solutes against the concentration gradient, a process that diffusion alone cannot do (**Figure 2.5**). Transport, which requires energy, ensures that the cytoplasm has sufficient concentrations of the nutrients it needs to perform biochemical reactions efficiently.

Transport proteins typically display high sensitivity and high specificity. If the concentration of a solute is high enough to saturate the transporter, which often occurs at the very low concentrations of nutrients found in nature, the rate of uptake can be near maximal (Figure 2.5). Some nutrients are transported by a low-affinity transporter when the nutrient is present at *high* external concentration and by a separate, typically higher-affinity, transporter when the nutrient is present at *low* concentration (Figure 2.5).

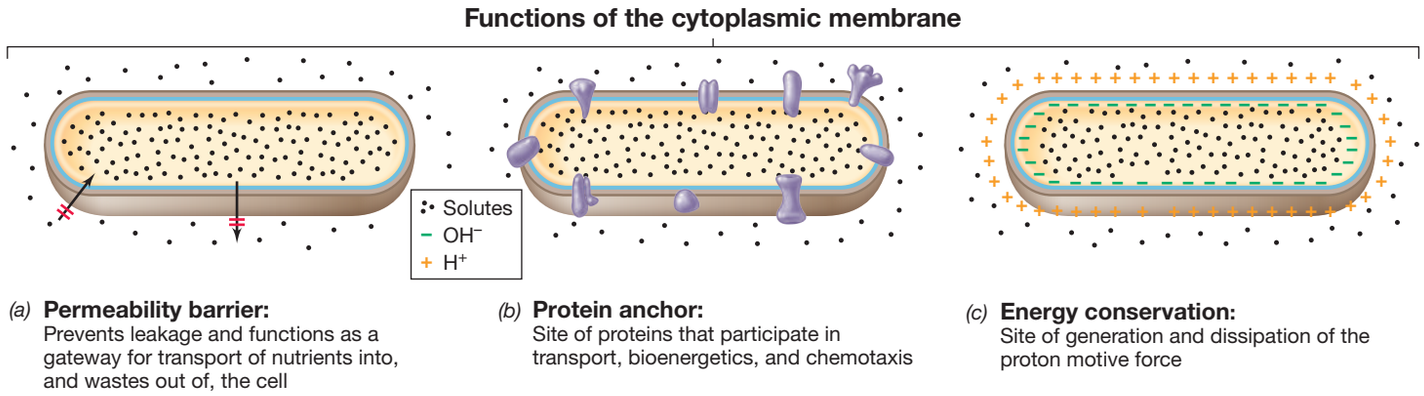


Figure 2.4 The major functions of the cytoplasmic membrane. Although physically weak, the cytoplasmic membrane controls at least three critically important cellular functions: maintaining selective permeability, anchoring proteins, and conserving energy.

Moreover, many transport proteins transport only a single kind of molecule while others carry a related class of molecules, such as different sugars or different amino acids. This economizing reduces the need for separate transport proteins for each different sugar or amino acid.

In addition to its permeability and transport functions, the cytoplasmic membrane of *Bacteria* and *Archaea* is a major site of both energy conservation and energy consumption. We discuss in Chapter 3 how the cytoplasmic membrane can be energized when protons (H^+) are separated from hydroxyl ions (OH^-) across the membrane surface (Figure 2.4*c*). This charge separation creates an energized state of the membrane called the *proton motive force*, analogous to the potential energy present in a charged battery. Dissipation of the proton motive force can be coupled to several energy-requiring reactions, such as transport, cell locomotion, and the biosynthesis of ATP. In eukaryotic microbial cells, although transport across the cytoplasmic membrane is just as necessary as it is in prokaryotic cells, energy conservation takes place in the membrane systems of the cell's key organelles, the mitochondrion (respiration) and chloroplast (photosynthesis), as we will see later in this chapter.

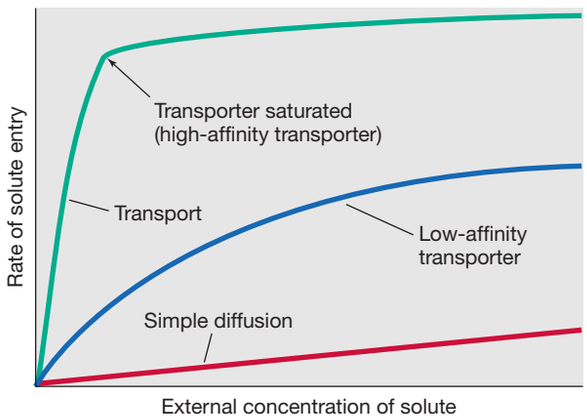


Figure 2.5 The importance of transport in membrane function. In both types of transport, the uptake rate shows saturation at relatively low external solute concentrations. Both high-affinity and low-affinity transport systems are depicted.

Check Your Understanding

- Draw the basic structure of a lipid bilayer and label the hydrophilic and hydrophobic regions. Why is the cytoplasmic membrane a good permeability barrier?
- How are the membrane lipids of *Bacteria* and *Archaea* similar, and how do they differ?
- Describe the major functions of the cytoplasmic membrane.
- Which molecule types can pass through the phospholipid bilayer directly by simple diffusion?

2.2 Transporting Nutrients into the Cell

In Section 2.1 we learned how the structure of the cytoplasmic membrane is an effective barrier to leakage; solutes leak neither into nor out of a living cell. However, selective transport is a major function of the cytoplasmic membrane. In order for cells to survive and grow, they must transport nutrients across the membrane and into the cell and they must export wastes out of the cell. Substances are transported across the cytoplasmic membrane through membrane-spanning integral membrane proteins (Figure 2.2). Some transporters also require the action of peripheral membrane proteins. We consider the most common of these transport systems here, with a focus on the well-studied transporters widespread in *Bacteria* and *Archaea*.

Active Transport and Transporters

Active transport is the process by which cells accumulate solutes against the concentration gradient. Three basic mechanisms of active transport are found in prokaryotic cells. A **simple transport system** consists only of a transmembrane transport protein, **group translocation** employs a series of proteins in the transport event, and **ABC transport systems** consist of three components: a binding protein, a transmembrane transporter, and an ATP-hydrolyzing protein (**Figure 2.6**). Each of these transport systems is energy-driven, be it from the proton motive force, ATP, or some other energy-rich compound.

The transmembrane component of virtually all transport systems is composed of a polypeptide containing 12 regions (called *domains*) that weave back and forth through the membrane to form a channel, and it is through this channel that the solute is transported into

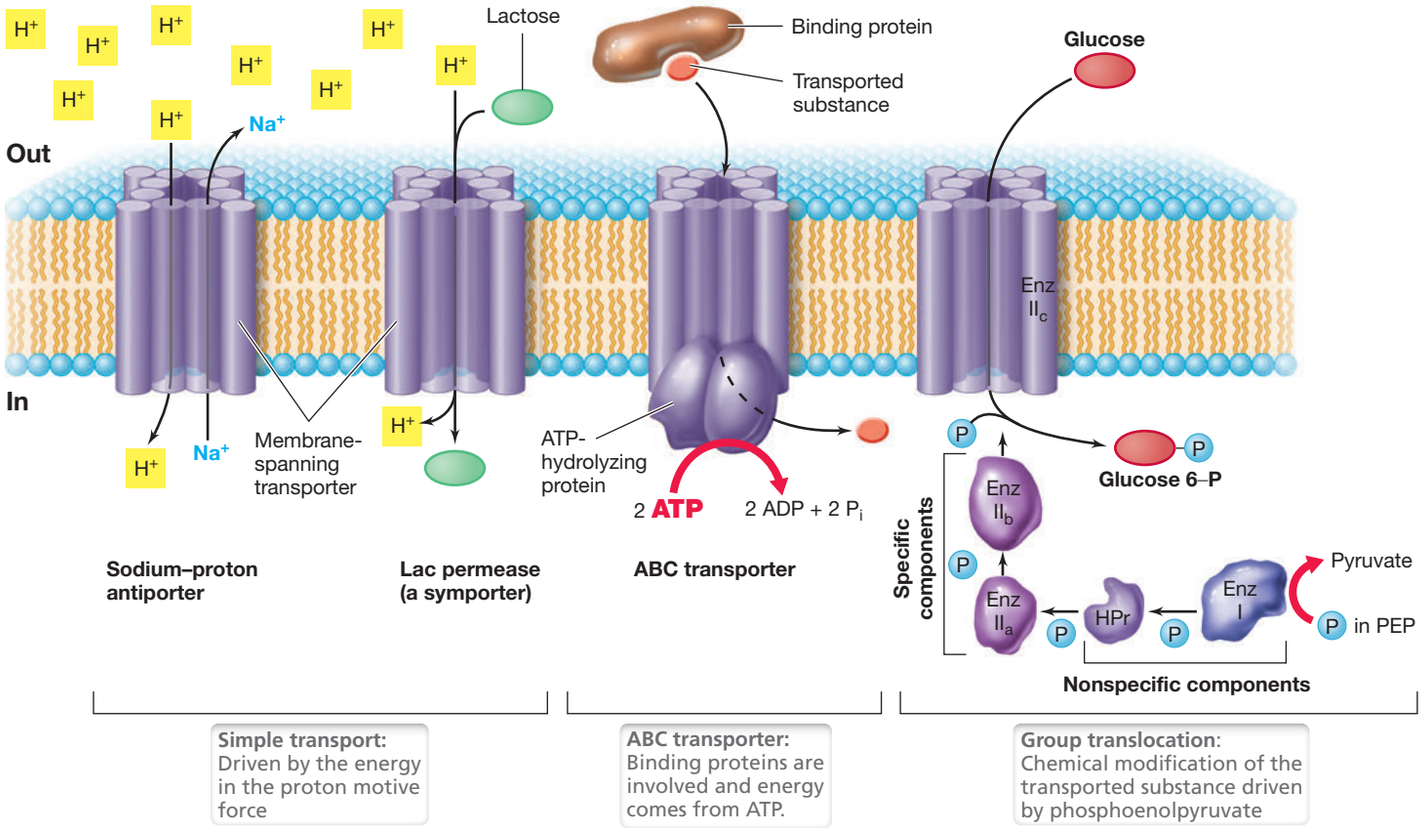


Figure 2.6 The three classes of transport systems. Transmembrane transporters are generally composed of a polypeptide that has 12 α-helices (each shown as a cylinder) that aggregate to form a channel through which solutes can cross the membrane. In simple transport the movement of a solute is coupled with the dissipation of an electrochemical gradient such as the proton motive force. ABC transporters have three components: a binding protein that has high affinity for a substrate, a transmembrane protein channel, and a cytoplasmic ATP-hydrolyzing protein, which supplies the energy required to drive substrate transport. In group translocation, the substance transported is chemically modified upon entering the cell. For example, the glucose group translocation system has five proteins: Enzyme (Enz) I, Enzymes II_a, II_b, and II_c, and HPr. A phosphate cascade occurs from phosphoenolpyruvate (PEP) to Enz II_c, and the latter protein actually transports and simultaneously phosphorylates the sugar. Proteins HPr and Enz I are nonspecific and participate in the transport of any sugar, while the three components of Enz II are specific for a particular sugar. Note how simple transporters and the ABC system transport substances without chemically modifying them, whereas group translocation results in chemical modification (in this case phosphorylation) of the transported substance.

the cell. Transport is linked to a conformational change in this transmembrane protein complex that occurs when it binds its specific solute. Like a gate swinging open, this conformational change sweeps the solute into the cell.

Simple Transporters and Group Translocation

Simple transport reactions are driven by the energy inherent in the proton motive force (Figure 2.4*c*). The two major transport events catalyzed are either *symport* reactions (where a solute and a proton are cotransported in the same direction) or *antiport* reactions (where a solute and a proton are transported in opposite directions) (Figure 2.6). A classic example of a simple transporter is the uptake of the sugar lactose by way of the *lac permease*, a well-studied symporter in *Escherichia coli*. As each lactose molecule enters the cell, the potential energy in the proton motive force is diminished slightly by the cotransport of a proton (Figures 2.4*c* and 2.6). The net result is the energy-driven accumulation of lactose in the cytoplasm against the concentration gradient. Many other solutes enter by the activity of their own simple symporters, including phosphate, sulfate, and several different organic compounds.

Group translocation differs from simple transport in two important ways: (1) the transported substance is *chemically modified* during the transport process, and (2) an energy-rich organic compound (rather than the proton motive force) drives the transport event. The best-studied group translocation systems transport the sugars glucose, mannose, and fructose in *E. coli*. During uptake, these compounds are phosphorylated by the *phosphotransferase system* (Figure 2.6). The phosphotransferase system consists of a family of five proteins that work in concert to transport any given sugar. Before the sugar is transported, the proteins in the phosphotransferase system are themselves alternately phosphorylated and dephosphorylated in a cascading fashion until Enzyme II_c phosphorylates the sugar as it enters the cytoplasm (Figure 2.6). A protein called HPr, the enzyme that phosphorylates HPr (Enzyme I), and Enzyme II_a are all cytoplasmic proteins. By contrast, Enzyme II_b is a peripheral membrane protein and Enzyme II_c is the transmembrane component.

In the phosphotransferase system, HPr and Enzyme I are *nonspecific* components and participate in the uptake of several different sugars. By contrast, distinct Enzyme II proteins exist, one set for each different sugar transported. Energy to drive the phosphotransferase system comes from phosphoenolpyruvate, an energy-rich intermediate in glycolysis (▶ Sections 3.4 and 3.6).

ABC Transporter Systems

ABC transporters are modular systems that have three components: a binding protein, a transmembrane protein channel, and an ATP-hydrolyzing protein (Figure 2.6). The ABC stands for *A*TP-*b*inding *c*assette, a structural feature of proteins that bind ATP. More than 200 different ABC transport systems are known, and these catalyze the uptake of a wide variety of organic and inorganic compounds.

Substrate-binding proteins are present outside of the cell, where they bind to a specific substrate and enable its transport into the cell. A characteristic property of binding proteins is their extremely high substrate affinity. These proteins can bind their specific substrate even when it is present at extremely low concentration; for example, less than 1 micromolar (10^{-6} M). Once its specific substrate is bound, the binding protein interacts with its respective transmembrane component to transport the substrate into the cell driven by the energy in ATP (Figure 2.6).

We move on now from our coverage of the cytoplasmic membrane to consider components of the cell envelope that confer structural strength on the cell, something the membrane cannot do.

Check Your Understanding

- Compare and contrast simple transporters, the phosphotransferase system, and ABC transporters in terms of (1) energy source, (2) chemical alterations of the solute during transport, and (3) number of proteins required.
- Which major characteristic of ABC transport systems makes them ideal for organisms living in nutrient-poor environments?

2.3 The Cell Wall

The cytoplasm of prokaryotic cells maintains a high concentration of dissolved solutes that creates significant osmotic pressure—about 2 atm (203 kPa); this is about the same as the pressure in an automobile tire. This osmotic pressure is sufficient to cause the cell membrane to burst and the cell to die—a process called *cell lysis*. To withstand this *turgor pressure*, the cell envelopes of most *Bacteria* and *Archaea* have a layer outside the cytoplasmic membrane called the *cell wall*. Besides protecting against osmotic lysis, cell walls also maintain cell shape and rigidity.

The cell envelopes of most *Bacteria* can be classified as being either *gram-positive* or *gram-negative* based on their organization and cell wall structures. The structures of gram-positive and gram-negative cell envelopes differ markedly as viewed in the electron microscope (**Figure 2.7**). The cell envelope of a gram-positive cell typically contains a cytoplasmic membrane and a thick cell wall, whereas a gram-negative cell has a cytoplasmic membrane, a thin cell wall, an outer membrane (Figure 2.7), and a periplasm, which is a compartment between the cytoplasmic and outer membranes. We will consider the outer membrane and periplasm in the next section. The names we use to describe the typical gram-positive and gram-negative cell envelopes are based on their Gram stain reactions (◀ Section 1.8). The Gram stain reaction is determined primarily by the thickness of the cell wall rather than the number of layers in the cell envelope, and so Gram stain reaction does not always correlate with cell envelope structure. However, the Gram stain reaction is sufficiently predictive of cell envelope structure in *Bacteria* that the names of the two most common bacterial cell envelopes—gram-positive and gram-negative—are based on their typical reactions to the Gram stain.

Knowledge of cell wall envelope structure and function is important not only for understanding the biology of microbial cells but also for medical reasons. Certain antibiotics, for example, the penicillins and cephalosporins, target bacterial cell wall synthesis, leaving the cell susceptible to osmotic lysis. Since human and other animal cells lack cell walls and are therefore not a target of such antibiotics, these drugs are of obvious benefit for treating bacterial infections. The major component of the bacterial cell wall, and a target of many antibiotics, is a molecule called *peptidoglycan*, and we consider this molecule in detail now.

Bacterial Cell Walls

The cell walls found in *Bacteria* contain a rigid polysaccharide called **peptidoglycan** that confers structural strength on the cell. Peptidoglycan is found in all *Bacteria* that contain a cell wall, but it is unique to *Bacteria* and is not found in *Archaea* or *Eukarya*. The sugar backbone of peptidoglycan is composed of alternating repeats of two modified glucose residues called *N-acetylglucosamine* and *N-acetylmuramic acid* joined by a β-1,4 linkage (**Figure 2.8**). Attached to the latter residue is a short peptide side chain. The amino acid composition of this peptide side chain can vary considerably between bacterial species. In *Escherichia coli* this peptide contains the amino acids L-alanine, D-alanine, D-glutamic acid, and diaminopimelic acid (DAP), though in other bacteria, L-lysine can be substituted for DAP. The presence of D stereoisomer amino acids, D-alanine and D-glutamic acid, is an unusual feature of peptidoglycan since proteins are always constructed of L-amino acids. These constituents are connected in an ordered way to form the *glycan tetrapeptide* (Figure 2.8), and long chains of this basic unit form peptidoglycan.

Strands of peptidoglycan run parallel to each other around the circumference of the cell (**Figure 2.9**). The peptide side chains of adjacent peptidoglycan strands are cross-linked together by covalent peptide bonds (Figure 2.9*a*), and in this way, the peptidoglycan forms one single enormous molecule. In gram-negative bacteria, the cross-links form primarily between the amino group of DAP on one glycan strand and the carboxyl group of the terminal D-alanine on the adjacent glycan strand (Figure 2.9*a*). The cell wall in the gram-negative cell envelope is 2–7 nm thick consisting primarily of a single layer of peptidoglycan, though it can be up to three layers thick in some places (Figure 2.9*c*).The peptidoglycan mesh so formed is flexible and porous, but strong enough to resist turgor pressure and prevent rupture of the cytoplasmic membrane and cell lysis. Additional strength against osmotic lysis in gram-negative bacteria is provided by the outer membrane, as discussed in the next section.

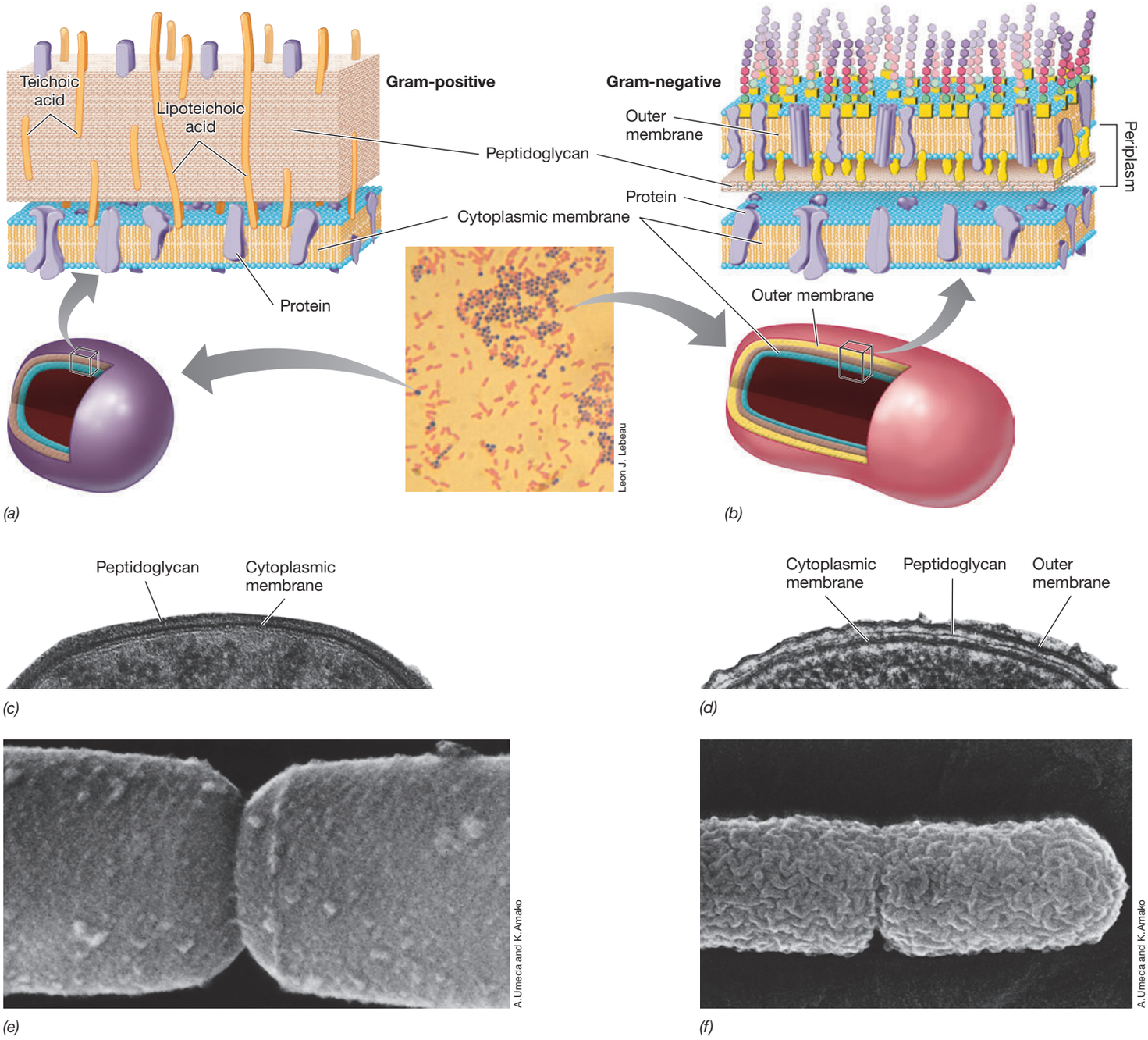


Figure 2.7 **Cell envelopes of *Bacteria*.** *(a, b)* Schematic diagrams of gram-positive and gram-negative cell envelopes; the Gram stain procedure was described in Section 1.8 and shown in Figure 1.23. The photo of Gram-stained bacteria in the center shows cells of *Staphylococcus aureus* (purple, gram-positive) and *Escherichia coli* (pink, gram-negative). *(c, d)* Transmission electron micrographs showing the cell wall of a gram-positive bacterium and a gram-negative bacterium, respectively. *(e, f)* Scanning electron micrographs of gram-positive and gram-negative bacteria, respectively. Note differences in surface texture. Each cell is about 1 μm wide.

The typical bacterial gram-positive cell envelope contains a thick peptidoglycan cell wall, which can measure 20 to 35 nm in thickness and is usually much thicker than the wall of gram-negative organisms. As much as 90% of the gram-positive cell envelope can consist of peptidoglycan. Whereas the gram-negative cell wall typically contains only a single layer of peptidoglycan, the gram-positive cell wall can be 15 or more layers thick (**Figure 2.10*a***). The peptidoglycan of the gram-positive cell wall is stabilized three-dimensionally by peptide cross-links, which form between adjacent peptidoglycan strands both horizontally and vertically. In gram-positive bacteria, peptide cross-links often contain a short peptide "interbridge," the kinds and numbers of amino acids in the interbridge varying between species. In the gram-positive bacterium *Staphylococcus aureus*, for example, the interbridge often consists of five glycines (Figure 2.9*b*).

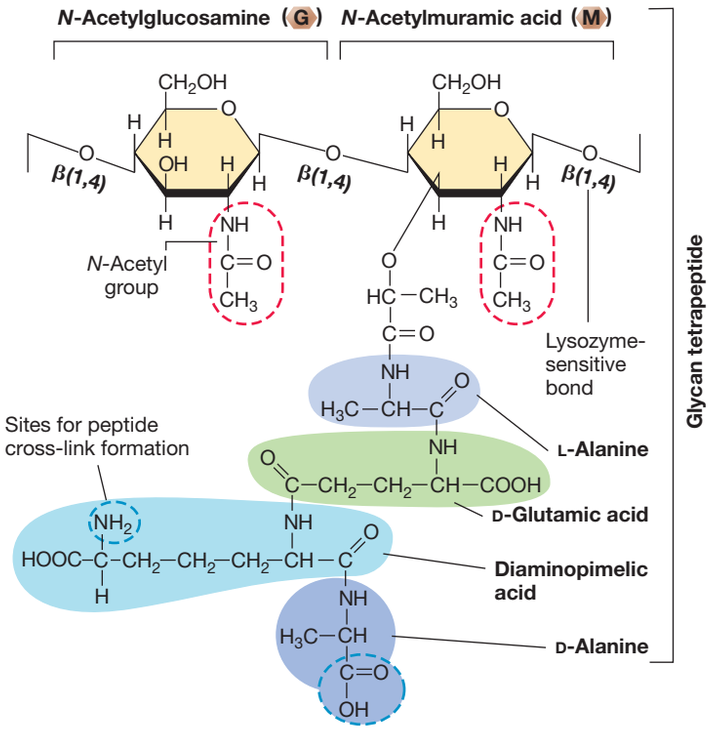


Figure 2.8 Structure of the repeating unit in peptidoglycan, the glycan tetrapeptide. The structure given is that for the peptidoglycan of *Escherichia coli* and most other gram-negative *Bacteria*. Cross-links can be formed between adjacent peptide side chains at residues having free amino and carboxyl groups (circled in blue). For example, cross-links in *E. coli* most commonly occur between the amino group of diaminopimelic acid on one peptide and the terminal carboxyl group of D-alanine on a different peptide.

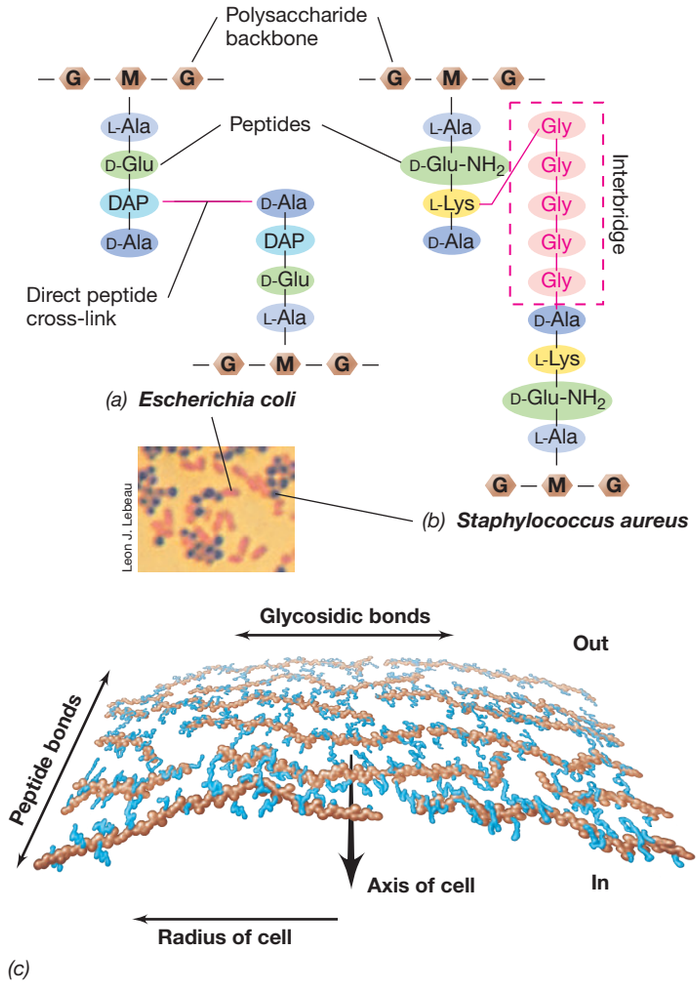


Figure 2.9 Peptidoglycan structure in the cell wall. *(a)* Gram-negative cells that have thin cell walls, such as the cell wall of *E. coli*, mostly have direct cross-links between peptide side chains. *(b)* Gram-positive cells that have thick cell walls, such as *S. aureus*, can also have peptide interbridges that extend between cross-linked peptide side chains. *(c)* Conformation of peptidoglycan in the gram-negative cell wall. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid. Note how glycosidic bonds confer strength on peptidoglycan around the circumference of the cell whereas peptide bonds confer strength along the axis of the cell.

Mastering Microbiology
Art Activity: Figure 2.12*c* Parts of the Gram-positive cell wall

In addition to peptidoglycan, many gram-positive bacteria produce acidic molecules called **teichoic acids** embedded in their cell wall (Figure 2.10). Teichoic acids are composed of glycerol phosphate or ribitol phosphate with attached molecules of glucose or D-alanine (or both). Individual alcohol molecules are then connected through their phosphate groups to form long strands, and these are then covalently linked to peptidoglycan (Figure 2.10*b*). Some teichoic acids are covalently bonded to membrane lipids rather than to peptidoglycan, and these are called *lipoteichoic* acids.

Peptidoglycan can be destroyed by *lysozyme*, an enzyme that cleaves the glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid (Figure 2.8). This weakens the peptidoglycan and can cause cell lysis. Lysozyme is present in human secretions including tears, saliva, and other bodily fluids, and functions as a major line of defense against bacterial infection. Many antibiotics, including penicillin, also target peptidoglycan. Whereas lysozyme destroys preexisting peptidoglycan, penicillin blocks the formation of peptide cross-links, which compromises the strength of the peptidoglycan, leading to cell lysis.

Archaeal Cell Walls

The cell envelopes of *Archaea* differ in fundamental ways from those of *Bacteria*. We have already learned that the cytoplasmic membranes of *Archaea*, while functionally analogous to those of *Bacteria*, differ in chemical structure (Section 2.1). Another major difference is that *Archaea* lack peptidoglycan. In addition, *Archaea* typically lack an outer membrane (see Section 2.4). One consequence of these differences is that the Gram stain reaction is not very useful for predicting the structures of archaeal cell envelopes and so we typically do not use the terms gram-positive and gram-negative to describe cells of *Archaea*. Most *Archaea* lack a polysaccharide-containing cell wall and instead have an *S-layer* (see Section 2.5), which is a rigid protein shell that functions to prevent osmotic lysis just as does the bacterial cell wall.

While some *Archaea* do have cell walls, these walls have unique chemical structures not found in *Bacteria*. For example, the cell walls of certain methane-producing *Archaea* (methanogens) contain a polysaccharide called *pseudomurein* (Figure 2.11), which is structurally remarkably similar to peptidoglycan (the term *murein* is from the Latin word for "wall" and was an old term for peptidoglycan). The backbone of pseudomurein is formed from alternating repeats

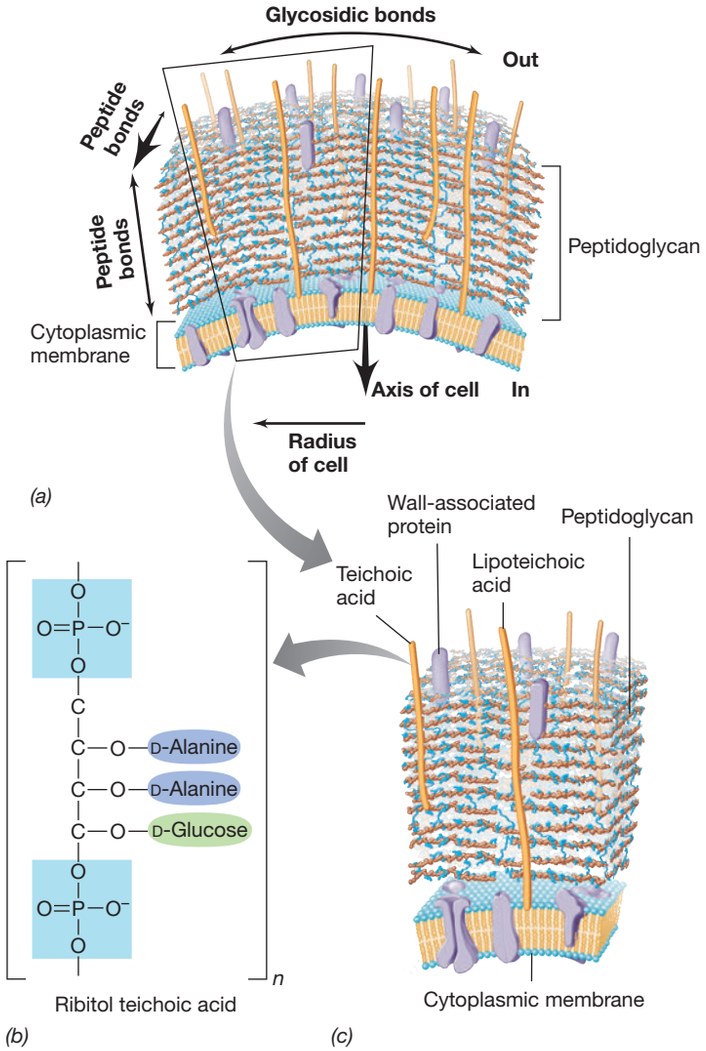


Figure 2.10 Structure of the gram-positive bacterial cell wall. *(a)* Schematic of a gram-positive cell wall showing the internal architecture of the peptidoglycan and its relationship to teichoic acids. Peptide cross-links form between peptidoglycan strands that are adjacent both horizontally and vertically, and peptidoglycan can also form covalent bonds to teichoic acids. *(b)* Structure of a ribitol teichoic acid. The teichoic acid is a polymer of the repeating ribitol unit shown here. *(c)* Summary diagram of the gram-positive bacterial cell wall. Lipoteichoic acids tether the cell wall to the cell membrane.

of *N*-acetylglucosamine (also present in peptidoglycan) and *N*-acetyltalosaminuronic acid; the latter replaces the *N*-acetylmuramic acid of peptidoglycan. Pseudomurein also differs from peptidoglycan in that the glycosidic bonds between the sugar derivatives are β-1,3 instead of β-1,4, and the amino acids are all of the L stereoisomer (compare Figures 2.9 and 2.11).

Because in many respects they are so similar, it is likely that peptidoglycan and pseudomurein are variants of a cell wall polysaccharide originally present in the common ancestor of *Bacteria* and *Archaea*. However, although they are structurally and functionally very similar, they differ sufficiently that pseudomurein is immune from destruction by both lysozyme and penicillin, molecules that destroy peptidoglycan.

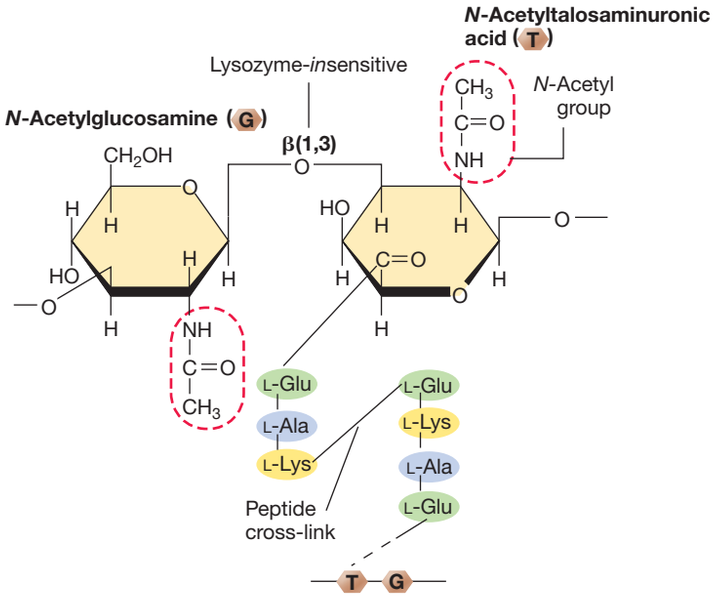


Figure 2.11 Pseudomurein. Structure of pseudomurein, the cell wall polymer of *Methanobacterium* species. Note the similarities and differences between pseudomurein and peptidoglycan (Figures 2.8 and 2.9).

Check Your Understanding

- Explain how peptidoglycan's structural arrangement resists turgor pressure.
- Describe the major differences between the cell walls of gram-negative and gram-positive bacteria.
- Explain whether you expect the enzyme lysozyme to be equally effective against *Bacteria* and *Archaea*.

2.4 LPS: The Outer Membrane

Most of the gram-negative cell envelope is composed of the **outer membrane**. The outer membrane is a second lipid bilayer found external to the cell wall (Figure 2.12), but its structure and function differs from that of the cytoplasmic membrane. The outer membrane and cytoplasmic membrane are similar in that they both contain phospholipid and protein, but a major difference is that the outer membrane also contains polysaccharide molecules covalently bound to lipids (Figure 2.12). Hence, the outer membrane is often called the **lipopolysaccharide** layer, or simply **LPS** for short.

Mastering Microbiology
Art Activity: Figure 2.14*a* Gram-negative cell wall structure

LPS molecules have several unique functions: They can facilitate surface recognition, they are important virulence factors for some bacterial pathogens, and they contribute to the mechanical strength of the cell. We will see that another major difference between the cytoplasmic and outer membranes is that the outer membrane contains *porins*, which are transmembrane proteins that allow for the nonspecific transport of solutes. Hence, we will see that the outer membrane is far more permeable than is the cytoplasmic membrane.

Structure and Activity of LPS

While the precise chemistry of LPS can vary among different species of bacteria, these molecules have several common features. As seen in Figure 2.13, LPS contains a polysaccharide that consists of two

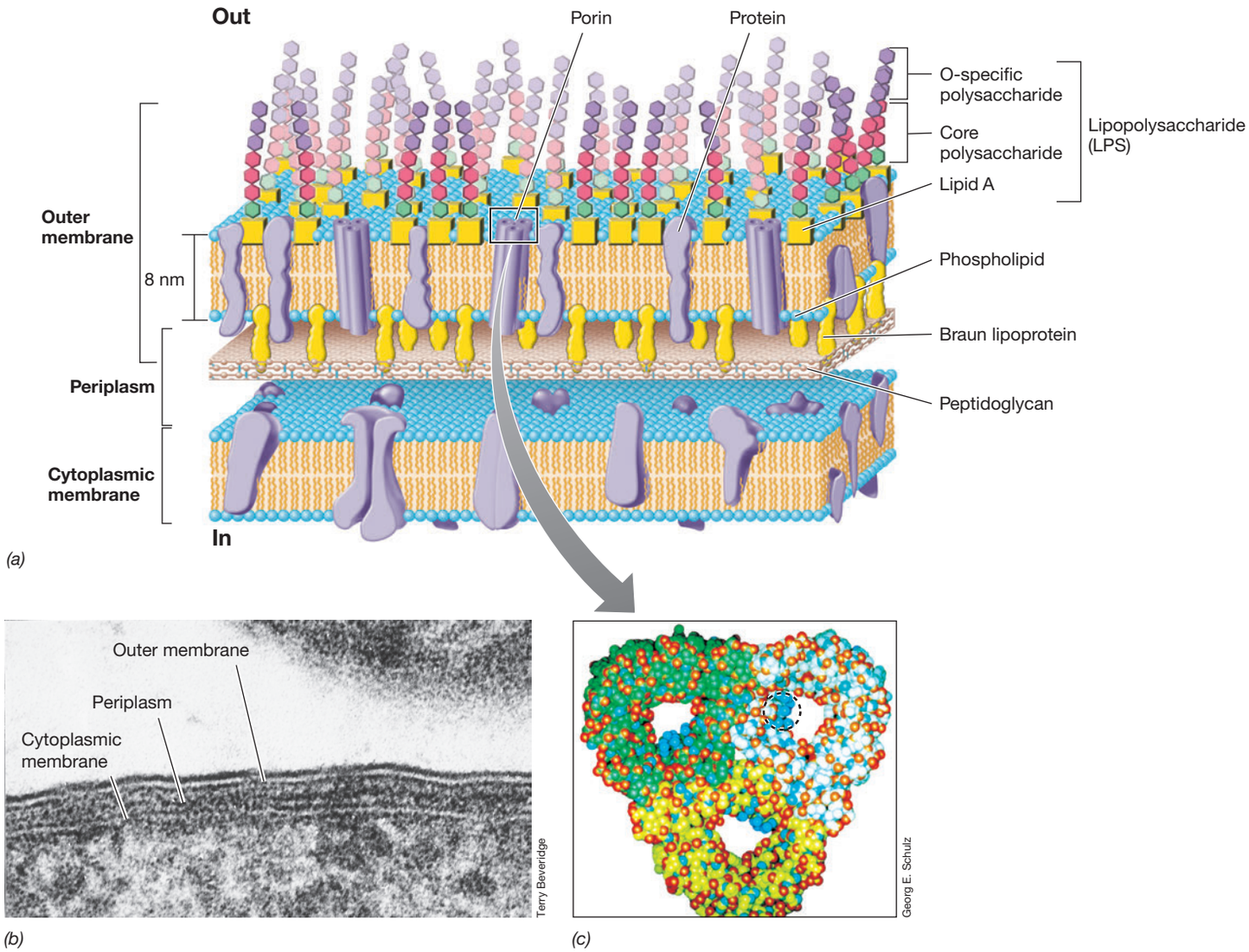


Figure 2.12 **The gram-negative bacterial cell envelope.** *(a)* Arrangement of lipopolysaccharide, lipid A, phospholipid, porins, and Braun lipoprotein in the outer membrane. See Figure 2.13 for details of the structure of LPS. *(b)* Transmission electron micrograph of a cell of *Escherichia coli* showing the cytoplasmic membrane and wall. *(c)* Molecular model of porin proteins showing their hollow pores that allow solute transport across the outer membrane. The view of the porin is perpendicular to the plane of the membrane. The black dashed circle highlights some of the hydrophilic amino acids that line the inside of the pore.

components, the *core polysaccharide* and the *O-specific polysaccharide* (see also Figure 2.12*a*). In *Salmonella* species, where LPS has been well studied, the core polysaccharide consists of ketodeoxyoctonate (KDO), various seven-carbon sugars (heptoses), the hexose sugars glucose and galactose, and *N*-acetylglucosamine. Connected to the core is the O-specific polysaccharide, which typically contains galactose, glucose, the hexoses rhamnose and mannose, and one or more dideoxyhexoses, such as abequose, colitose, paratose, or tyvelose. These sugars are connected in four- or five-membered sequences, which often are branched. When the sequences repeat, the long O-specific polysaccharide is formed. Within the outer membrane these negatively charged polysaccharides can be linked together tightly when adjacent LPS molecules mutually form ionic bonds to divalent cations (such as Ca^{2+} and Mg^{2+}). The presence of these ionic bonds confers considerable strength to the outer membrane, which rivals the gram-negative cell wall in its mechanical strength.

The lipid portion of the LPS, called *lipid A*, is not a typical glycerol lipid (see Figure 2.1*c*); instead the fatty acids are bonded through the amine groups from a disaccharide composed of glucosamine phosphate. The disaccharide is attached to the core polysaccharide through KDO (Figure 2.13). Fatty acids typically found in lipid A include caproic (C_6), lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), and stearic (C_{18}) acids. LPS replaces much of the phospholipid in the outer half of the outer membrane, and although the outer membrane is technically a lipid bilayer, its many unique components distinguish it from the cytoplasmic membrane. The outer membrane is anchored to the peptidoglycan layer by the *Braun lipoprotein*, a molecule that spans the gap between the LPS layer and the peptidoglycan layer (in the periplasm, discussed in the next subsection) (Figure 2.12*a*).

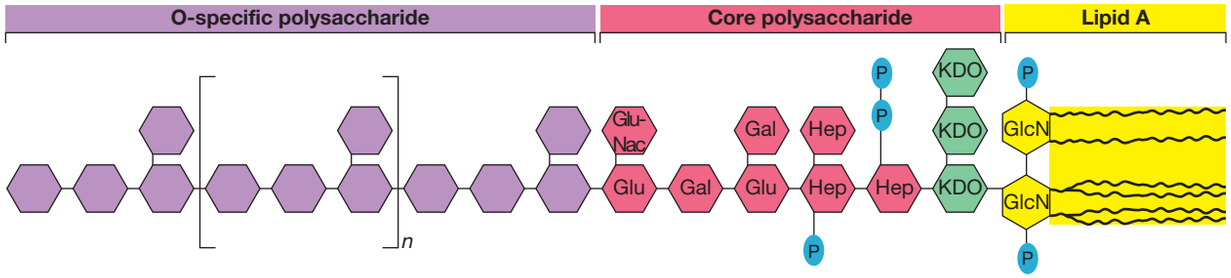


Figure 2.13 Structure of bacterial lipopolysaccharide. The chemical structures of lipid A and polysaccharides can vary among gram-negative *Bacteria*, but the major components (lipid A–KDO–core–O-specific) are typically invariant. The O-specific polysaccharide is highly variable among species. KDO, ketodeoxyoctonate; Hep, heptose; Glu, glucose; Gal, galactose; GluNac, *N*-acetylglucosamine; GlcN, glucosamine; P, phosphate. Glucosamine and the lipid A fatty acids are linked through the amine groups of GlcN. The lipid A portion of LPS can be toxic to animals and comprises the endotoxin complex. Compare the art here with that of Figure 2.12 and follow the LPS components by their color-coding.

An important biological activity of LPS is its toxicity to animals. Common gram-negative pathogens for humans include species of *Salmonella*, *Shigella*, and *Escherichia*, among many others, and some of the gastrointestinal symptoms these pathogens elicit are due to their toxic outer membrane components. Toxicity is specifically linked to the LPS layer, in particular, to lipid A. The term *endotoxin* refers to this toxic component of LPS. Some endotoxins cause violent symptoms in humans, including gas, diarrhea, and vomiting, and the endotoxins produced by *Salmonella* and enteropathogenic strains of *Escherichia coli* transmitted in contaminated foods are classic examples of this. We discuss major gram-negative enteric pathogens in Chapter 33 and endotoxin in Section 25.8.

The Periplasm and Porins

The outer membrane is impermeable to proteins and other very large molecules. In fact, a major function of the outer membrane is to prevent cellular proteins whose activities must occur outside the cytoplasm from diffusing away from the cell. These extracellular proteins reside in the **periplasm**, a space of about 15 nm located between the *outer surface* of the cytoplasmic membrane and the *inner surface* of the outer membrane (Figure 2.12*a*, *b*).

The periplasm may contain several different classes of proteins. These include hydrolytic enzymes, which function in the initial degradation of polymeric substances; binding proteins, which begin the process of transporting substrates (Section 2.2 and Figure 2.6); chemoreceptors, which are proteins that govern the chemotaxis response (Section 2.11); and proteins that construct extracellular structures (such as peptidoglycan and the outer membrane) from precursor molecules secreted through the cytoplasmic membrane. Most periplasmic proteins reach the periplasm by way of a protein-exporting system present in the cytoplasmic membrane (▶ Sections 6.12 and 6.13).

The outer membrane is relatively permeable to small molecules because of proteins called *porins* that function as channels for the entrance and exit of solutes (Figure 2.12*a*, *c*). Porins are unique to the outer membrane of *Bacteria* and should not be confused with *aquaporins*, which are a different class of proteins (aquaporins facilitate water transport across the cytoplasmic membrane). Several porins are known, including both specific and nonspecific classes. Nonspecific porins form water-filled channels through which most very small hydrophilic substances can pass. By contrast, specific porins contain a binding site for one or a group of structurally related substances. Porins are transmembrane proteins composed of three identical polypeptides; the proteins are arranged to form channels through which solutes can diffuse (Figure 2.12*c*).

While the cell envelopes of many *Bacteria* conform to either the gram-positive or the gram-negative model, we have already learned that *Archaea* have cell envelopes that diverge from both of these model structures. In the next section we will see that there is considerable diversity in cell envelope structure across the microbial world.

Check Your Understanding

- Describe and contrast the cell envelope structure of gram-negative and gram-positive bacteria.
- What is the function of porins, and where are they located in the gram-negative cell envelope?
- What component of the gram-negative cell envelope has endotoxin properties?

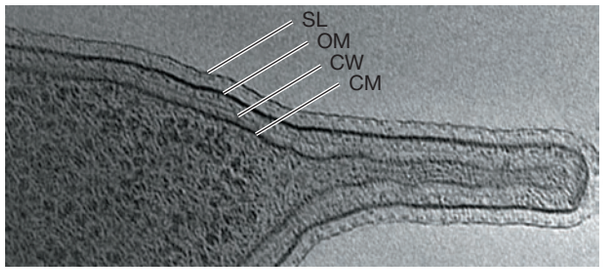
2.5 Diversity of Cell Envelope Structure

While many *Bacteria* have a gram-positive or gram-negative cell envelope organization (Figure 2.7), a variety of cell envelope structures are known. One way in which cell envelopes can vary between cells is in the presence of an *S-layer*.

S-Layers

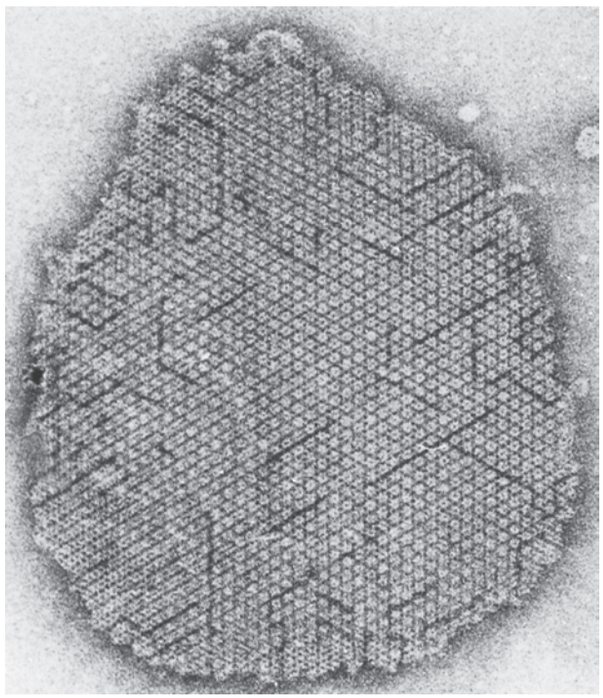
S-layers are found in many *Bacteria* and in nearly all *Archaea*. An S-layer consists of a paracrystalline monolayer of interlocking molecules of protein or glycoprotein (**Figure 2.14**). When an S-layer is present, it is always the *outermost* layer of the cell envelope (Figure 2.14*a*). The S-layer is usually composed of only one or a few subunits self-organized into repeating structures, which can have hexagonal, tetragonal, or trimeric symmetry. These repeating units form a rigid yet permeable paracrystalline lattice (Figure 2.14*b*), which can be as much as 5–20 nm thick in *Bacteria* and up to 70 nm thick in some *Archaea*.

S-layers have many important functions. In many *Archaea*, thick S-layers can take on the role of the cell wall and are responsible for providing structural strength, protecting the cell from osmotic lysis,



Tanmay A.M. Bharat

(a) **Cell envelope of *Caulobacter crescentus***



Susan F. Koval

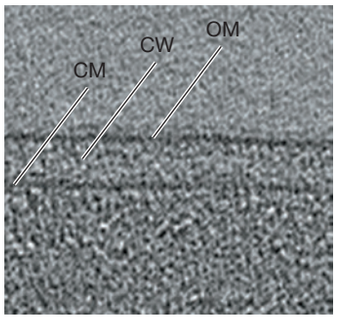
(b) **S-layer fragment from *Aquaspirillum***

Figure 2.14 S-layers. *(a)* The S-layer (SL), outer membrane (OM), peptidoglycan cell wall (CW), and cytoplasmic membrane (CM) can be clearly seen in this electron tomographic slice through a cell of *Caulobacter crescentus*, a stalked bacterium that has a gram-negative cell envelope plus an outer S-layer. *(b)* Transmission electron micrograph of a portion of an S-layer removed from the bacterium *Aquaspirillum* and flattened to show the paracrystalline nature and hexagonal symmetry common to S-layers. The stalk in *a* has an outer diameter of about 150 nm and both images are at the same scale.

and conferring cell shape. The S-layer can also create a periplasmic-like space in *Archaea*. S-layers function as molecular sieves and have pore sizes in the range of 2–10 nm in diameter. These pores are large enough to allow low-molecular-weight compounds to pass but small enough to trap large molecules—such as most proteins—in the space between the S-layer and the cytoplasmic membrane. The compartment formed between the cytoplasmic membrane and the S-layer thus functions much as the periplasm does in gram-negative bacteria, including forming a site near the cytoplasmic membrane where reactions important to cellular metabolism can occur. As the outermost layer of the cell, S-layers can also facilitate cell surface interactions, such as attachment. S-layers can also increase the ability of some bacterial pathogens to cause disease by either promoting adhesion or protecting the cell from host defenses.

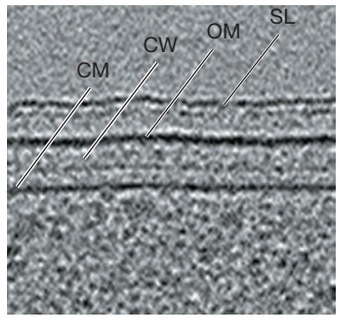
Alternative Configurations of the Cell Envelope

We have now learned about the most common components found in the cell envelope: the cytoplasmic membrane (CM), the cell wall (CW), the outer membrane (OM), and S-layers (SL). While certain configurations of these structures are common, a range of different configurations are possible (**Figure 2.15**). A common variation on cell envelope structure is to find an outer S-layer surrounding an otherwise gram-positive or gram-negative bacterium (compare Figure 2.15*a* with Figure 2.15*b*). In addition, many *Archaea* have *only* an S-layer outside of their cytoplasmic membrane, and these layers, while always constructed of some type of paracrystalline protein or



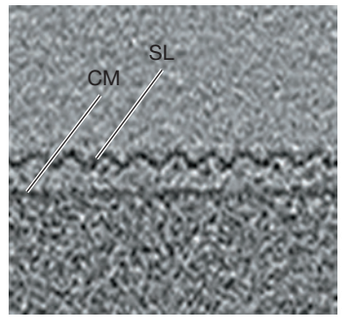
Y-W. Yang, G. Jensen Lab

(a) ***Vibrio cholerae***



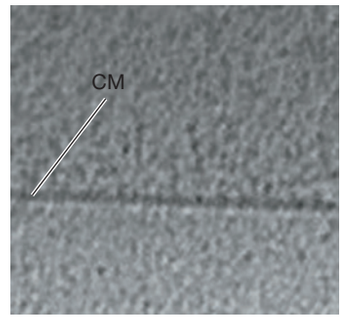
Q. Yao, G. Jensen Lab

(b) ***Caulobacter crescentus***



R. Ramdasi, G. Jensen Lab

(c) ***Nitrosopumilus maritimus***



J. Shi, G. Jensen Lab

(d) ***Mycoplasma pneumoniae***

Figure 2.15 Alternative cell envelope structures. Cell envelope structures including cytoplasmic membranes (CM), cell walls (CW), outer membranes (OM), and S-layers (SL) can be found in both bacterial and archaeal species. *(a) Vibrio cholerae* has a classic gram-negative type bacterial cell envelope. *(b) Caulobacter crescentus* is a bacterium with a gram-negative envelope and an S-layer (see also Figure 2.14*a*). *(c) Nitrosopumilus maritimus* has a typical archaeal cell envelope containing a CM and an SL. *(d) Mycoplasma pneumoniae* is a pathogenic bacterium whose cell envelope consists of only a CM. S-layers, while typically composed of a paracrystalline protein or glycoprotein layer (see also Figure 2.14*b*) can vary considerably in molecular structure (compare the S-layers in *b* and *c*). All images are transmission electron tomographs, a technique in which a special transmission electron microscope passes electrons through a specimen at different angles and then consolidates the views to form a final three-dimensional image.

glycoprotein, can vary considerably in their molecular structures (compare Figure 2.15*b* with Figure 2.15*c*). Some methanogenic *Archaea* also have cell walls made of pseudomurein, and such cell walls may or may not have an outer S-layer as well. Finally, though unusual, *Archaea* such as the heat-loving *Ignicoccus* actually have an outer membrane. This structure in *Ignicoccus* is unlike that of gram-negative bacteria in that it is composed largely of archaeal isoprenoid lipids and lacks LPS.

Although this is uncommon, a few *Bacteria* and *Archaea* lack cell walls altogether. These include in particular the mycoplasmas (Figure 2.15*d*) and other pathogenic *Bacteria* that grow within a host cell, and *Archaea* such as *Thermoplasma* and its relatives. Lacking a cell wall, these cells would be expected to contain unusually tough cytoplasmic membranes, and chemical analyses show that they do. For example, most mycoplasmas contain *sterols* in their cytoplasmic membranes; these molecules function to add strength and rigidity to the membrane as they do in the cytoplasmic membranes of eukaryotic cells. Mycoplasmas may also have little need for a cell wall because they experience little osmotic pressure when living within the cytoplasm of another cell. In addition, the loss of peptidoglycan may help mycoplasmas evade the host immune system because host defenses recognize bacterial cell wall components as one of many signals of bacterial invasion (Chapter 26).

Check Your Understanding

- What is the structure of an S-layer, and what are its functions?
- What are some alternate arrangements of cell envelope structure?

II • Cell Surface Structures and Inclusions

Many prokaryotic cells contain a cell surface layer that can have a variety of functions. Cytoplasmic inclusions may also be present and function as food reserves or bestow upon the cell a unique capacity of ecological value.

The cell envelope governs many aspects of how microbes interact with their environments and with other cells. Likewise, many other structures visible to microscopy can have profound impacts on cellular function. In particular, structures found on the cell surface and inclusions—structures present within the cell's cytoplasm—have many important functions that govern microbial interactions with the world around them.

2.6 Cell Surface Structures

Many *Bacteria* and *Archaea* secrete sticky or slimy materials on their cell surface that consist of either polysaccharide or protein. However, these are distinct from and external to the cell envelope. The terms "capsule" and "slime layer" are used to describe these layers. These outer layers can mediate attachment, they can protect the cell from attack and from environmental stresses, and they can alter the diffusive environment of the cell.

Capsules and Slime Layers

The terms *capsule* and *slime layer* are used to describe a sticky coat of polysaccharide formed outside of the cell envelope. If the polysaccharide layer is organized in a tight matrix that excludes small particles and is tightly attached to the cell, it is called a **capsule**. Capsules are readily visible by light microscopy if cells are treated with India ink, which contains particulates that stain the background but cannot penetrate the capsule; capsules can also be seen in the electron microscope (**Figure 2.16*b–d***). By contrast, if the surface

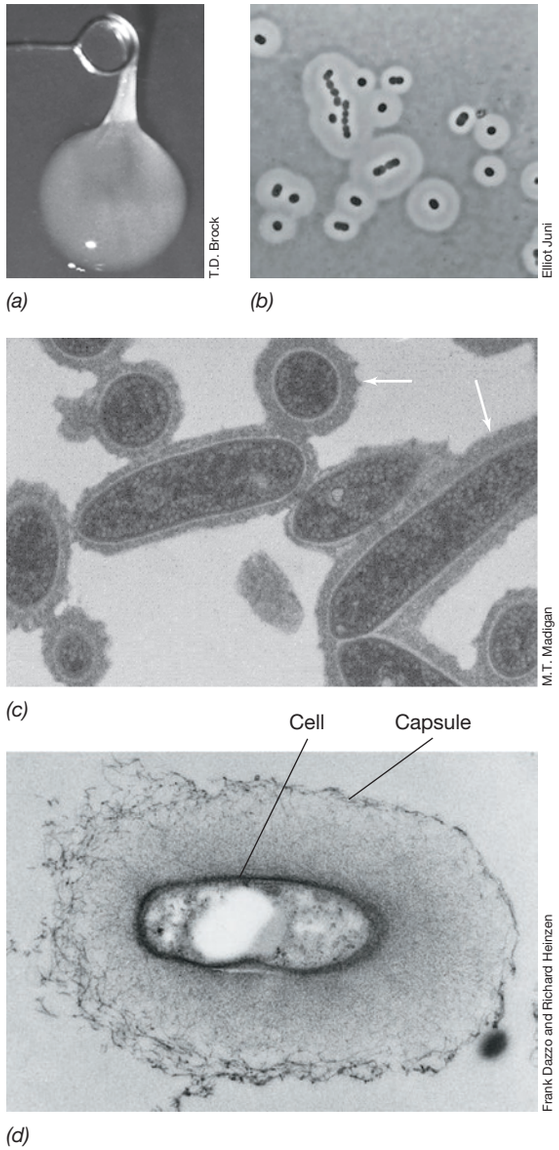


Figure 2.16 Bacterial capsules and slime formation. *(a)* A semisolid colony of the bacterium *Leuconostoc mesenteroides* (lifted up by an inoculating loop) contains a thick dextran (glucose polymer) slime layer formed by the cells. *(b)* Capsules of *Acinetobacter* species observed by phase-contrast microscopy after negative staining with India ink. India ink does not penetrate the capsule and so the capsule appears as a light area surrounding the cell, which appears black. *(c)* Transmission electron micrograph of a thin section of cells of *Rhodobacter capsulatus* with capsules (arrows) clearly evident; cells are about 0.9 μm wide. *(d)* Transmission electron micrograph of *Rhizobium leguminosarum* biovar *trifolii* stained with ruthenium red to reveal the capsule. The cell is about 0.7 μm wide.

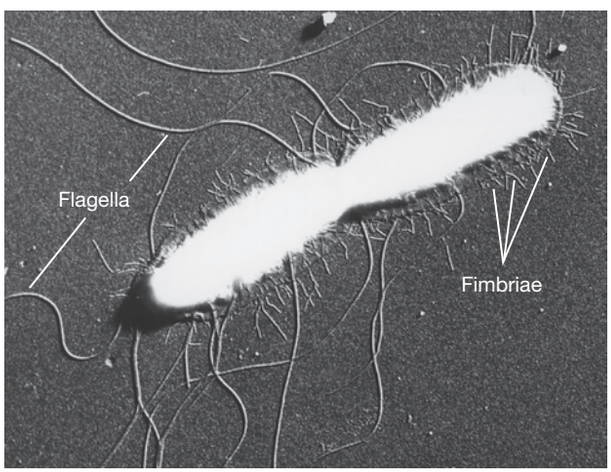
layer is easily deformed and loosely attached, it will not exclude particles and is more difficult to see microscopically. Such a loosely attached polysaccharide coat is called a *slime layer*, and it is easily detected in colonies of slime-forming species such as the lactic acid bacterium *Leuconostoc* (Figure 2.16*a*).

Outer surface layers have several functions. Surface polysaccharides assist in the attachment of microorganisms to solid surfaces. As we will see later, pathogenic microorganisms that enter the body by specific routes usually do so by first binding to specific surface components of host tissues; this binding is often facilitated by bacterial cell surface polysaccharides. When the opportunity arises, many bacteria will bind to solid surfaces, often forming a thick layer of cells called a *biofilm* (▶ Section 4.9). Extracellular polysaccharides play a key role in the development and maintenance of biofilms as well.

Besides attachment, outer surface layers have other functions. These include contributing to the infectivity of a bacterial pathogen and preventing dehydration. For example, the causative agents of the diseases anthrax and bacterial pneumonia—*Bacillus anthracis* and *Streptococcus pneumoniae*, respectively—each contain a thick capsule of either protein (*B. anthracis*) or polysaccharide (*S. pneumoniae*). Encapsulated cells of these bacteria avoid destruction by the host's immune system because the immune cells that would otherwise recognize these pathogens as foreign and destroy them are blocked from doing so by the bacterial capsule. In addition to this role in disease, bacterial outer surface layers bind water, and this helps protect the cell from desiccation in periods of dryness.

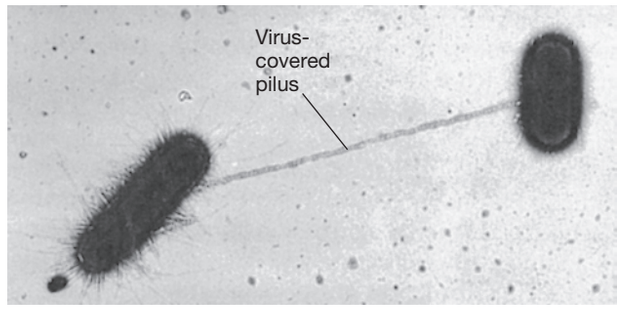
Fimbriae, Pili, and Hami

Pili are thin (2–10 nm in diameter) filamentous structures made of protein that extend from the surface of a cell and can have many functions. Short pili that mediate attachment are often called *fimbriae* (**Figure 2.17**). Pili enable bacterial cells to stick to surfaces, including animal tissues, or to form pellicles (thin sheets of cells on a liquid surface) or biofilms on solid surfaces. All gram-negative bacteria produce pili of one sort or another, and many gram-positive bacteria also contain these structures. Pili, by allowing bacteria to attach to other cells, often contribute to the virulence of pathogens.



J.P. Duguid and J.F. Wilkinson

Figure 2.17 Fimbriae. Electron micrograph of a dividing cell of *Salmonella enterica* (*typhi*), showing flagella and fimbriae. A single cell is about 0.9 μm wide.



Charles C. Brinton, Jr. and Judith Carnahan

Figure 2.18 Pili. The pilus on an *Escherichia coli* cell that is undergoing conjugation. The cells are about 0.8 μm wide. The visibility of the pilus in this electron micrograph has been improved because it is coated with viral particles that bind to the pilin protein.

Many classes of pili are known, and they can have diverse functions. As already mentioned, pili can enable bacteria to adhere to surfaces and this function can allow pathogens to target and invade specific host tissues. However, pili are diverse and they can have several other important functions as well. For example, *conjugative pili* facilitate genetic exchange by causing cell-to-cell attachment (**Figure 2.18**) during a process called *conjugation* (▶ Section 9.8). In addition, *electrically conductive pili* (also known as *nanowires*, ▶ Section 23.3) can conduct electrons toward or away from the cell and in so doing play an important role in the energy metabolism of diverse microbes (▶ Sections 14.13 and 15.13). Lastly, a type of pili called *type IV pili* not only facilitate adhesion but also support an unusual form of cell movement called *twitching motility* in certain bacterial species (see Section 2.10).

Twitching motility allows cells to move along a solid surface. In twitching motility, pili are extended away from the cell, attach to a surface, and are subsequently retracted, dragging the cell forward. ATP supplies the energy necessary for extension and retraction of the pilus. On rod-shaped cells that move by twitching, type IV pili are present only at the cell poles. Type IV pili assist in infectivity by certain pathogens, including the gram-negative bacteria *Vibrio cholerae* (cholera) and *Neisseria gonorrhoeae* (gonorrhea) and the gram-positive bacterium *Streptococcus pyogenes* (strep throat and scarlet fever). The twitching motility of these organisms assists them in locating specific sites for attachment to initiate the disease process. Type IV pili are also widespread in the *Archaea*, functioning in surface adhesion and cell aggregation events that lead to biofilm formation.

An unusual group of *Archaea*, the SM1 group, forms a unique attachment structure called a *hamus* (plural, hami) that resembles a tiny grappling hook (**Figure 2.19*a, b***). The SM1 group inhabits anoxic groundwater in Earth's deep subsurface, and hami function to affix cells to a surface to form a networked biofilm (Figure 2.19*c*). Hami structurally resemble type IV pili except for their barbed terminus, which functions to attach cells both to surfaces and to each other (Figure 2.19*c*). The biofilms formed by SM1 *Archaea* are likely an ecological strategy that allows these microbes to more efficiently trap the scarce nutrients present in their deep subsurface habitat.

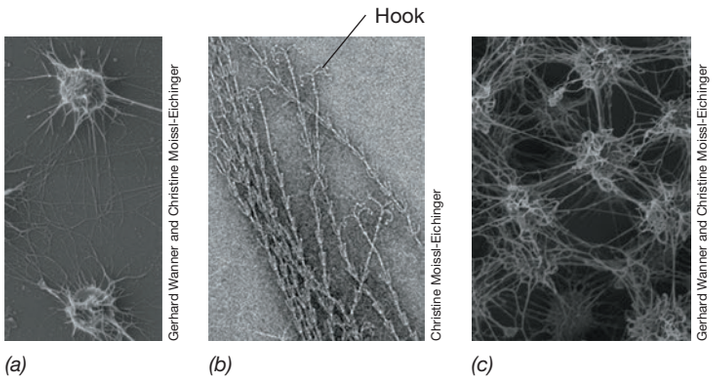


Figure 2.19 Unique attachment structures in the SM1 group of *Archaea*: Hami. *(a)* Cells of SM1 *Archaea* showing the pili-like surface structures called hami. *(b)* Transmission electron micrograph of isolated hami. A hamus "grappling hook" (labeled "Hook" in the micrograph) is about 60 nm in diameter. *(c)* A biofilm of SM1 cells showing the network of hami connecting individual cells.

Although cells of the SM1 group are not as small as the groundwater ultramicrobacterial cells described in Chapter 1 (see Explore the Microbial World, "Tiny Cells"), they are less than 1 μm in diameter and live in a similar nutrient-limiting habitat. Thus, their hami likely play an important role in preventing cells from being washed away in groundwater flowage.

In addition to the cell surface, the *cytoplasm* of a prokaryotic cell may contain enclosed and sometimes rigid structures that benefit the cell in one way or another, and we consider these now.

Check Your Understanding

- How does the thick capsule help encapsulated bacteria establish an infection in a host?
- How do fimbriae differ from pili, both structurally and functionally?
- How would inactivating the conjugative pili mechanism in bacteria help prevent antibiotic resistance genes from spreading between bacteria?

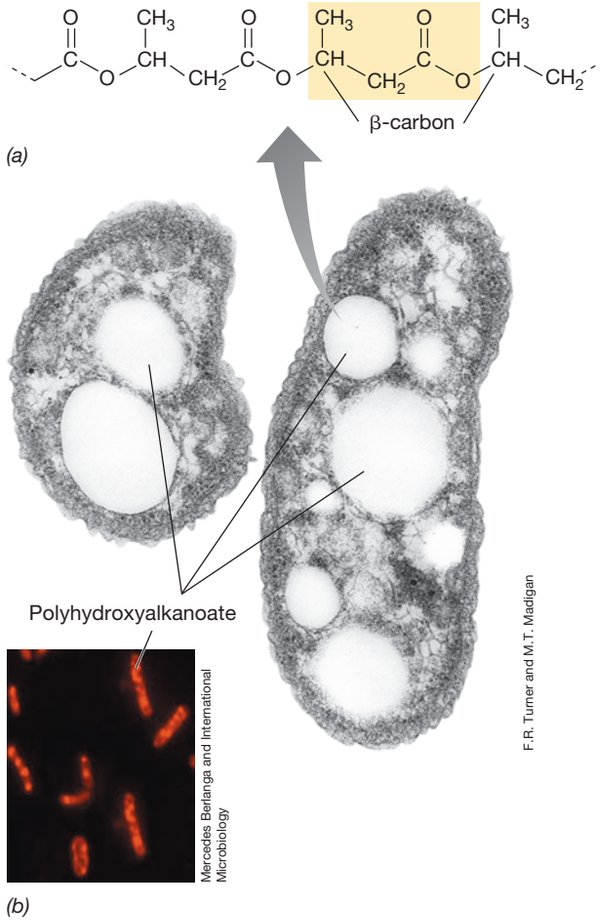


Figure 2.20 Poly-β-hydroxyalkanoates (PHAs). *(a)* Chemistry of poly-β-hydroxybutyrate, a common PHA. A monomeric unit is highlighted in color. Other PHAs are made by substituting longer-chain hydrocarbons for the $-CH_3$ group on the β-carbon. *(b)* Electron micrograph of a thin section of cells of a bacterium containing granules of PHB. Color photo: Nile red–stained cells of a PHA-containing bacterium.

2.7 Cell Inclusions

Prokaryotic cells often contain inclusions of one sort or another. Many inclusions store energy or nutrients (such as carbon or phosphorus), but some have other highly specialized functions that confer unique properties on the cells that contain them. Inclusions are often visible in cells with the light microscope and are enclosed by a single-layer (as opposed to a unit) membrane composed of proteins that partitions off the inclusion in the cytoplasm. Storing carbon or other substances in an insoluble form within the cytoplasm reduces osmotic stress and takes up less space compared with storing these substances in a soluble form.

Carbon Storage Polymers

One of the most common inclusion bodies in prokaryotic organisms is **poly-β-hydroxybutyric acid (PHB)**, a lipid that is formed from β-hydroxybutyric acid units. The monomers of PHB polymerize by ester linkage and then the polymer aggregates into granules; the granules can be seen by either light or electron microscopy (**Figure 2.20**). The monomer in the polymer is usually hydroxybutyrate (C_4) but can vary in length from as short as C_3 to as long as C_{18}. Thus, the more generic term *poly-β-hydroxyalkanoate* (PHA) is often used to describe this class of carbon- and energy-storage polymers. PHAs are synthesized by cells when there is an excess of carbon and are broken down as carbon or energy sources when conditions warrant.

Another carbon storage inclusion is *glycogen*, which is a polymer of glucose; like PHA, glycogen is a reservoir of both carbon and energy and is produced when carbon is in excess. Glycogen resembles starch, the major storage reserve of plants, but differs slightly from starch in the manner in which the glucose units are linked together.

Polyphosphate, Sulfur, and Carbonate Minerals

Many prokaryotic and eukaryotic microbes accumulate inorganic phosphate (PO_4^{3-}) in the form of *polyphosphate* granules (**Figure 2.21*a***). These granules are formed when phosphate is in excess and can be drawn upon as a source of phosphate for nucleic acid and phospholipid biosynthesis when phosphate is limiting. In

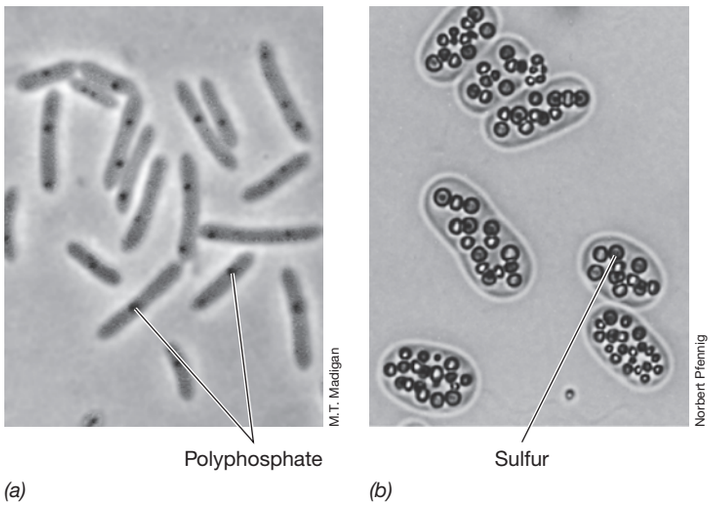


Figure 2.21 **Polyphosphate and sulfur storage products.** *(a)* Phase-contrast photomicrograph of cells of *Heliobacterium modesticaldum* showing polyphosphate as dark granules; a cell is about 1 μm wide. *(b)* Bright-field photomicrograph of cells of the purple sulfur bacterium *Isochromatium buderi*. The periplasmic inclusions are sulfur globules formed from the oxidation of hydrogen sulfide (H_2S). A cell is about 4 μm wide.

addition, in some organisms, polyphosphate can be broken down to synthesize the energy-rich compound ATP from ADP.

Many gram-negative *Bacteria* and several *Archaea* oxidize reduced sulfur compounds, such as hydrogen sulfide (H_2S); these organisms are the "sulfur bacteria," discovered by the great Russian microbiologist Sergei Winogradsky (◀ Section 1.13). The oxidation of sulfide generates electrons for use in energy metabolism (chemolithotrophy) or CO_2 fixation (autotrophy). In either case, *elemental sulfur* (S^0) from the oxidation of sulfide may accumulate in the cell in microscopically visible granules (Figure 2.21*b*). This sulfur remains as long as the source of reduced sulfur from which it was derived is still present. However, as the reduced sulfur source becomes limiting, the S^0 in the granules is oxidized to sulfate (SO_4^{2-}), and the granules slowly disappear. Interestingly, although sulfur globules appear to reside in the cytoplasm (Figure 2.21*b*), they are actually present in the periplasm (Section 2.4). In these cells the periplasm expands outward to accommodate the growing globules as H_2S is oxidized to S^0 and then contracts inward as S^0 is oxidized to SO_4^{2-}.

Filamentous cyanobacteria have long been known to form carbonate minerals on the external surface of their cells. However, some cyanobacteria also form carbonate minerals *inside* the cell, as cell inclusions. For example, the unicellular cyanobacterium *Gloeomargarita* forms intracellular granules of benstonite, a carbonate mineral that contains barium, strontium, and magnesium (**Figure 2.22**). The microbiological process of forming minerals is called *biomineralization*. It is unclear why benstonite is formed by *Gloeomargarita*, although it might function as ballast to maintain cells of this cyanobacterium in their habitat, deep in an alkaline lake. Alternatively (or in addition), the mineral could be a way to sequester carbonate (a source of CO_2) to support autotrophic growth.

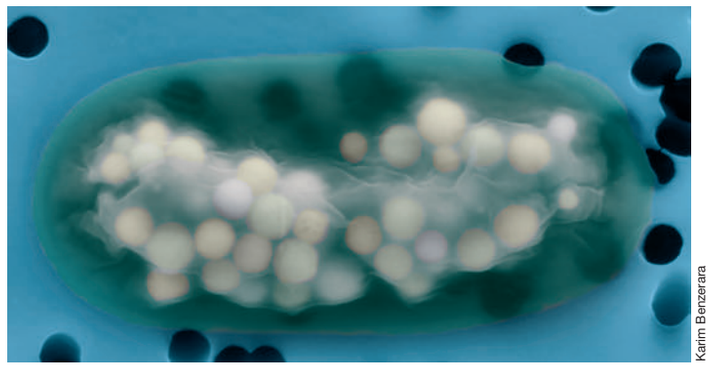


Figure 2.22 **Biomineralization by a cyanobacterium.** Electron micrograph of a cell of the cyanobacterium *Gloeomargarita* containing granules of the mineral benstonite [$(Ba,Sr)_6(Ca,Mn)_6Mg(CO_3)_{13}$]. A cell is about 2 μm wide.

Gas Vesicles

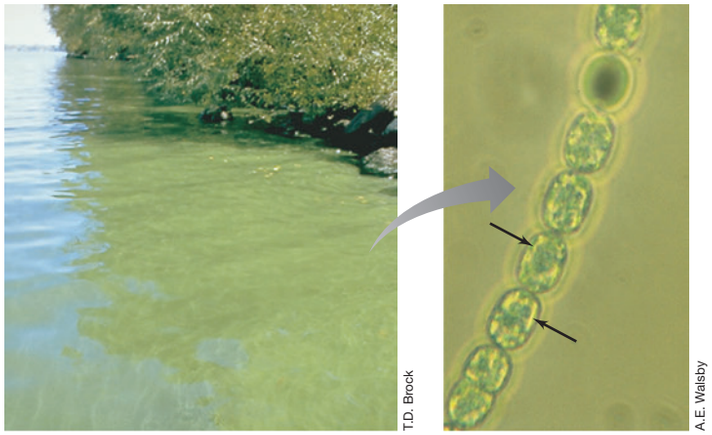
Some *Bacteria* and *Archaea* can float because they contain **gas vesicles**, structures that confer buoyancy and allow the cells to position themselves in regions of the water column that best suit their metabolisms. The most dramatic examples of gas-vesiculate microbes are those cyanobacteria that form massive accumulations called *blooms* in lakes or other bodies of water. These blooms are commonly on or near the lake surface (**Figure 2.23*a***) where sunlight is most intense and photosynthesis can occur at maximal rates.

Gas vesicles are conical-shaped structures composed of two different proteins; they are hollow yet rigid and of variable length and diameter (Figure 2.23*b*, *c*). Gas vesicles in different species vary from 300 to more than 1000 nm in length and from 45 to 120 nm in width. Gas vesicles may number from a few to hundreds per cell and are impermeable to water and solutes but permeable to gases. The composition and pressure of the gas inside a gas vesicle is that in which the organism is suspended. This could be air at 1 atm in cyanobacteria on a lake surface (Figure 2.23*a*), or a mixture of gases such as N_2, CO_2, and H_2 at greater than 1 atm in gas vesiculate species that inhabit anoxic zones deeper in the lake. The presence of gas vesicles in cells can be detected either by light microscopy, where clusters of vesicles, called *gas vacuoles*, appear as irregular bright inclusions (Figure 2.23*b*), or by transmission electron microscopy of cell thin sections (Figure 2.23*c*).

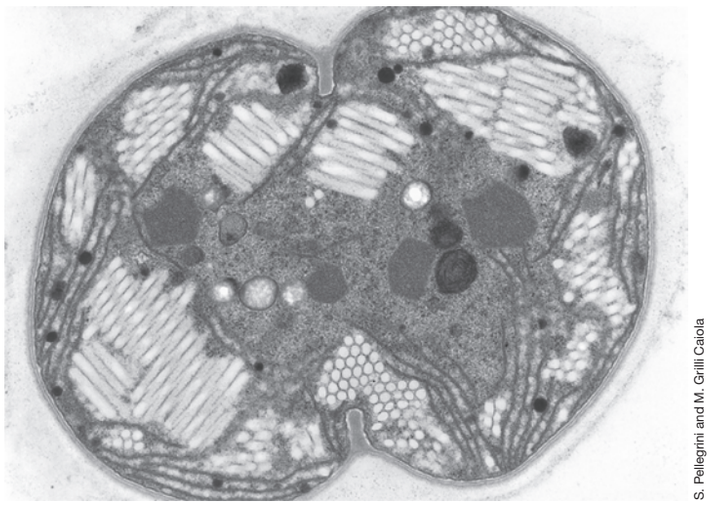
Magnetosomes

Some bacteria can orient themselves within a magnetic field because they contain **magnetosomes**. These structures are biomineralized particles of the magnetic iron oxides magnetite [$Fe(II)Fe(III)_2O_4$] or greigite [$Fe(II)Fe(III)_2S_4$] (**Figure 2.24**). Magnetosomes impart a magnetic dipole on a cell, allowing it to orient itself in a magnetic field. This allows the cell to undergo *magnetotaxis*, the process of migrating along Earth's magnetic field lines (see Section 2.12).

Magnetosome synthesis begins with insertion of magnetosome-specific proteins into the cytoplasmic membrane followed by invagination of the membrane to form a vesicle. The vesicle is then filled with iron—primarily iron in the Fe(II) oxidation state—and biomineralization proceeds through the activities of the magnetosome



(a) (b)



(c)

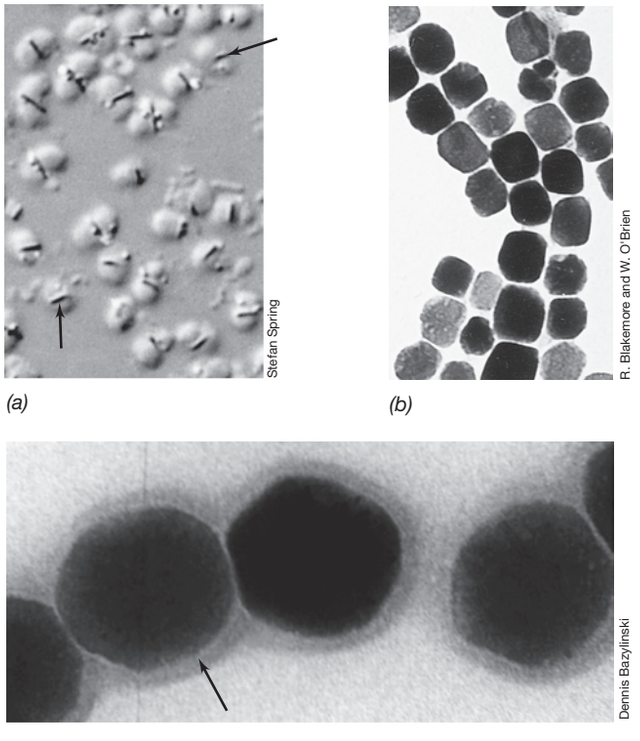
Figure 2.23 Buoyant cyanobacteria and their gas vesicles. *(a)* Flotation of a bloom of gas-vesiculate cyanobacteria in a freshwater lake. *(b)* Phase-contrast photomicrograph of *Anabaena*. Clusters of gas vesicles form phase-bright gas vacuoles (small arrows). *(c)* Transmission electron micrograph of a dividing cell of *Microcystis*. Gas vesicles are arranged in bundles, here seen in both longitudinal and cross section. A cell of *Microcystis* is about 5 μm wide.

proteins, which includes an iron oxidase enzyme that catalyzes formation of the Fe(III) needed to form the magnetic minerals. The morphology of magnetosomes varies and appears to be species-specific; several morphologies are possible, but square, rectangular, or spike-shaped magnetosomes are most common.

We now consider a special case of a cytoplasmic enclosed cell structure—the endospore.

Check Your Understanding

- Under what nutritional conditions would you expect PHAs or glycogen to be produced?
- Why would it be impossible for gram-positive bacteria to store sulfur as gram-negative sulfur-oxidizing chemolithotrophs can?
- How are magnetosomes and the *Gloeomargarita* inclusions similar and how do they differ? What are gas vesicles made of and what is inside them?



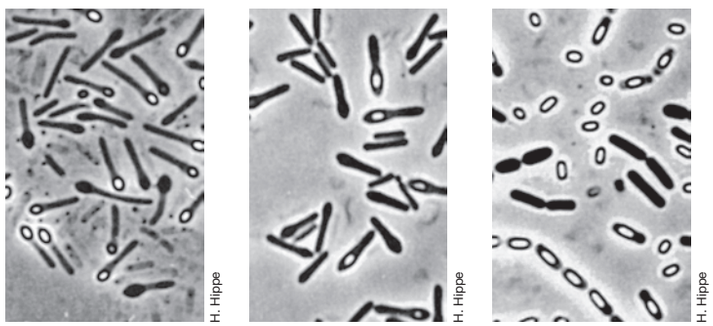
(a) (b)

(c)

Figure 2.24 Magnetotactic bacteria and magnetosomes. *(a)* Differential interference contrast micrograph of coccoid magnetotactic bacteria; note chains of magnetosomes (arrows). A cell is 2.2 μm wide. *(b)* Magnetosomes isolated from the magnetotactic bacterium *Magnetospirillum magnetotacticum*; each particle is about 50 nm wide. *(c)* Transmission electron micrograph of magnetosomes from an unnamed magnetic coccus. The arrow points to the membrane that surrounds each magnetosome. A single magnetosome is about 90 nm wide.

2.8 Endospores

Many microbes produce spore structures that allow them to survive unfavorable conditions. However, certain species of *Bacteria* produce specialized spores called **endospores** (Figure 2.25). Endospores (the prefix *endo-* means "within") are highly differentiated dormant cells that function as survival structures and can tolerate harsh



(a) Terminal endospores (b) Subterminal endospores (c) Central endospores

Figure 2.25 The bacterial endospore. Phase-contrast photomicrographs showing different intracellular locations of endospores in different species of bacteria. Endospores appear bright by phase-contrast microscopy.

environmental conditions, including extreme heat, radiation, chemical exposure, drying, and nutrient depletion. Endospores are not reproductive structures, such as the spores of fungi, but are rather the dormant stage of a bacterial life cycle: vegetative cell → endospore → vegetative cell (**Figure 2.26**).

Many microbes can form spores or spore-like structures, including the myxospores of myxobacteria (▶ Section 15.16), the arthrospores of actinomycetes (▶ Section 16.12), and various forms of cysts, but endospores are exceptional for their extreme resistance to environmental challenges. Most notably, endospores are the only type of spore that can tolerate high heat, being able to survive for hours in boiling water. And, because of their unique structure, endospores can remain dormant for hundreds and perhaps even thousands of years, only to germinate and grow when conditions become favorable. Endospores are easily dispersed by wind, water, or through the animal gut, and hence endospore-forming bacteria are widely distributed in nature.

Endospores are only produced by two groups of bacteria, the *Bacillales* and *Clostridiales*, both of which are gram-positive bacteria of the phylum *Firmicutes*. These bacteria share an ancestor and so it is likely that the ability to form endospores evolved only once, though many species within these groups have lost the ability to form endospores over time. The best-studied endospore formers are in the genera *Bacillus* and *Clostridium*. These bacteria are found widely in soil and other environments, and some are well-known pathogens of humans and other animals. In particular, endospore-forming bacteria are a major cause of food spoilage and foodborne disease. Botulism, tetanus, and several foodborne bacterial infections are caused by species of endospore-forming bacteria (Chapters 32 and 33).

Endospore Formation and Germination

The process of cellular differentiation that results in endospore formation is called *sporulation*. During endospore formation, a vegetative cell is converted through a process of cellular differentiation into a nongrowing, heat-resistant, and light-refractive structure (Figure 2.26). Sporulation is typically triggered when some nutrient becomes limiting. When the cell senses this, the developmental process that converts a vegetative cell into an endospore begins and is controlled by a complex regulatory system we will consider later (▶ Section 8.6). Our focus here is not on regulation but instead on the major steps in the process.

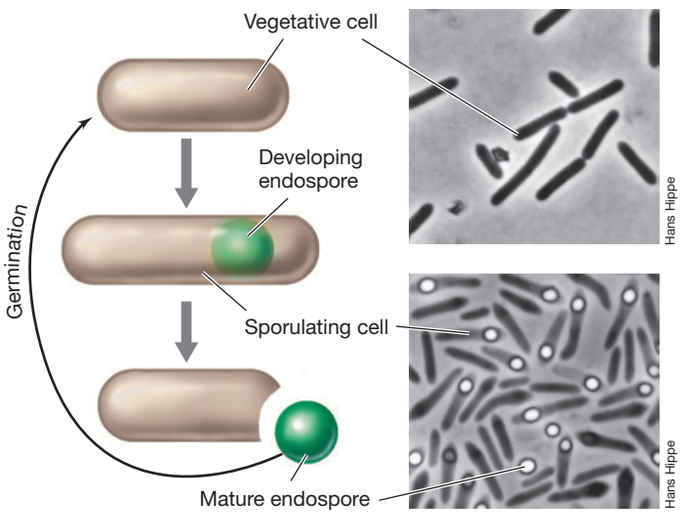


Figure 2.26 The life cycle of an endospore-forming bacterium. The phase-contrast photomicrographs are of cells of *Clostridium pascui*. A vegetative cell is about 0.8 μm wide.

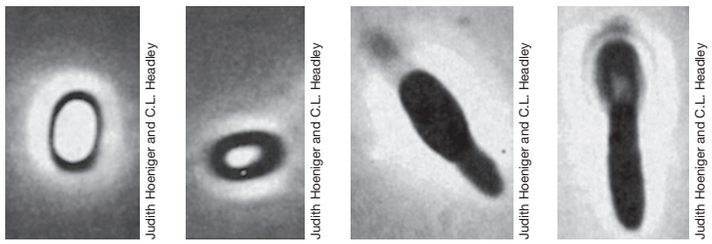


Figure 2.27 Endospore germination in *Bacillus*. Conversion of an endospore into a vegetative cell. This series of phase-contrast photomicrographs shows the sequence of events starting from *(a)* a highly refractile free endospore. *(b) Activation*: The spore becomes less refractile as the spore is hydrated. *(c) Germination*: the spore begins to develop into a vegetative cell. *(d) Outgrowth*: the vegetative cell emerges and begins to divide.

An endospore can remain dormant for years, but when conditions are favorable for growth, it can convert back to a vegetative cell rapidly through the process of *germination* (Figure 2.26). Germination is usually triggered by the availability of nutrients, such as certain amino acids or sugars. This process occurs in three steps: *activation*, *germination*, and *outgrowth* (**Figure 2.27**). The overall process occurs over a period of just a few minutes, and it is characterized by hydration of the spore, which results in loss of its heat and chemical resistance, the loss of specific spore structures and the regeneration of vegetative cell structures, and ultimately, the onset of vegetative growth by binary fission.

Endospore Structure and Features

Endospores differ in many ways from vegetative cells (**Table 2.1**). Endospores are visible by light microscopy as strongly refractile structures (Figures 2.25 and 2.26). Endospores are impermeable to most dyes, so occasionally they are seen as unstained regions within cells that have been stained with basic dyes such as methylene blue. To stain endospores, special stains and procedures must be used. In the classical endospore-staining protocol, the stain malachite green is used and is infused into the spore with steam.

The structure of the endospore as seen with the electron microscope differs distinctly from that of the vegetative cell (**Figure 2.28**). The endospore contains many layers absent from the vegetative cell. The innermost region of the endospore is called the *core*; this contains DNA and ribosomes and develops from the cytoplasm of the vegetative cell. Surrounding the core is the *inner membrane*, the *cortex*, and the *outer membrane* (Figure 2.28). The inner membrane develops from the cytoplasmic membrane of the vegetative cell, the cortex is composed of peptidoglycan, and the outer membrane is a special membrane formed during sporulation (and should not be confused with the LPS-containing outer membrane of

TABLE 2.1 Differences between endospores and vegetative cells

Characteristic	Vegetative cell	Endospore
Microscopic appearance	Nonrefractile	Refractile
Calcium content	Low	High
Dipicolinic acid	Absent	Present
Enzymatic activity	High	Low
Respiration rate	High	Low or absent
Macromolecular synthesis	Present	Absent
Heat resistance	Low	High
Radiation resistance	Low	High
Resistance to chemicals	Low	High
Lysozyme	Sensitive	Resistant
Water content	High, 80–90%	Low, 10–25% in core
Small acid-soluble spore proteins	Absent	Present

gram-negative cells). Beyond the outer membrane is the *endospore coat* (Figure 2.28), composed of layers of spore-specific proteins, and some (but not all) endospores also have an outer proteinaceous layer called the *exosporium* (Figure 2.28).

The secret to the endospore toughness, and the reasons that endospores are so highly refractile, lies in the dehydration of the core. The endospore core contains less than one-quarter of the water found in the vegetative cell (Table 2.1). Dehydration greatly increases heat and chemical resistance and causes enzymes in the core to become inactive (but not denatured). Dehydration of the core is facilitated by the accumulation of a substance called **dipicolinic acid**, a distinctive characteristic of endospores. Endospores also contain large amounts of calcium (Ca^{2+}), most of which is complexed with dipicolinic acid. The calcium–dipicolinic acid complex forms about 10% of the dry weight of the endospore and functions to bind water, helping to dehydrate the developing endospore. In addition, the complex inserts between bases in DNA, which helps stabilize DNA against heat denaturation.

The endospore core also contains high levels of *small acid-soluble spore proteins* (SASPs). These proteins are only made during the sporulation process and have at least two functions. SASPs bind tightly to DNA in the core and protect it from potential damage from ultraviolet radiation, desiccation, and dry heat. Ultraviolet resistance is conferred when SASPs alter the physical structure of DNA, causing it to become more compact. This change in DNA structure causes it to be more resistant to mutations and other forms of potential damage caused by harsh chemicals or UV radiation (▶ Section 12.4) and also increases its resistance to thermal denaturation. In addition, SASPs function as a carbon and energy source for the outgrowth of a new vegetative cell from the endospore during germination.

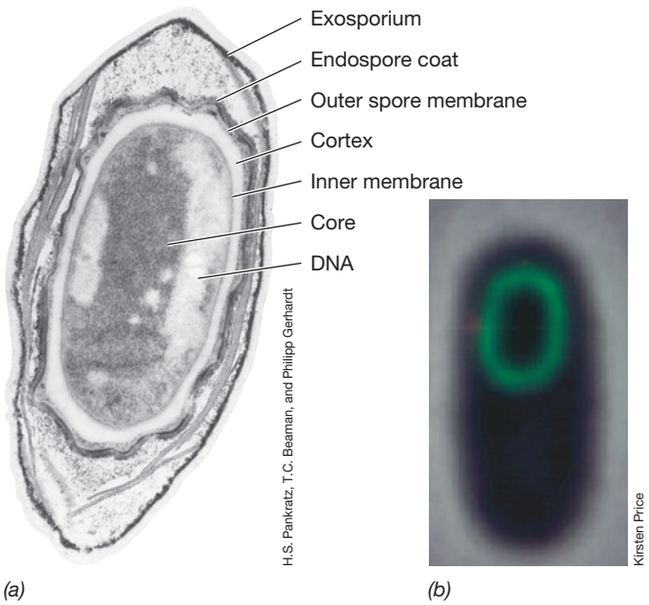


Figure 2.28 Structure of the bacterial endospore. *(a)* Transmission electron micrograph of a thin section through an endospore of *Bacillus megaterium*. Note that the composition of the endospore "outer membrane" is not the same as the outer membrane (LPS layer) of gram-negative bacteria shown in Figure 2.12. *(b)* Fluorescent photomicrograph of a cell of *Bacillus subtilis* undergoing sporulation. The green color is a fluorescent dye that specifically stains a sporulation protein in the endospore coat.

The Sporulation Cycle

Sporulation is a form of cellular differentiation (◀ Figure 1.5), and many genetically directed events occur during the conversion from vegetative growth to sporulation. The structural changes in sporulating cells of *Bacillus* are shown in **Figure 2.29**. In *Bacillus subtilis*, which has been studied in detail, the conversion of a vegetative cell into an endospore takes about 8 hours and begins with asymmetric cell division and the formation of a *forespore* (Figure 2.29). Engulfment of the forespore by the mother cell results in the formation of the outer membrane that surrounds the developing endospore; the outer membrane forms from part of the mother cell's cytoplasmic membrane. Key events in endospore formation such as asymmetric cell division, cortex formation, and SASP production take place in a defined sequence and at specific times in the sporulation cycle (Figure 2.29). Genetic studies of mutants of *Bacillus subtilis*, each blocked at one of the stages of sporulation, indicate that more than 200 sporulation-specific genes exist. These genes are turned on and off in a genetic program that governs cellular differentiation, a process we will consider in detail when we examine the genetic regulation of sporulation (▶ Section 8.6).

Prokaryotic cells are not static entities, and so we now turn our attention to how and why cell movements occur.

Check Your Understanding

- What features differentiate an endospore from a vegetative cell?
- Ingestion of honey by infants can cause botulism as it often contains *Clostridium botulinum* endospores. Why wouldn't heating honey prevent this from occurring?
- How is the outer membrane of an endospore formed, and how does this structure differ from the outer membrane in the gram-negative cell envelope?

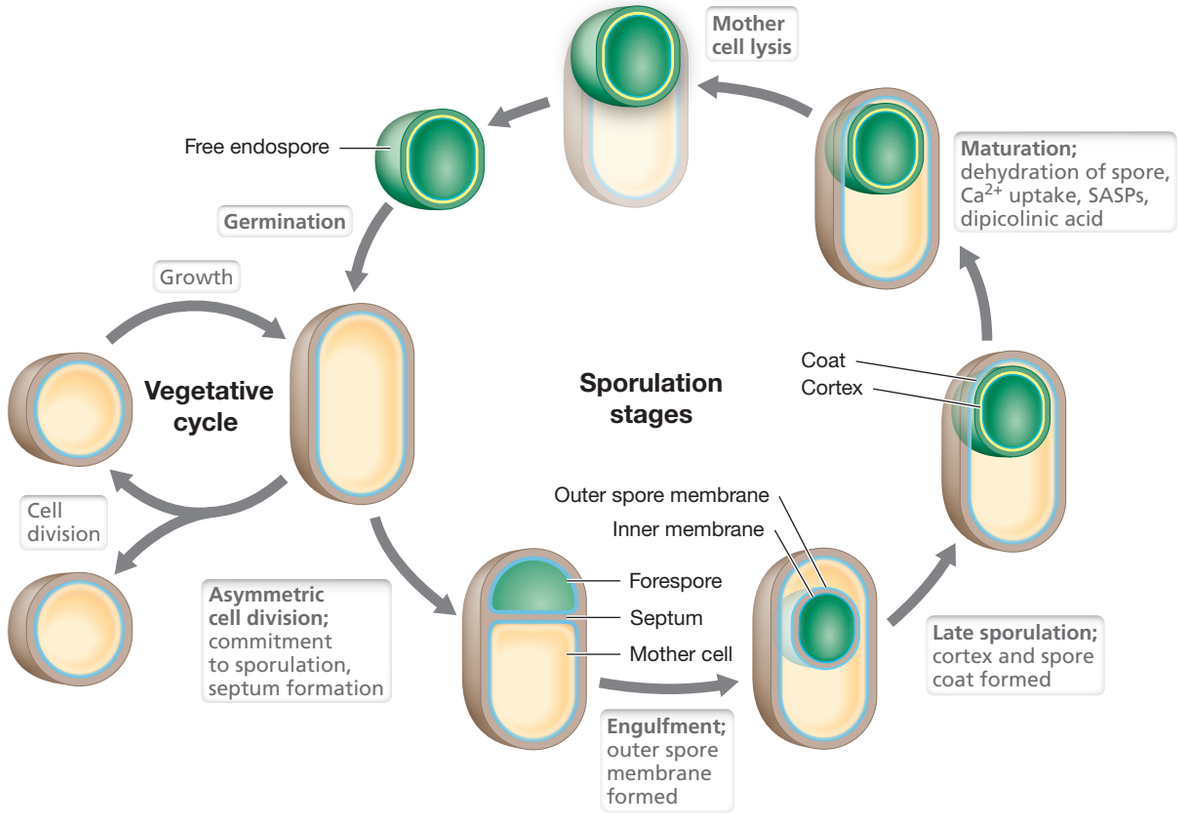


Figure 2.29 Major events in endospore formation. The steps depicted are those defined from genetic and microscopic analyses of sporulation in *Bacillus subtilis*, the model organism for studies of sporulation. SASPs, small acid-soluble proteins.

III • Cell Locomotion

Prokaryotic cells move about their environments by powering motility devices that allow them to swim or crawl, and they can move in specific directions in response to chemical or physical signals.

We finish our survey of prokaryotic cell structure and function by examining cell locomotion. Many microbial cells can move under their own power. Motility allows cells to reach different parts of their environment, and in nature, a new location may offer a cell additional resources or protection from harmful substances and ultimately spell the difference between life and death.

We examine here the two major types of prokaryotic cell movement, *swimming* and *gliding*. We then consider how motile cells are able to move in a directed fashion toward or away from particular stimuli (a phenomenon called *taxis*) and present examples of these simple behavioral responses.

2.9 Flagella, Archaella, and Swimming Motility

Mastering Microbiology Microbiology Animation: Flagella: Movement

Many *Bacteria* are motile by swimming due to a structure called the **flagellum** (plural, flagella) (Figure 2.30); an analogous structure called the **archaellum** is present in many *Archaea*. Flagella and archaella are tiny rotating machines that function to push or pull the cell through a liquid.

Flagella and Flagellation

Bacterial flagella are long, thin appendages (15–20 nm wide, depending on the species) free at one end and anchored into the cell at the other end. Flagella can be stained and observed by light microscopy (Figure 2.30) or electron microscopy (Figure 2.31).

Flagella can be anchored to a cell in different locations. In **polar flagellation**, the flagella are attached at one or both ends of a cell (Figure 2.30*b*). Occasionally, a group of many flagella (called a *tuft*) may arise at one end of the cell, a type of polar flagellation called *lophotrichous* (Figure 2.30*c*). Tufts of flagella can sometimes be seen in large unstained cells by dark-field or phase-contrast microscopy (Figure 2.32). When a tuft of flagella emerges from both poles of the cell, flagellation is called *amphitrichous*. In contrast to these more

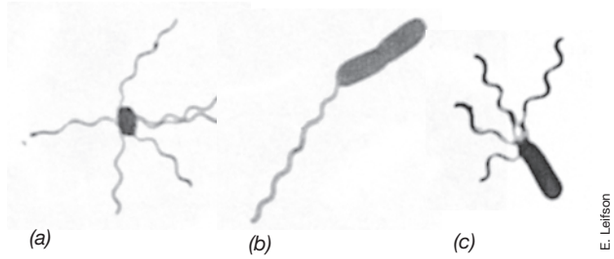


Figure 2.30 Bacterial flagella. Classic light photomicrographs taken by Einar Leifson of bacteria containing different arrangements of flagella. Cells are stained with the Leifson flagella stain. *(a)* Peritrichous. *(b)* Polar. *(c)* Lophotrichous.

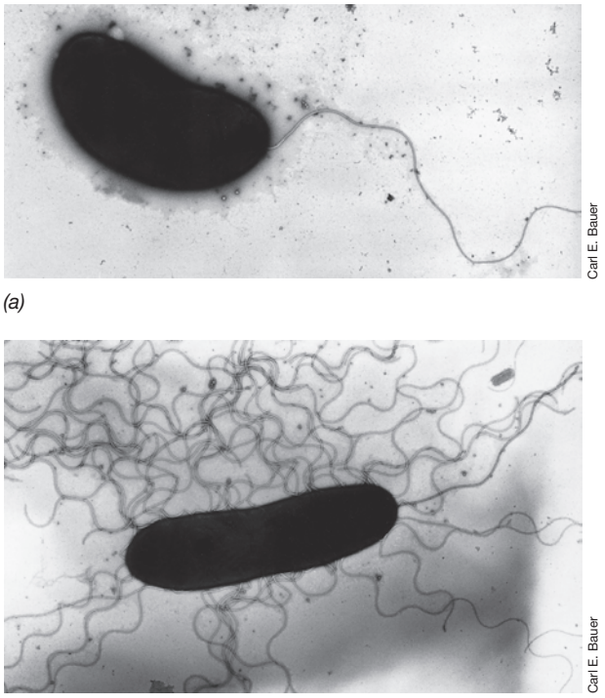


Figure 2.31 Bacterial flagella as observed by negative staining in the transmission electron microscope. *(a)* A single polar flagellum. *(b)* Peritrichous flagella. Both micrographs are of cells of the phototrophic bacterium *Rhodocista centenaria*, which are about 1.5 μm wide. Cells of *R. centenaria* are normally polarly flagellated but under certain growth conditions form peritrichous flagella. See Figure 2.41*b* for a photo of colonies of *R. centenaria* cells that move toward an increasing gradient of light (phototaxis).

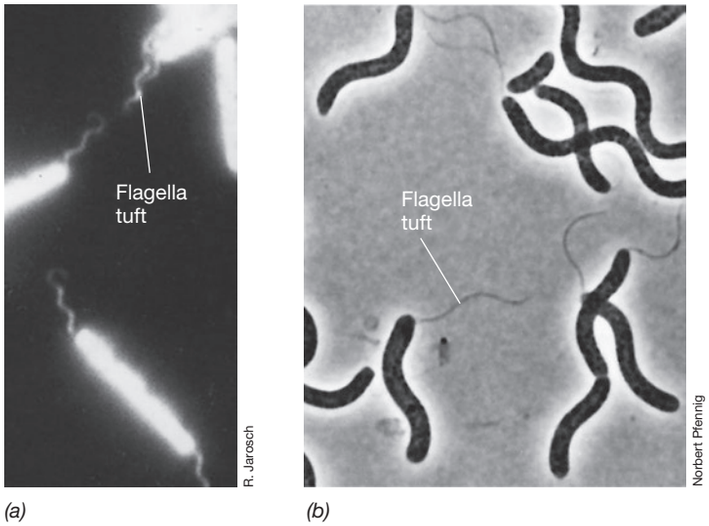


Figure 2.32 Bacterial flagellar tufts observed in living cells. *(a)* Dark-field photomicrograph of a group of large rod-shaped bacteria with flagellar tufts at each pole (a condition called *amphitrichous flagellation*). A single cell is about 2 μm wide. *(b)* Phase-contrast photomicrograph of cells of the large phototrophic purple bacterium *Rhodospirillum photometricum* that contain a tuft of lophotrichous flagella emerging from one of the poles. A cell measures about 4 × 25 μm.

specific sites of flagellation, in **peritrichous flagellation** (Figures 2.30*a* and 2.31*b*), flagella are inserted around the cell surface.

Flagella do not rotate at a constant speed but increase or decrease their rotational speed in relation to the strength of the proton motive force. Flagella can rotate at up to 1000 revolutions per second to support a swimming speed of up to 60 cell-lengths/sec. The fastest known land animal, the cheetah, can move at about 25 body-lengths/sec. Thus, a bacterium swimming at 60 cell-lengths/sec is actually moving over twice as fast—relative to its size—as the fastest animal!

The swimming motions of polarly and lophotrichously flagellated organisms differ from those of peritrichously flagellated organisms, and these can be distinguished microscopically (**Figure 2.33**). Peritrichously flagellated organisms typically move slowly in a straight line, stop and then head off in a new direction. By contrast, polarly flagellated organisms often move more rapidly and continuously, and some are able to reverse their direction. The different behavior of flagella on polar and peritrichous organisms, including differences in reversibility of the flagellum, is illustrated in Figure 2.33.

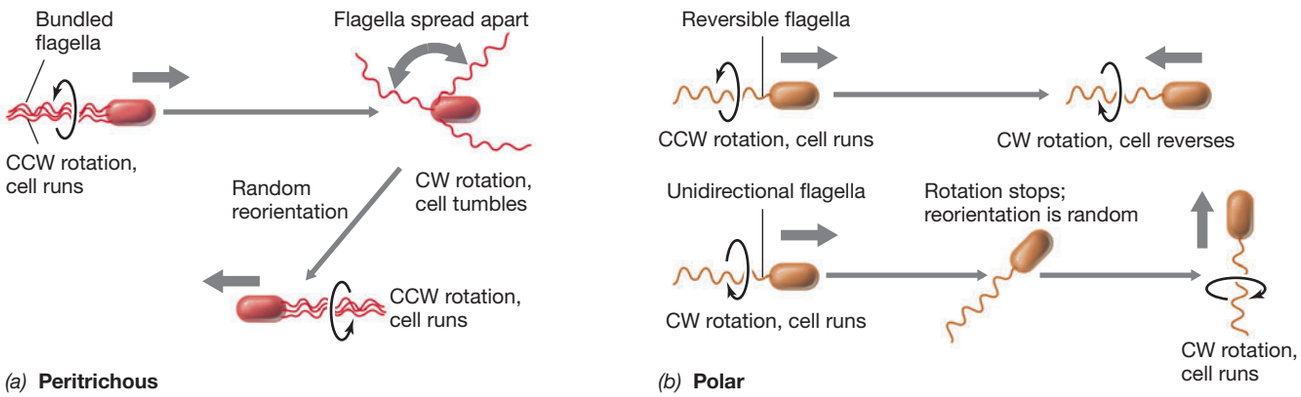


Figure 2.33 Movement in peritrichously and polarly flagellated prokaryotic cells. *(a)* Peritrichous: Forward motion is imparted by all flagella forming into a bundle and rotating counterclockwise (CCW). Clockwise (CW) rotation causes the bundle to break apart and the cell to tumble. A return to counterclockwise rotation leads the cell off in a new direction. *(b)* Polar: Cells change direction by reversing flagellar rotation (thus pulling instead of pushing the cell) or, with unidirectional flagella, by stopping periodically to reorient and then moving forward by clockwise rotation of its flagella. The gray arrow above each cell shows the direction the cell is traveling.

Flagella Structure and Activity

Bacterial flagella are rigid and helical (unlike eukaryal flagella, which are whiplike). The main part of the flagellum, called the *filament*, is composed of many copies of a protein called *flagellin*. The amino acid sequence of flagellin is highly conserved in *Bacteria*, suggesting that flagellar motility evolved early and has deep roots within this domain. In addition to the filament, a flagellum consists of several other components. A wider region at the base of the filament called the *hook* consists of a single type of protein and connects the filament to the flagellum motor in the *basal body* (**Figure 2.34**).

Mastering Microbiology
Microbiology Animation: Flagella: Structure

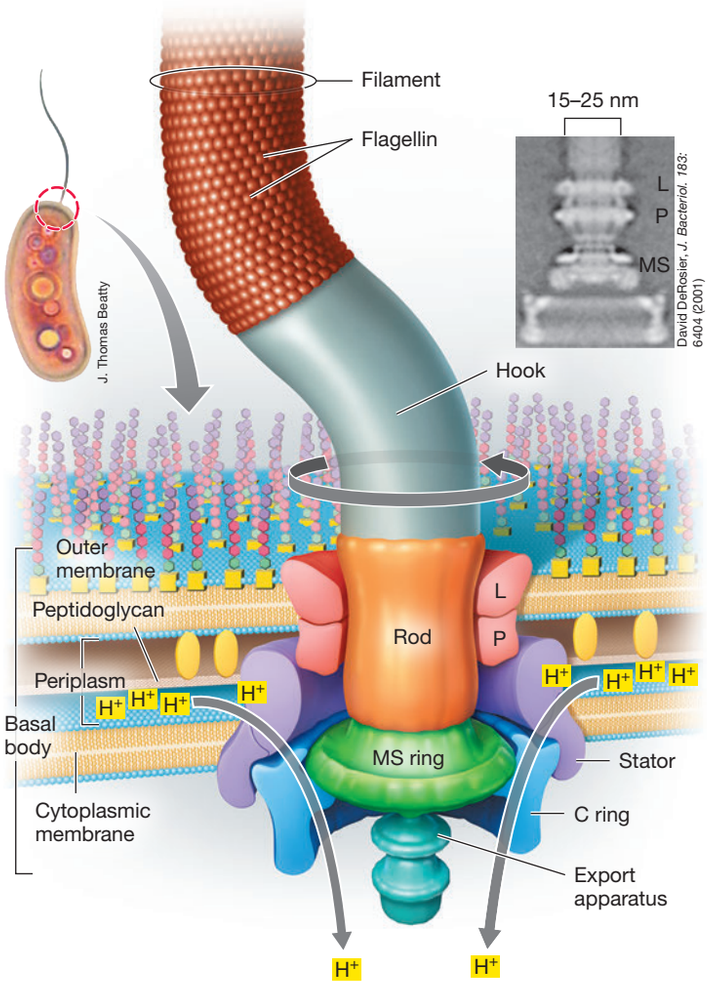


Figure 2.34 Structure and function of the flagellum in gram-negative *Bacteria*. The L ring is embedded in the LPS and the P ring in peptidoglycan. The MS ring is embedded in the cytoplasmic membrane and the C ring is found within the cytoplasm. The stator (Mot proteins) is embedded both within the cytoplasmic membrane and the peptidoglycan. Proton translocation through channels within the stator cause the MS ring to rotate, thereby driving rotation of the attached rod and flagellum. Flagellin molecules move from the export apparatus through a narrow channel in the rod and filament to reach the site of flagellar synthesis at the filament tip. The Mot proteins function as the flagellar motor. The flagellar motor rotates the filament to propel the cell through the medium. Inset photos: Top left, a cell of the purple sulfur bacterium *Chromatium* containing a tuft of polar flagella; Top right, transmission electron micrograph of a flagellar basal body from a cell of *Salmonella enterica* with the various rings labeled.

The flagellum motor is a reversible rotating machine composed of more than 25 proteins and anchored in the cytoplasmic membrane and cell wall. The motor consists of a central rod that passes through a series of rings. In gram-negative bacteria, an outer ring, called the *L ring*, is anchored in the outer membrane (Section 2.4). A second ring, called the *P ring*, is anchored in the peptidoglycan layer (Section 2.3). A third set of rings, called the *MS* and *C rings*, are located within the cytoplasmic membrane (Section 2.1) and the cytoplasm, respectively (Figure 2.34). In gram-positive bacteria, which lack an outer membrane, only the inner pair of rings is present. Surrounding the inner rings and anchored in the cytoplasmic membrane and the peptidoglycan is the *stator*, which is composed of *Mot proteins*. On the cytoplasmic side of the MS ring is the *export apparatus*, a type III secretion system (▶ Section 6.13) that facilitates synthesis of the flagellum.

The flagellum motor contains two main components: the *rotor* and the *stator*. The rotor consists of the central rod and the L, P, C, and MS rings. The stator is comprised of Mot proteins, which surround the rotor and function to generate torque. Collectively, these structures make up the flagellar **basal body** (Figure 2.34). Rotation of the flagellum occurs at the expense of the proton motive force (Section 2.1), and it is thought that rotation is caused by a type of "proton turbine" process. In this model, proton translocation through channels within the stator complex cause the MS ring to rotate, thereby driving rotation of the attached rod and flagellum. The L and P rings act like bushings within which the rod rotates. Protons flowing through the Mot proteins exert electrostatic forces on helically arranged charged residues on rotor proteins and cause the MS ring to rotate. About 1200 protons are translocated by each rotation of the flagellum. The rotational speed of the flagellum is set by the proton flow rate through the Mot proteins, which is a function of the intensity of the proton motive force. The flagellar motors of different microbes are able to generate different amounts of torque, causing significant differences in swimming speed; such adaptations are driven by adding or subtracting subunits from the stator and C ring, which presumably changes the "gear ratio" of the motor.

Flagellar Synthesis

Several genes encode the motility apparatus of *Bacteria*. In *Escherichia* and *Salmonella* species, in which motility studies have been extensive, over 50 genes are linked to motility in one way or another. These genes encode the structural proteins of the flagellum and motor apparatus, of course, but also encode proteins that export the structural proteins through the cytoplasmic membrane to the outside of the cell and proteins that regulate the synthesis of new flagella.

A flagellar filament grows not from its base, as does an animal hair, but from its tip. The MS ring is synthesized first and inserted into the cytoplasmic membrane. Then other anchoring proteins are synthesized along with the hook and the cap before the filament forms (**Figure 2.35**). Flagellin molecules synthesized in the cytoplasm are exported through the *export apparatus* (Figure 2.34) present on the cytoplasmic side of the basal body. The export apparatus shuttles flagellin molecules into a 3-nm channel that runs through the center of the basal body and the hollow flagellar filament. The flagellin

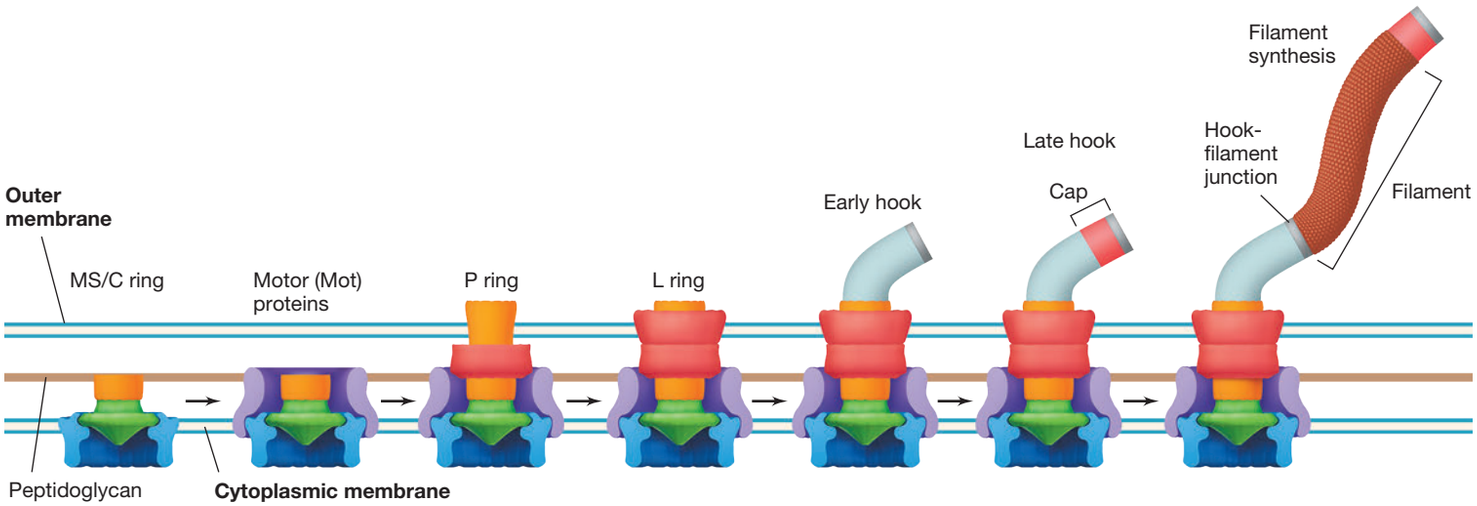


Figure 2.35 Flagella biosynthesis. Synthesis begins with assembly of MS and C rings in the cytoplasmic membrane, followed by the other rings, the hook, and the cap. Flagellin protein flows through the hook to form the filament and is guided into position by cap proteins.

subunits are ultimately passed to the end of the filament where they attach to the terminus of the growing flagellum. Cap proteins assist flagellin molecules to assemble in the proper fashion at the flagellum tip (Figure 2.35). Approximately 20,000 flagellin protein molecules are needed to make one filament. The flagellum grows more or less continuously until it reaches its final length. Broken flagella still rotate and can be repaired with new flagellin units passed through the filament channel to replace the lost ones.

Archaella

As in *Bacteria*, swimming motility is widespread among species of *Archaea* and is driven by the rotation of their flagellum analog, the *archaellum*. However, despite their similar function, archaella proteins are unrelated to those of flagella, and in evolutionary terms are more closely related to type IV pili (Section 2.6). Archaella are somewhat smaller than flagella, measuring about 10–13 nm in width (**Figure 2.36**), which is only slightly wider than type IV pili. Archaella are not hollow and are assembled from their bases much like type IV pili, whereas flagella are hollow and are assembled at their tips (Figure 2.35). Archaellar rotation, like the extension and retraction of type IV pili, is driven by ATP hydrolysis, whereas the energy for flagellar rotation is derived from the proton motive force. In addition, the archaellar motor is structurally simpler than the flagellar motor (compare Figure 2.36 with Figure 2.34), and only 7–12 genes are required to encode the major proteins that make up the archaellar motor, in contrast to the more than 25 genes that encode components of the flagellar motor.

Studies of swimming *Archaea*, such as *Halobacterium*, show that they swim at speeds only about one-tenth that of *Escherichia coli*, and in general, *Archaea* swim much more slowly than *Bacteria*. This could be due to the smaller diameter of the archaellum compared to the flagellum, which should reduce the torque the structure can generate. It could also be due to differences in the construction of the motor and how it is powered. However, some *Archaea* have found a way to overcome these limitations. For example, cells of *Methanocaldococcus* (Figure 2.36*c*) swim nearly 50 times faster than cells of *Halobacterium* and 10 times faster than cells of *Escherichia coli*. In fact, *Methanocaldococcus* swims at nearly 500 cell-lengths per second, which in a relative sense makes it one of the fastest organisms on Earth!

We now compare swimming motility with mechanistically quite different forms of cell movement but ones that still allow the cell to move about and explore its environment.

Check Your Understanding

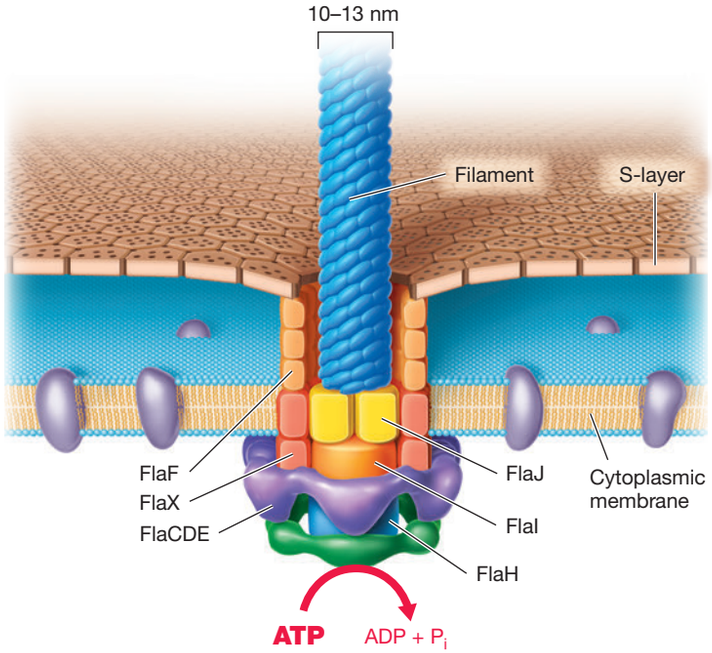
- Cells of *Salmonella* are peritrichously flagellated, those of *Pseudomonas* polarly flagellated, and those of *Spirillum* lophotrichously flagellated. Using a sketch, show how each organism would appear in a flagella stain.
- Compare flagella and archaella in terms of their structure, function, and energy source.
- The genes for flagellin are highly conserved between bacterial species. What does this imply about the importance of motility during bacterial evolution?

2.10 Surface Motility

Many motile microbes are unable to swim and instead crawl over surfaces in various ways. These forms of surface motility require attachment to a surface and are independent of flagella or archaella. Surface motility results in distinctive colony morphology because cells can move out and away from the center of the colony (**Figure 2.37**). All forms of surface motility are considerably slower than swimming motility, usually less than 10 μm/sec. Finally, whereas nearly all swimming motility in *Bacteria* and *Archaea* is driven by flagella and archaella (Section 2.9), surface motility can be produced by a diversity of different systems, the most well characterized of which are *twitching motility* and *gliding motility*.

Twitching Motility

Twitching motility requires type IV pili (Section 2.6), which extend from one pole of the cell, attach to a surface, and then retract to pull



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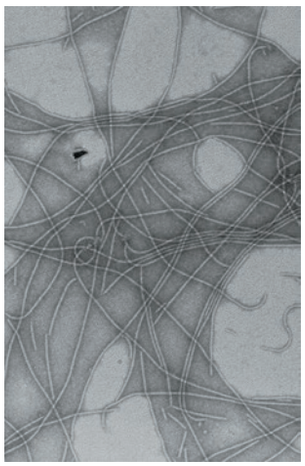

S.I. Aizawa and K.F. Jarrell

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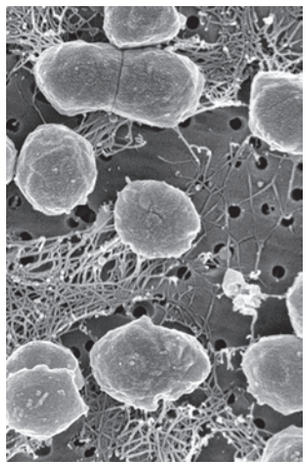
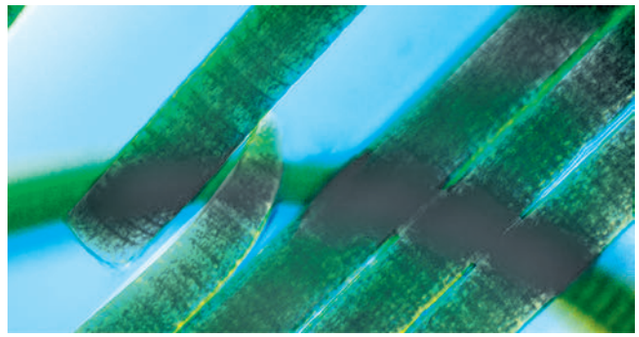


(c)

Figure 2.36 Archaella. *(a)* Depiction of an archaellum embedded in an S-layer and cytoplasmic membrane; compare with the bacterial flagellum in Figure 2.34. The archaellum and its motor differ from flagella in that the archaellum has a smaller diameter, it is not hollow, its motor has fewer proteins, and the energy for rotation comes from ATP hydrolysis and not from consumption of the proton motive force. *(b)* Negatively stained transmission electron micrograph of purified archaella. *(c)* Scanning electron micrograph of cells of *Methanocaldococcus* containing multiple archaella.



Richard W. Castenholz

(a)

Richard W. Castenholz

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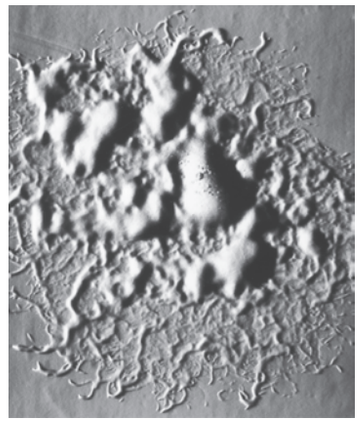

Mark J. McBride

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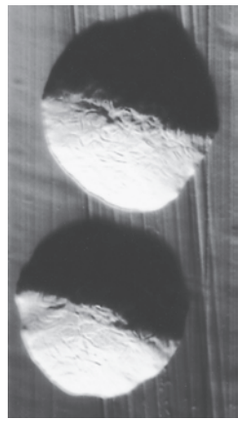


Mark J. McBride

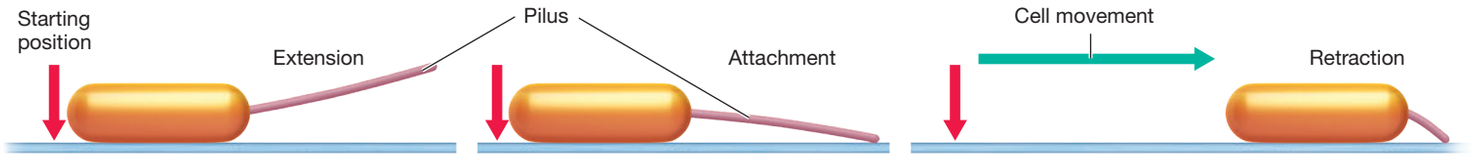
(d)

Figure 2.37 Gliding bacteria. *(a, b)* The large filamentous cyanobacterium *Oscillatoria* has cells about 35 μm wide. *(b) Oscillatoria* filaments gliding on an agar surface. *(c)* Masses of the bacterium *Flavobacterium johnsoniae* gliding away from the center of the colony (the colony is about 2.7 mm wide). *(d)* Nongliding mutant strain of *F. johnsoniae* showing typical colony morphology of nongliding bacteria (the colonies are 0.7–1 mm in diameter). See also Figure 2.41.

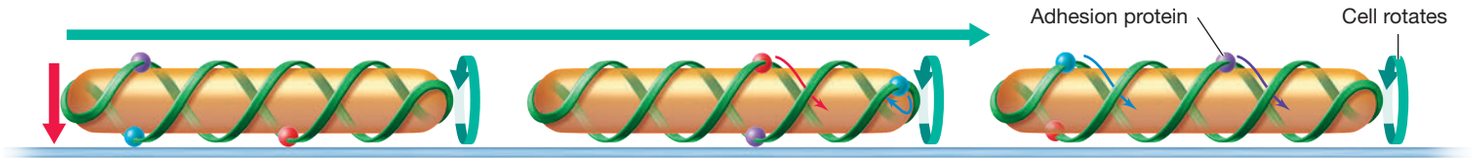
the cell forward (**Figure 2.38*a***). The energy required to catalyze the activity of type IV pili comes from ATP hydrolysis. Twitching motility has been described in many *Bacteria* and some *Archaea*. For example, species of the gram-negative bacterium *Pseudomonas* are often capable of twitching motility and this trait is important to their ability to form biofilms (▶ Section 4.9). Twitching motility has also been well described in myxobacteria, which are social predatory bacteria whose cells can work together to consume other bacteria. Myxobacteria exhibit two forms of motility: social motility, which is caused by twitching, and adventurous motility, which is caused by gliding (see below). Twitching motility allows cells to move together in groups, and this trait is facilitated by the production of both type IV pili and the secretion of extracellular polysaccharides that aid in cellular cohesion.

Gliding Motility

Gliding motility (Figure 2.38*b*) is defined as a smooth motion along the long axis of a cell without the aid of external propulsive



(a) **Twitching**



(b) **Gliding**

Figure 2.38 Surface motility. *(a)* Twitching motility employs type IV pili. These structures require ATP hydrolysis to extend (up to several micrometers) and then retract, causing the cell to move forward; movement occurs in discrete increments. *(b)* Gliding motility requires a helical intracellular protein track that interacts with gliding motors and extracellular adhesion proteins. The proton motive force drives rotation of gliding motors that translate this force to the helical track, causing adhesion proteins to move in a helical pattern; this results in continuous forward motion and clockwise rotation of the cell.

structures (such as pili or attachment organelles). Gliding motility is a continuous form of movement, very unlike swimming motility, in which cells frequently stop and then swim off in a random new direction. Gliding bacteria are typically filamentous or rod-shaped in morphology, and while gliding has been observed among diverse species of *Bacteria*, no gliding *Archaea* are known. The adventurous motility of myxobacteria is a form of gliding that has been well studied, and many species of the *Bacteroidetes* such as *Flavobacterium* also exhibit gliding motility.

Gliding has been best studied in *Myxococcus* and *Flavobacterium* (Figure 2.37*c*), and the mechanism of gliding in these two organisms is likely to be quite similar. These organisms both possess a helical intracellular track made of proteins that run in a continuous loop around the cell (Figure 2.38*b*). Associating with this track are "gliding motors," rotary motors that are driven by the proton motive force, and which are evolutionarily and operationally similar to the flagellar motor (see Figure 2.34). These organisms also have adhesion proteins that grab onto surfaces on the outside of the cell. While the exact mechanism of gliding remains unknown, it is thought that the gliding motor associates with the helical track, and rotation of the motor causes displacement of the track relative to the motor (or of the motor relative to the track). This movement is in some way transduced to the surface adhesion proteins, causing them to move in a helical direction around the surface of the cell. The net result is that the cell is propelled forward over the surface and caused to rotate around its axis as it moves forward (Figure 2.38*b*).

We now consider how these various forms of prokaryotic cell motility can move cells in specific directions, which allows them to both exploit useful nutrients and avoid harmful substances.

Check Your Understanding

- How does gliding motility differ from swimming motility in both mechanism and requirements?
- Contrast the mechanism of twitching motility with that of gliding motility.

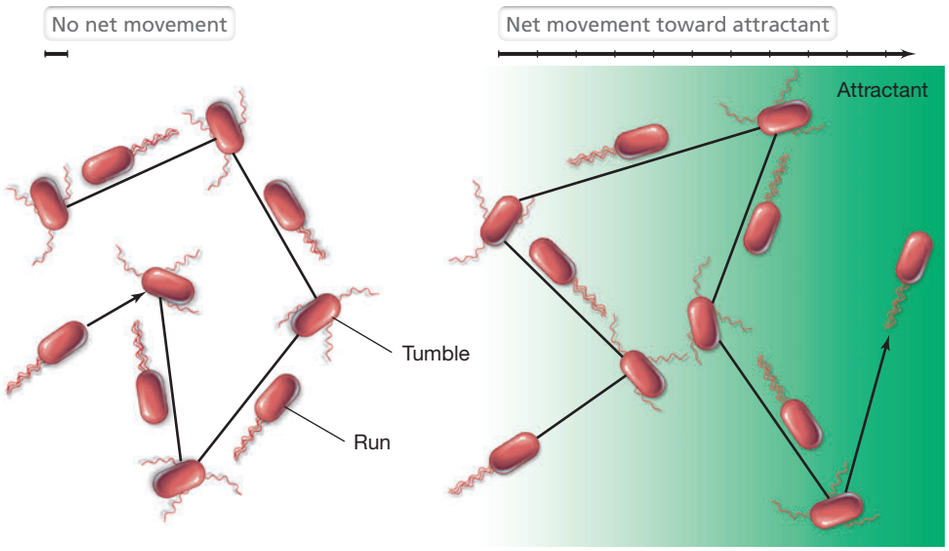
2.11 Chemotaxis

Cells of *Bacteria* and *Archaea* have evolved numerous systems that allow them to sense and respond to chemical and physical stimuli in their environments. For example, microbes are able to sense and move toward a food source. Such a directed movement is called *taxis* (plural, taxes). **Chemotaxis**, a response to chemicals, and **phototaxis**, a response to light, are two well-studied forms of taxis. The ability of a cell to move toward or away from various stimuli has ecological significance in that the directed movement may enhance a cell's access to resources or allow it to avoid harmful substances that could damage or kill it.

Chemotaxis has been well studied in *Bacteria* that use swimming motility; these organisms sense environmental stimuli and transmit signals to the flagellum causing it to alter its rotation. In addition, most *Archaea* that exhibit swimming motility are also chemotactic, and several of the proteins that control chemotaxis in *Bacteria* are similar to those found in *Archaea*. Gliding bacteria are also often chemotactic and can alter their direction in response to environmental stimuli. Phototaxis is often observed in filamentous cyanobacteria (Figure 2.37*a*, *b*) which move toward or away from a light source. In Section 7.6 we examine in detail the molecular mechanism of chemotaxis and its genetic regulation in the model bacterium *Escherichia coli*. For now, we will focus on the general principles by which chemotaxis operates.

Chemotaxis in Peritrichously Flagellated *Bacteria*

Much research on chemotaxis has been done with the peritrichously flagellated bacterium *E. coli*. In the absence of an attractant, cells move in a random fashion that includes *runs*, in which the cell is swimming forward in a smooth fashion, and *tumbles*, when the cell stops and jiggles about randomly (**Figure 2.39**). During a run, the flagellar motor rotates *counterclockwise*, causing the flagella to coil together into a bundle that propels the cell forward. By contrast, when flagella rotate *clockwise*, the bundle of flagella pushes apart, forward motion ceases, and the cells tumble randomly (Figure 2.39*a*). Following a tumble, the cell will begin a new run in a random direction. Thus, in the absence of an



(a) **No attractant present: Random movement** *(b)* **Attractant present: Directed movement**

Figure 2.39 Chemotaxis in a peritrichously flagellated bacterium. *(a)* In the absence of a chemical attractant, the cell swims randomly in runs, changing direction during tumbles. *(b)* In the presence of an attractant, runs become biased, and the cell moves up the gradient of the attractant. The attractant gradient is depicted in green, with the highest concentration where the color is most intense.

attractant, the cell moves about its environment in random fashion through a series of runs and tumbles.

To understand chemotaxis, consider what happens when *E. coli* encounters a chemical attractant, such as the sugar glucose (Figure 2.39*b*). Because of diffusion, the concentration of the attractant will increase when the cell is moving toward the attractant. During chemotaxis cells will tend to move toward the attractant over time, but cells do not move continuously nor directly toward the attractant; instead, they exhibit a behavior known as a *biased random walk*. The key to this mechanism is that cells sample the concentration of attractant not over space, but rather *over time*. As the cell is swimming in a random direction, it continues to sense the concentration of attractant over time. If the concentration goes up, runs become longer and tumbles less frequent, but if the concentration goes down, runs become shorter and tumbles more frequent. The biased random walk causes net movement toward the attractant (Figure 2.39*b*), and this mechanism is remarkably effective for bringing cells to the source of an attractant. For a repellent, the same mechanism applies, but the response is reversed, with longer runs triggered by a *decrease* in concentration of the repellent. Attractants and repellents are sensed by a series of membrane proteins called *chemoreceptors*. Chemoreceptors sense the concentration of particular chemicals and transduce this information to flagella, causing them to alter their rotation (▶ Section 7.6).

Chemotaxis in Polarly Flagellated *Bacteria*

Chemotaxis in polarly flagellated cells is similar but not identical to chemotaxis in peritrichously flagellated cells such as *E. coli*. Many polarly flagellated bacteria, such as *Pseudomonas* species, which have a single polar flagellum rather than a tuft, do not tumble when they reverse their flagellum. Instead, they swim backwards when their flagellum reverses its direction of rotation (Figure 2.33*b*). In contrast, the phototrophic bacterium *Rhodobacter* has a single polar flagellum that is not reversible; it can only start and stop its rotation. Both of these polarly flagellated bacteria rely on Brownian motion when stopped to randomly reorient the cell (Figure 2.33*b*). Then as the flagellum begins to rotate again, the cell moves off in a new direction. Despite this seemingly random activity, cells of *Rhodobacter* are strongly chemotactic to various organic compounds and to oxygen and light. Chemotactic organisms, by employing a *biased* random walk, can navigate effectively through their environments toward conditions that favor growth and away from those that could inhibit growth or otherwise cause harm.

Measuring Chemotaxis

Bacterial chemotaxis can be measured in a chemotaxis assay in which a small glass capillary tube containing an attractant is immersed into a suspension of motile bacteria. A chemical gradient extends from the tip of the capillary into the surrounding medium, with the chemical concentration decreasing with distance from the tip (**Figure 2.40**). When an attractant is present, chemotactic bacteria will move toward it, forming a swarm around the open tip (Figure 2.40*c*) with many of the bacteria swimming into the capillary tube. Of course, because of random movements some chemotactic bacteria will swim into the capillary even if it does not contain an attractant (control solution, Figure 2.40*b*). However, when an attractant is present, the number of cells within the capillary will increase significantly (Figure 2.40*e*). If the capillary contains a repellent, the number of bacteria within the capillary will be fewer than in the control (Figure 2.40*d*). This capillary method can be used to screen chemicals to see if they are attractants or repellents for a given bacterium.

Chemotaxis can also be observed microscopically. It is possible to quantify chemotactic behavior using a video camera that captures the positions of bacterial cells with time and records the motility tracks of each cell (Figure 2.40*f*). This method has been used to study chemotaxis of microbes in natural environments. In nature, nutrients are often excreted from larger microbial cells whether they are living or dead. Algae, for example, produce both organic compounds and oxygen (O_2, from photosynthesis), which can trigger chemotactic movements of bacteria toward the algal cell (Figure 2.40*f*). Chemotaxis can result in complex cellular interactions in nature including competition for resources and cooperation by recycling metabolic products.

Check Your Understanding

- Describe how bacteria use a biased random walk to move toward an attractant.
- Contrast the motility of a peritrichously flagellated bacterium such as *Escherichia coli* with that of a polarly flagellated bacterium such as a species of *Pseudomonas*.
- In a capillary tube assay, why is it necessary to use a control that lacks attractant?

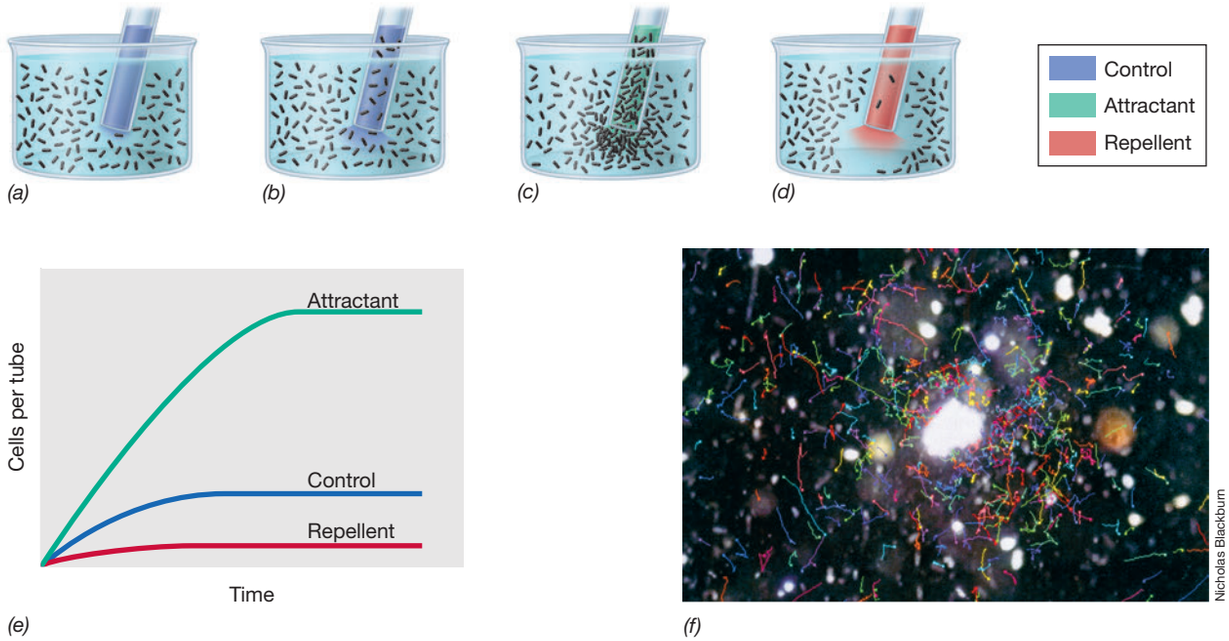


Figure 2.40 **Measuring chemotaxis using a capillary tube assay.** *(a)* Insertion of the capillary into a bacterial suspension. As the capillary is inserted, a gradient of the chemical begins to form. *(b)* A control capillary contains a salt solution that is neither an attractant nor a repellent. Cell concentration inside the capillary becomes the same as that outside. *(c)* Accumulation of bacteria in a capillary containing an attractant. *(d)* Repulsion of bacteria by a repellent. *(e)* Time course showing cell numbers in capillaries containing various chemicals. *(f)* Tracks of motile bacteria in seawater swarming around an algal cell (large white spot, center) photographed with a tracking video camera system attached to a microscope. The bacterial cells are showing positive aerotaxis by moving toward the oxygen-producing algal cell. The alga is about 60 μm in diameter.

2.12 Other Forms of Taxis

Chemotaxis is a directed movement with respect to a chemical gradient, but many other forms of sensory response can govern microbial motility. For example, *osmotaxis* is directed movement with respect to a gradient of ionic strength, *hydrotaxis* is directed movement with respect to a gradient of available water, *aerotaxis* is directed movement with respect to gradients of O_2, and *phototaxis* is directed movement with respect to a gradient in light intensity. Here we will consider phototaxis and aerotaxis, two taxes that have been particularly well studied.

Phototaxis

Many phototrophic microorganisms can move toward light, a process called *phototaxis*. Phototaxis allows a phototrophic organism to position itself most efficiently to receive light for photosynthesis. For example, if motile phototrophic purple bacteria are placed on a microscope slide that is illuminated with a spectrum of light they will move preferentially toward certain wavelengths (**Figure 2.41*a***). The wavelengths of light at which the bacteria accumulate correspond to the wavelengths of light that are absorbed by their photosynthetic pigments. These pigments include, in particular, bacteriochlorophylls and carotenoids (Chapter 14).

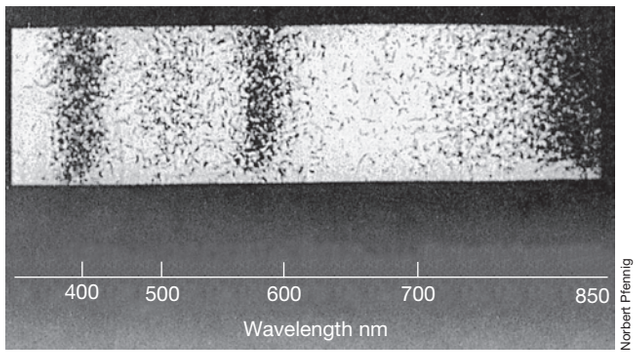
Two different light-mediated taxes are observed in phototrophic bacteria. One, called *scotophobotaxis*, is easily observed in the microscope. Scotophobotaxis occurs when a phototrophic bacterium happens to swim into darkness outside the illuminated field of view of the microscope. Entering darkness negatively affects photosynthesis, and thus cellular energy levels, and this signals the cell to tumble with greater frequency until it reenters the light by a biased random walk. Scotophobotaxis is a mechanism to prevent phototrophic cells from swimming away from a lighted zone into darkness, and this likely improves their competitive success in nature.

Phototaxis differs from scotophobotaxis in that cells move with respect to a *gradient* of light intensity, whereas scotophobotaxis is a response only to the absence of light. Phototaxis is analogous to chemotaxis except that the attractant is light instead of a chemical. In some phototactic organisms, such as the highly motile phototrophic purple bacterium *Rhodocista centenaria* (Figure 2.31), *entire colonies* of cells show phototaxis and move in unison toward the light (Figure 2.41*b*).

Several components of the regulatory system that control chemotaxis also control phototaxis. A *photoreceptor*, a protein that functions similarly to a chemoreceptor but which senses a gradient of light instead of chemicals, is the initial sensor in the phototaxis response. The photoreceptor then interacts with the same cytoplasmic proteins that control flagellar rotation in chemotaxis, maintaining the cell in a run if it is swimming toward an increasing intensity of light. Section 7.6 describes the activities of these proteins in more detail.

Aerotaxis and Magnetotaxis

Aerotaxis is directed motility with respect to O_2 (see Figure 2.40*f*). Aerobic organisms require O_2 and may exhibit a positive aerotactic response, swimming toward increasing concentrations of O_2. We will see in the next chapter, however, that O_2 is toxic to many



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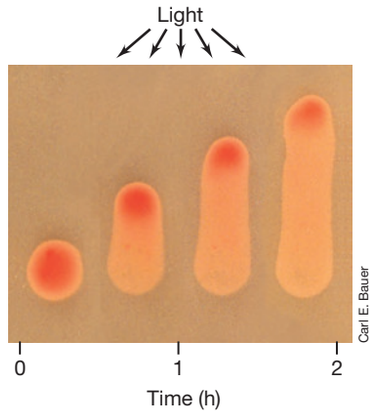


(b)

Figure 2.41 Phototaxis of phototrophic bacteria. *(a)* Scotophobic accumulation of the phototrophic purple bacterium *Thiospirillum jenense* at wavelengths of light at which its pigments absorb. A light spectrum was displayed on a microscope slide containing a dense suspension of the bacteria; after a period of time, the bacteria had accumulated selectively and the photomicrograph was taken. The wavelengths at which the bacteria accumulated are those at which the photosynthetic pigment bacteriochlorophyll *a* absorbs. *(b)* Phototaxis of an entire colony of the purple phototrophic bacterium *Rhodocista centenaria*. These strongly phototactic cells move in unison toward the light source at the top. See Figure 2.31 for electron micrographs of flagellated *R. centenaria* cells.

microbes. *Microaerophiles* are aerobic organisms that require O_2 but are killed when the concentration of O_2 is too high. Microaerophiles often use aerotaxis to position themselves at an optimum concentration of O_2 (usually between 1% and 5% O_2). Microaerophily can be a challenging lifestyle because either too much or too little O_2 can mean death, and O_2 gradients are highly dynamic because many organisms either produce or consume O_2. Hence, some microaerophiles have evolved aerotactic mechanisms that help them better navigate dynamic gradients of O_2. Indeed, a movement of only a few tens of micrometers may be necessary to place aerotactic cells in a more suitable gaseous environment.

Magnetosomes (Section 2.7) are found in specialized microaerophiles, referred to as *magnetotactic bacteria*, which are found in sediments, lakes, and ponds where concentrations of O_2 are naturally low. Because Earth is a sphere, its magnetic field lines have a significant vertical component where they intersect the surface. Magnetosomes allow magnetotactic bacteria to align themselves with these magnetic field lines. This causes the bacterial cells to point up or down so that they can swim either toward or away from O_2 at the surface. These so-called magnetotactic bacteria do not actually exhibit directed motility toward magnetic fields but instead are exhibiting aerotaxis; their magnetosomes allow them to reduce a three-dimensional biased random walk to a two-dimensional biased random walk and this vastly improves their ability to position themselves in their environment with respect to O_2.

We now segue from our consideration of the structure and function of major components of prokaryotic cells to examine structure–function issues in eukaryotic cells, many of which are microbial and coexist with prokaryotic cells in the microbial world.

Check Your Understanding

- How does scotophobotaxis differ from phototaxis?
- Explain how magnetosomes contribute to the motility of magnetotactic bacteria.

IV • Eukaryotic Microbial Cells

The cytoplasm of eukaryotic cells contains membrane-bound organelles including the nucleus, mitochondria, and chloroplasts, and several other distinct structures. During cell division, the nucleus divides by mitosis and reproductive structures are formed by the process of meiosis.

Compared with prokaryotic cells, microbial eukaryotes typically have structurally more complex and much larger cells. Microbial eukaryotes include the fungi and a vast diversity of protists as we will see in Chapter 18. Many aspects of eukaryotic cell structure set *Eukarya* apart from *Archaea* and *Bacteria*, but the defining feature of the eukaryotic cell is its nucleus.

2.13 The Nucleus and Cell Division

A double membrane–enclosed nucleus is a universal feature of the eukaryotic cell. Mitochondria are also nearly universal among eukaryotic cells, although a few unusual protists lack mitochondria. Chloroplasts are found only in phototrophic eukaryotic cells, including plants and diverse algae. Other characteristic eukaryotic structures include the Golgi complex, lysosomes, endoplasmic reticula, and microtubules and microfilaments (Figure 2.42). Some microbial eukaryotes have flagella or cilia—structures that confer motility—and a cell wall is present in many eukaryotic cells, such as the fungi and algae.

Eukaryotic cell membranes contain *sterols*. These rather rigid organic molecules, which are a subclass of steroids and absent from all but a few prokaryotic cells, lend structural strength to the eukaryotic cell, something especially important to those eukaryotes that lack a cell wall, such as many protists and animal cells.

The Nucleus

The **nucleus** contains the chromosomes of the eukaryotic cell. DNA within the nucleus is wound around basic (positively charged)

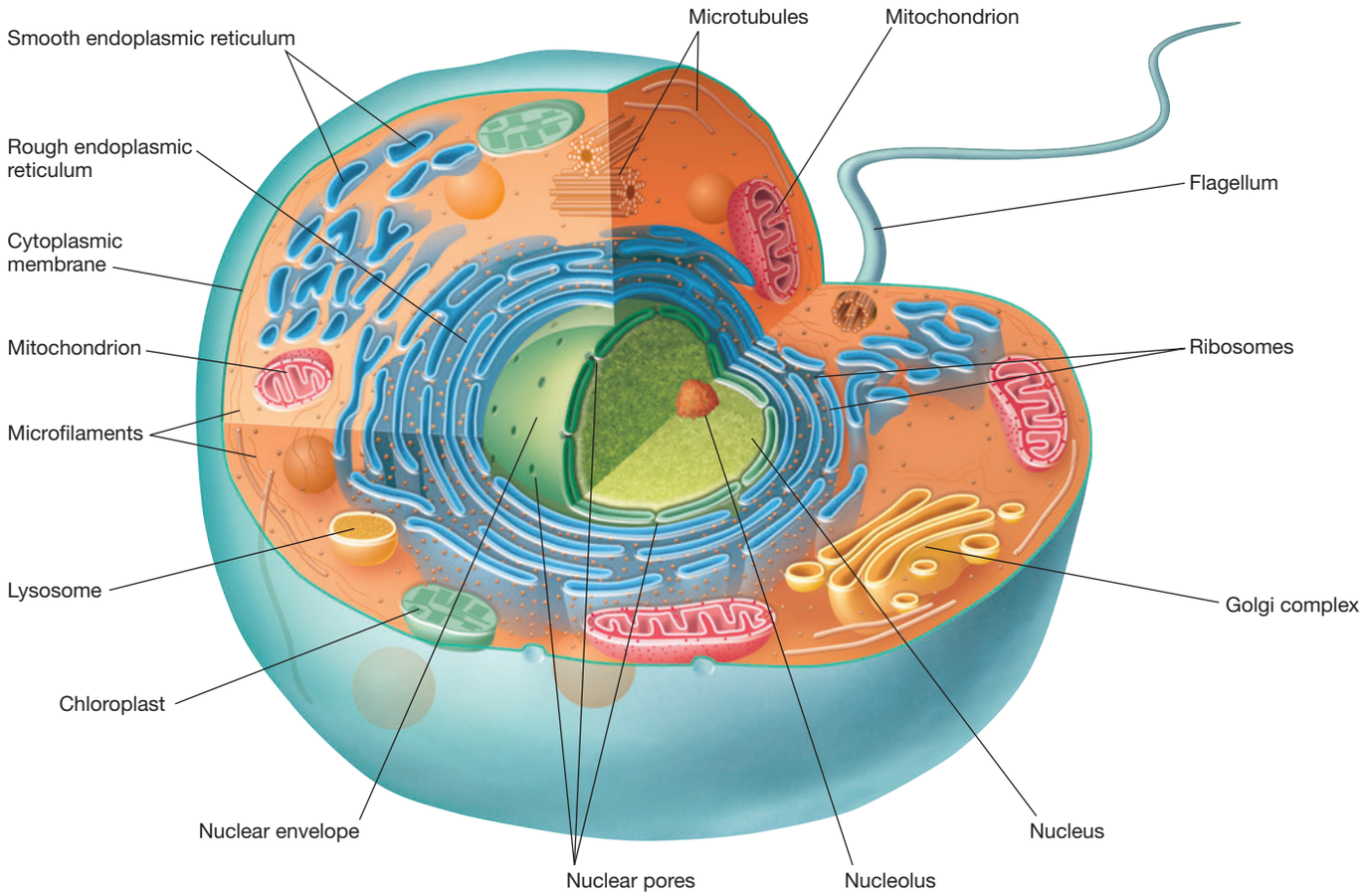


Figure 2.42 **Cutaway schematic of a cell of a microbial eukaryote.** Although all eukaryotic cells contain a nucleus, not all organelles and other structures shown are present in all microbial eukaryotes. Not shown is the cell wall, found in fungi, algae, plants, and a few protists.

proteins called **histones**, which tightly pack the negatively charged DNA to form *nucleosomes* (**Figure 2.43**); the latter are then organized into chromosomes. *Archaea* also contain histones and nucleosomes, and these are evolutionarily related to those found in *Eukarya*. Moreover, a few *Bacteria* also contain histone-like proteins that function to help organize their DNA.

Only cells of *Eukarya* contain a nucleus. The nucleus is enclosed by a *pair* of membranes, each with its own function, separated by a space. The innermost membrane is a simple sac while the outermost membrane is in many places continuous with the endoplasmic reticulum. The inner and outer nuclear membranes specialize in interactions with the nucleoplasm and the cytoplasm, respectively. The nuclear membranes contain pores (Figures 2.42 and 2.43*a*), formed from holes where the inner and outer membranes are joined. The pores allow transport proteins to import and export other proteins and nucleic acids into and out of the nucleus, a process called *nuclear transport*.

Within the nucleus is found the *nucleolus* (Figure 2.42), the site of ribosomal RNA (rRNA) synthesis. The nucleolus is rich in RNA, and ribosomal proteins synthesized in the cytoplasm are transported into the nucleolus and combine with rRNA to form the small and large subunits of eukaryotic ribosomes. These are then exported to the cytoplasm, where they associate to form the intact ribosome and function in protein synthesis.

Cell Division

Eukaryotic cell division requires a special process, called **mitosis**, in which the chromosomes are replicated, the nucleus is disassembled, the chromosomes are segregated into two sets, and a nucleus is reassembled in each daughter cell (**Figure 2.44**). Whereas many (though not all) prokaryotic cells are genetically haploid, microbial eukaryotes often alternate between haploid and diploid states. *Diploid* cells have two copies of each chromosome whereas *haploid* cells have only one. For example, the brewer's yeast *Saccharomyces cerevisiae* can exist in the haploid state (in which cells contain 16 chromosomes) as well as in the diploid state (in which cells contain 32 chromosomes). However, regardless of its genetic state, during cell division the chromosome number is first doubled and later halved to give each daughter cell its correct complement of chromosomes. During mitosis, the chromosomes condense, divide, and are separated into two sets, one for each daughter cell. Each of these distinct stages of nuclear division has a common name used widely in biology: *prophase*, *metaphase*, *anaphase*, and *telophase* (Figure 2.44*a–d*, respectively).

In contrast to mitosis, **meiosis** converts a diploid cell into several haploid cells. Meiosis consists of two successive cell divisions. In the first meiotic division, pairs of chromosomes segregate into separate cells, changing the genetic state from diploid to haploid. The second meiotic division is essentially the same as mitosis, as the two

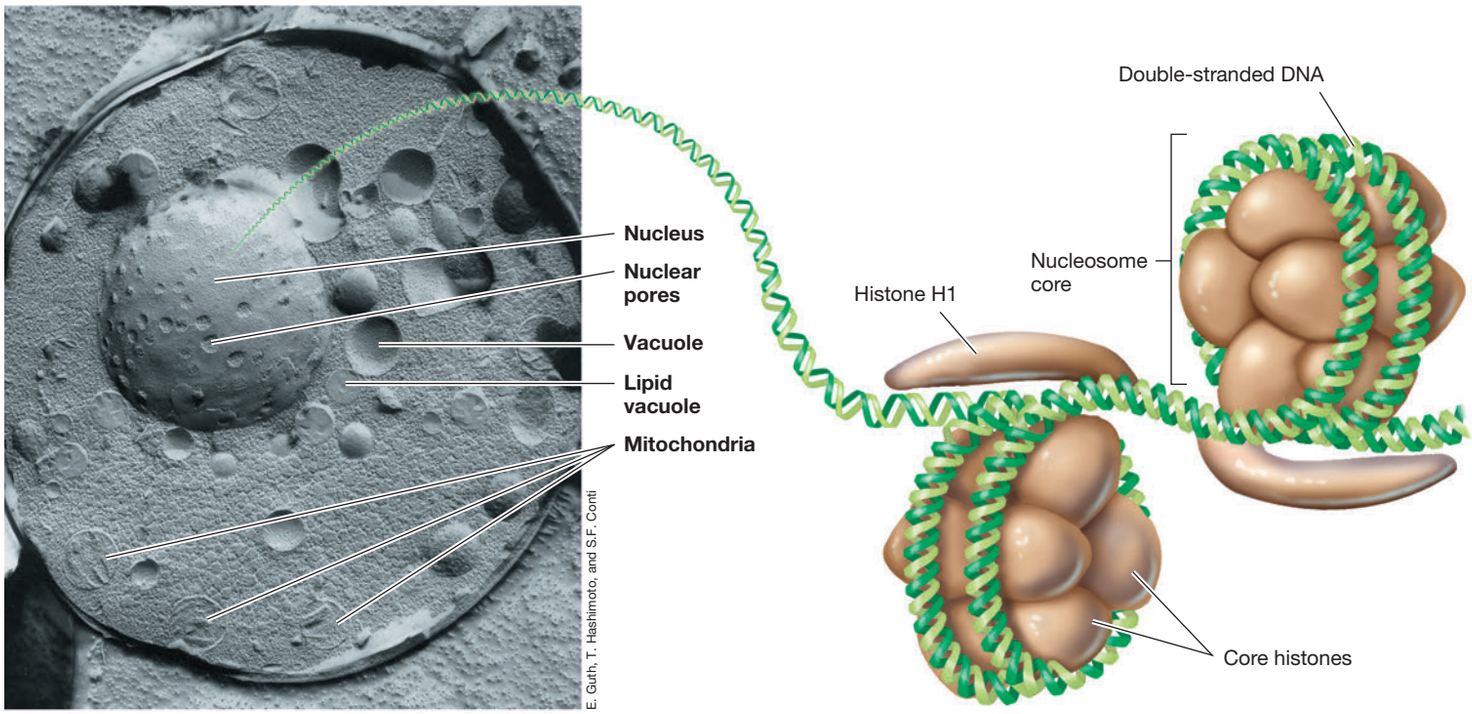


Figure 2.43 The nucleus and DNA packaging in eukaryotes. *(a)* Electron micrograph of a yeast cell prepared in such a way as to reveal a surface view of the nucleus. The cell is about 8 μm wide. *(b)* Packaging of DNA around histone proteins to form a nucleosome. Nucleosomes are arranged along the DNA strand like beads on a string and aggregate to form chromosomes during the process of mitosis (see Figure 2.44).

haploid cells divide to form a total of four haploid cells called *gametes*. This form of cell division is typically used in organisms that reproduce sexually. In animals, these gametes are the eggs and sperm; in eukaryotic microorganisms, they can be reproductive spores or other reproductive structures.

We now consider the classic organelles of the eukaryotic cell: the mitochondrion and the chloroplast.

Check Your Understanding

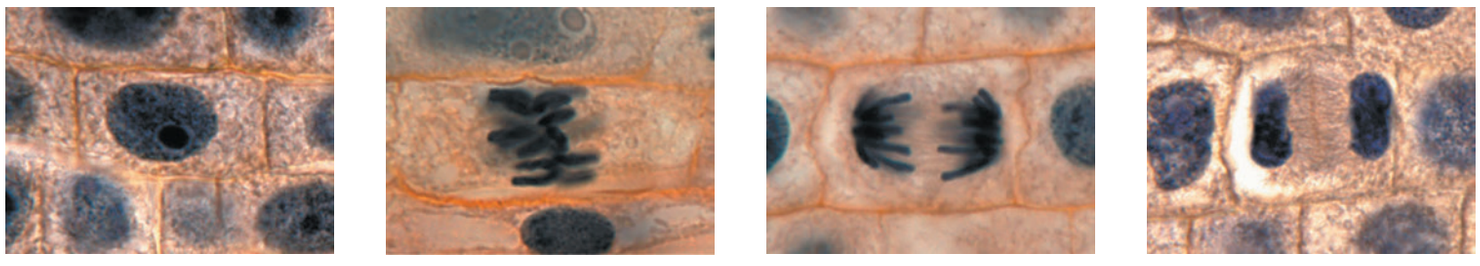
- How is DNA arranged in the chromosomes of eukaryotes?
- What are histones and what do they do?
- What are the major differences between mitosis and meiosis?

2.14 Mitochondria and Chloroplasts

Organelles that specialize in energy metabolism in eukaryotes include the mitochondrion and the chloroplast. These organelles have evolutionary roots within the *Bacteria* and provide ATP to the eukaryotic cell either from the oxidation of organic compounds or from light.

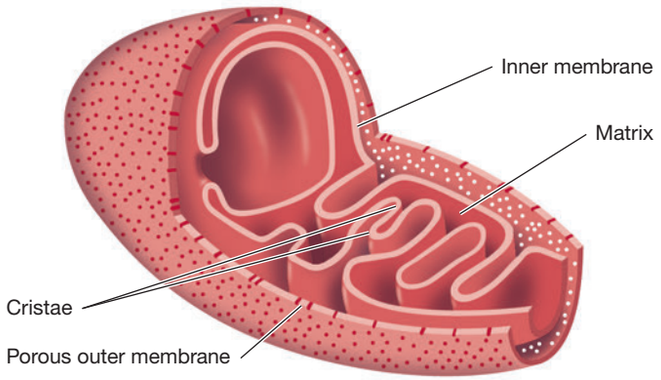
Mitochondria

In aerobic eukaryotic cells, respiration occurs in the mitochondrion. **Mitochondria** are the size of a typical bacterium, they have their own DNA and ribosomes, they can take on many shapes (Figure 2.45), and they reproduce independently of the cell. The number of



(a) **Interphase** (b) **Metaphase** (c) **Anaphase** (d) **Telophase**

Figure 2.44 Light micrograph of eukaryotic cells undergoing mitosis. *(a)* Interphase, distinct chromosomes are not apparent. *(b)* Metaphase. Homologous chromosomes are lining up along the cell center; compare with Figure 2.47*b*. *(c)* Anaphase. Homologous chromosomes are pulling apart. *(d)* Telophase. Chromosomes have separated into the newly forming daughter cells.



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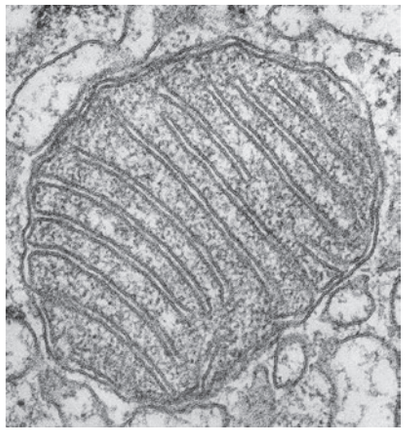
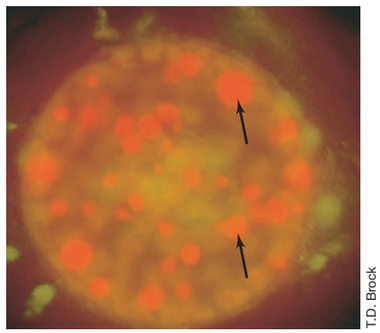


(b)

(c)

Figure 2.45 Structure of the mitochondrion. *(a)* Diagram showing the overall structure of the mitochondrion; note the inner and outer membranes. *(b, c)* Transmission electron micrographs of mitochondria from rat tissue showing the variability in morphology; note the cristae. Both mitochondria are about 0.7 μm wide.



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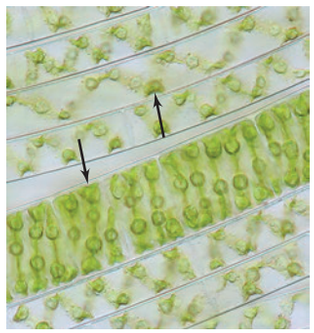

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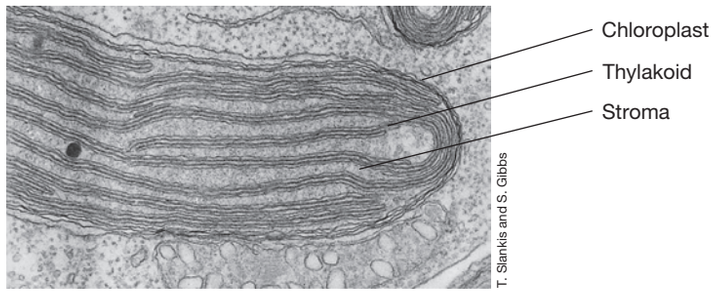

(c)

Figure 2.46 Chloroplasts of a diatom and a green alga cell. *(a)* Fluorescence photomicrograph of a diatom shows chlorophyll fluorescence; arrows, chloroplasts. The cell is about 40 μm wide. *(b)* Phase-contrast photomicrograph of the filamentous green alga *Spirogyra* showing the characteristic spiral-shaped chloroplasts (arrows) of this phototroph. A cell is about 20 μm wide. *(c)* Transmission electron micrograph showing a chloroplast of a diatom; note the thylakoids.

mitochondria per cell varies depending on the cell type, its size, and its physiological state. A yeast cell may have only a few mitochondria per cell, whereas an animal cell may have over a thousand. The mitochondrion is enclosed by a double membrane system. Like the nuclear membrane, the outermost mitochondrial membrane is somewhat permeable and contains pores that allow the passage of small molecules. The innermost membrane is much less permeable, and its structure more closely resembles that of the cytoplasmic membrane of *Bacteria*.

Mitochondria also contain folded internal membranes called **cristae**. These membranes, formed by invagination of the inner membrane, contain the enzymes needed for respiration and ATP production. Cristae also contain transport proteins that regulate the passage of key molecules such as ATP into and out of the *matrix*, the innermost compartment of the mitochondrion (Figure 2.45*a*). The matrix contains enzymes for the oxidation of organic compounds, in particular, enzymes of the citric acid cycle, the major pathway for the combustion of organic compounds to CO_2 (▶ Section 3.6).

Chloroplasts

Chloroplasts are the chlorophyll-containing organelles found in plants and algae and are the site of photosynthesis. Chloroplasts are about the size of unicellular cyanobacteria and are readily visible with the light microscope (**Figure 2.46**). Like mitochondria, the number of chloroplasts per cell varies among species, and chloroplasts contain their own DNA and ribosomes.

Also like mitochondria, chloroplasts are enclosed by a double membrane composed of a permeable outer membrane and a less-permeable inner membrane. The innermost membrane surrounds the **stroma**, analogous to the matrix of the mitochondrion (Figure 2.46*c*). The stroma contains large levels of the enzyme *ribulose bisphosphate carboxylase* (RuBisCO), the key enzyme of the *Calvin cycle*, which is the series of biosynthetic reactions by which phototrophs convert CO_2 to organic compounds (▶ Section 3.12). The permeability of the outermost chloroplast membrane allows glucose and ATP produced during photosynthesis to diffuse into the cell cytoplasm where they are consumed in biosynthesis.

Chlorophyll and all other components needed for ATP synthesis in chloroplasts are located in a series of flattened membrane discs called **thylakoids** (Figure 2.46*c*). Like the cytoplasmic membrane, the thylakoid membrane is highly impermeable, and its major function is to form a proton motive force (Figure 2.4*c*) that results in ATP synthesis.

The Endosymbiotic Origin of Organelles

On the basis of their relative autonomy, size, and morphological resemblance to bacteria, it was hypothesized over 100 years ago that mitochondria and chloroplasts were descendants of respiratory

and phototrophic bacterial cells, respectively. According to this hypothesis, when these symbiotic bacteria associated with nonphototrophic hosts, the hosts gained new forms of energy metabolism while the bacterial partners received a stable and supportive growth environment inside the host cell. Then, over time, these originally free-living symbionts became an intimate part of the eukaryotic cell. The idea that symbiotic bacteria are ancestors to the mitochondrion and chloroplast is called the **endosymbiotic theory** and is well accepted in biology today (▶ Sections 13.4 and 18.1). Several lines of evidence support the endosymbiotic theory. These include, most notably, the fact that mitochondria and chloroplasts contain their own genomes and ribosomes whose structures are similar to the genomes and ribosomes of *Bacteria* but not to those of *Eukarya*. These organellar genomes are also circular, typical of bacterial chromosomes (▶ Section 10.4).

Eukarya are a distinct domain of life, a domain that evolved after *Bacteria* and *Archaea*. Nevertheless, eukaryotic cells share many genes with both *Bacteria* and *Archaea*. In this respect, *Eukarya* are genetic chimeras that contain genes from two domains of life. The domain *Eukarya* is hypothesized to have originated from a symbiotic fusion between an archaeal host cell and a mitochondrial endosymbiont (derived from *Bacteria*). Sometime later, a second major symbiotic event occurred, in which a eukaryotic host cell acquired a chloroplast endosymbiont (derived from cyanobacteria), becoming the ancestor of all phototrophic eukaryotes (including plants and algae).We consider these important concepts in detail in Chapter 13.

Check Your Understanding

- What key reactions occur in the mitochondrion and in the chloroplast, and what key products are made in each? Why is it important to keep these reactions behind a double membrane?
- What is the endosymbiotic theory, and what evidence is there to support it?

2.15 Other Eukaryotic Cell Structures

Several other complex structures are present within the cytoplasm of microbial eukaryotes in addition to mitochondria and chloroplasts (Figure 2.42). These include the endoplasmic reticulum, the Golgi complex, lysosomes, and a dynamic cytoskeleton. Unlike the nucleus, mitochondria, and chloroplasts, these structures lack both DNA and a double membrane and are not of endosymbiotic origin. In addition, these structures control the transport of proteins and nutrients within the cell and govern cell shape and movement. Cell walls are also present in certain microbial eukaryotes (such as fungi and algae), though there is great diversity in cell wall structure among eukaryotic cells.

Cytoskeleton

The eukaryotic cytoplasm is crisscrossed by a series of dynamic protein filaments, which are used to transport substances, to position cell structures, and to control cell movement. This internal support network consists of *microtubules, microfilaments*, and *intermediate filaments*; together, these structures form the cell **cytoskeleton** (Figure 2.42). Some cytoskeleton-like elements are also important for determining the shape and molecular organization of prokaryotic cells, but the eukaryotic cytoskeleton is notable for its complexity and dynamism.

Microtubules are hollow tubes about 25 nm in diameter and are composed of the proteins *α-tubulin* and *β-tubulin*. Microtubules (**Figure 2.47*a***) have many functions including maintaining cell shape and facilitating cell motility, moving chromosomes during mitosis (Figures 2.44 and 2.47*b*), and in the movement of organelles within the cell. **Microfilaments** (Figure 2.47*c*) are smaller than microtubules,

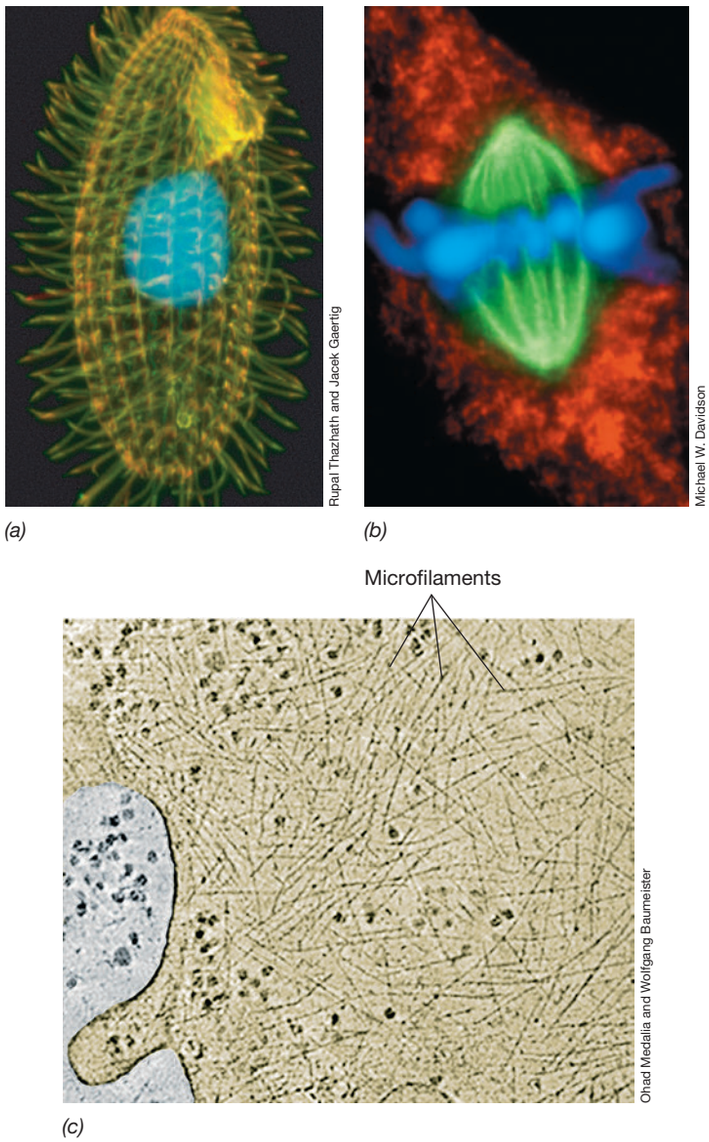

Figure 2.47 Tubulin and microfilaments. *(a)* Fluorescence photomicrograph of a cell of the ciliated protozoan *Tetrahymena thermophila* with red- and green-labeled antitubulin antibodies (the two dyes combine to give yellow) and with DAPI, which stains DNA (blue, nucleus). A cell is about 10 μm wide. *(b)* A mouse cell showing the role of tubulin (green) in separating chromosomes (blue) during metaphase of mitosis (cytoplasmic proteins stain red). Compare with Figure 2.44*c*. *(c)* Electron microscopic image of the cellular slime mold *Dictyostelium discoideum* showing the network of actin microfilaments that along with microtubules functions as the cell cytoskeleton. Microfilaments are about 7 nm in diameter.

about 7 nm in diameter, and are polymers of two intertwined strands of the protein *actin*. Microfilaments function in maintaining or changing cell shape, in cell motility by cells that move by amoeboid movement, and during cell division. **Intermediate filaments** are fibrous keratin proteins that are arranged into fibers 8–12 nm in diameter and function in maintaining cell shape and positioning organelles in the cell.

Endoplasmic Reticulum, the Golgi Complex, and Lysosomes

The endoplasmic reticulum (ER) is a network of membranes continuous with the nuclear membrane. Two types of endoplasmic reticulum exist: *rough* ER, which contains attached ribosomes, and *smooth* ER, which does not (Figure 2.42). Smooth ER participates in the synthesis of lipids and in some aspects of carbohydrate metabolism. Rough ER, through the activity of its ribosomes, is a major producer of glycoproteins and also produces new membrane material that is transported throughout the cell to enlarge the various membrane systems before cell division.

The Golgi complex is a stack of membrane-bound sacs. In the Golgi complex, products of the ER are chemically modified and sorted into those destined for secretion versus those that will function in other membranous structures in the cell. Many of the modifications made in the Golgi complex are glycosylations (addition of sugar residues) that convert the proteins into glycoproteins that can then be targeted to specific locations in the cell.

Lysosomes (Figure 2.42) are membrane-enclosed compartments that contain digestive enzymes that hydrolyze proteins, fats, and polysaccharides. The lysosome fuses with food that enters the cell in vacuoles and then releases digestive enzymes that break down the foods for biosynthesis and energy generation. Lysosomes also function in degrading damaged cellular components and recycling these materials for new biosyntheses. The lysosome thus allows the cell's lytic activities to be partitioned away from the cytoplasm proper. Following the degradation of macromolecules in the lysosome, the resulting nutrients pass from the lysosome into the cytoplasm for use by cytoplasmic enzymes.

Flagella and Cilia

Flagella and cilia are present on the surface of many eukaryotic microbes and function as motility structures, allowing cells to move by swimming. As we learned when we considered motility in prokaryotic cells (Sections 2.9–2.12), motility has survival value, as the ability to move allows motile organisms to move about their habitat and exploit new resources. *Cilia* are essentially short flagella that beat in synchrony to propel the cell—usually quite rapidly—through the medium. *Flagella*, by contrast, are long appendages present singly or in groups that propel the cell along—typically more slowly than

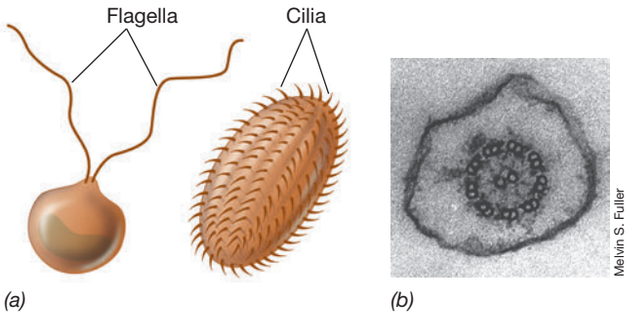

Figure 2.48 Motility organelles in eukaryotic cells: Flagella and cilia. *(a)* Flagella can be present as single or multiple filaments. Cilia are structurally similar to flagella but much shorter. Eukaryotic flagella move in a whiplike motion. *(b)* Cross section through a flagellum of the fungus *Blastocladiella* showing the outer sheath, the outer nine pairs of microtubules, and the central pair of microtubules.

by cilia—through a whiplike motion (**Figure 2.48*a***). In cross section, eukaryotic cilia and flagella appear similar. Each contains a bundle of nine pairs of microtubules surrounding a central pair of microtubules (Figure 2.48*b*). A protein called *dynein* is attached to the microtubules and uses ATP to drive motility. Movement of flagella and cilia is similar. In both cases, movement is the result of the coordinated sliding of microtubules against one another in a direction toward or away from the base of the cell. This movement confers the whiplike motion on the flagellum or cilium that results in cell propulsion.

Eukaryotic flagella differ structurally and functionally from the flagella of *Bacteria* and the archaella of *Archaea*, and these three structures should not be confused (contrast Figure 2.48 with Figure 2.34). Eukaryotic flagella are much larger than flagella or archaella, are enclosed by the cytoplasmic membrane and contain a cytoskeleton, and do not rotate but rather whip back and forth through the activity of their cytoskeleton proteins.

Armed with the major principles of microbial cell structure and function, we move on to Chapter 3 where the metabolic events that provide the energy necessary to build cellular components will unfold as a prelude to our consideration of the building process itself.

Check Your Understanding

- Why are the activities in the lysosome partitioned from the rest of the cytoplasm?
- Describe the structural elements that comprise the cytoskeleton.
- In terms of structure and function, how do the flagella of eukaryotic cells differ from those of prokaryotic cells?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • The Cell Envelope

2.1 The cytoplasmic membrane is a highly selective permeability barrier constructed of lipids and proteins that form a bilayer, with a hydrophobic interior and hydrophilic exterior. In contrast to *Bacteria* and *Eukarya*, where fatty acids are ester-linked to glycerol, *Archaea* contain ether-linked lipids, and some form monolayer instead of bilayer membranes. The major functions of the cytoplasmic membrane are permeability, transport, and energy conservation, and nutrient accumulation requires energy.

Q Does the cytoplasmic membrane of prokaryotic cells provide shape and rigid support to the cell? Contrast the typical structure of the cytoplasmic membranes of *Bacteria* and *Archaea*.

2.2 The active transport of nutrients into the cell is an energy-requiring process driven by ATP (or some other energy-rich compound) or by the proton motive force. Three transporters are employed by prokaryotic cells: simple, group translocation, and ABC systems. Each mechanism accumulates solutes against the concentration gradient.

Q Cells of *Escherichia coli* transport lactose via lac permease, glucose via the phosphotransferase system, and maltose via an ABC-type transporter. For each of these sugars describe: (1) the components of the transport system and (2) the source of energy that drives the transport event.

2.3 Peptidoglycan is a polysaccharide found only in *Bacteria* that consists of an alternating repeat of *N*-acetylglucosamine and *N*-acetylmuramic acid, the latter cross-linked by tetrapeptides in adjacent strands. The enzyme lysozyme and the antibiotic penicillin both destroy peptidoglycan, leading to cell lysis.

Q Why is the rigid layer of the bacterial cell wall called peptidoglycan? What are the structural reasons for the rigidity that is conferred on the cell wall by the peptidoglycan structure?

2.4 Gram-negative *Bacteria* have an outer membrane consisting of LPS, protein, and lipoprotein. Porins allow for permeability across the outer membrane. The gap between the outer and cytoplasmic membranes is called the periplasm and contains proteins that function in transport, sensing chemicals, and other important cell functions.

Q Is the outer membrane more selective or less selective than the cytoplasmic membrane? Describe the periplasm and its possible functions.

2.5 Cell envelopes can exhibit a range of different structures. One common variation is the presence of an outer S-layer composed of protein or glycoprotein. S-layers function as the cell wall for many *Archaea* and they are also found in many *Bacteria*.

Q What functions can S-layers provide to those cells that make them?

II • Cell Surface Structures and Inclusions

2.6 Many prokaryotic cells have capsules, slime layers, or pili. These structures have several functions, including attachment, genetic exchange, and twitching motility. Hami, present on the surface of certain *Archaea*, function as miniature grappling hooks to attach cells to a surface or to one another.

Q What function(s) do polysaccharide layers outside the cell wall have in prokaryotic cells?

2.7 Prokaryotic cells can contain inclusions of sulfur, polyphosphate, carbon polymers, various minerals formed by biomineralization, or atmospheric gases. These substances function as nutrient storage materials, control magnetotaxis (in the case of magnetosomes), or confer buoyancy (in the case of gas vesicles).

Q What are some of the roles of poly-β-hydroxyalkanoates (PHAs)?

2.8 The endospore is a highly resistant and differentiated structure produced by certain gram-positive *Bacteria*. Endospores are highly dehydrated and contain calcium dipicolinate and small acid-soluble spore proteins, both of which are absent from vegetative cells. Endospores can remain dormant indefinitely but can germinate quickly when conditions warrant.

Q Is an endospore still the same bacterial cell? What are the different types of endospores as to their location in the cell? Describe the structure of endospores.

III • Cell Locomotion

2.9 Swimming motility in prokaryotic cells is due to flagella (*Bacteria*) or archaella (*Archaea*). Both structures are composed of several proteins, are anchored in the cell wall and cytoplasmic membrane, and cause cells to swim by their rotation. However, flagella and archaella differ in both structure and mechanism of operation.

Q Compare and contrast the differences in structure and mechanism between the flagella of *Bacteria* and the archaella of *Archaea*.

2.10 Some microbes are capable of surface motility in which they move along solid surfaces by one of several mechanisms including twitching or gliding.

Q Contrast the mechanisms of gliding motility in *Flavobacterium* with twitching motility in *Pseudomonas* and swimming motility in *Escherichia coli*.

2.11 Swimming bacteria respond to chemical and physical gradients in their environment by controlling the lengths of runs and frequency of tumbles, resulting in a biased random walk. Tumbles are controlled by the direction of rotation of the flagellum, which in turn is controlled by a network of sensory and response proteins.

Q Chemotaxis causes motile bacteria to either move towards attractants or move away from repellents. Movement is determined by coordinated runs and tumbles, leading to biased random walks in response to the concentration of the chemotactic chemical. How would simple diffusion of an attractant over time affect the biased random walk?

2.12 Phototaxis describes directed motility in response to light intensity, while scotophobotaxis describes directed motility away from the dark. Aerotaxis describes directed motility in response to O_2 gradients.

Q Magnetotactic bacteria do not exhibit directed motility in response to magnetic fields and hence their name is a misnomer. Describe the role of magnetosomes and aerotaxis in the directed movements of magnetotactic bacteria.

IV • Eukaryotic Microbial Cells

2.13 Microbial eukaryotes contain several organelles including the nucleus, mitochondria, and chloroplasts. The nucleus contains the cell's DNA wrapped around histone proteins. Cells of microbial eukaryotes divide following the process of mitosis and may also undergo meiosis if a haploid/diploid life cycle occurs.

Q Eukaryotic cell division's aim is to create two identical, functional daughter cells. Hence, the DNA in the parent cell is replicated so that both daughter cells receive a full set of chromosomes. Do other organelles such as mitochondria, endoplasmic reticulum, etc. in the cytoplasm and cell membrane need to be replicated too? Explain.

2.14 Mitochondria and chloroplasts are energy-generating organelles. According to the endosymbiotic theory, these organelles were once free-living *Bacteria* that later established symbiotic relationships within ancestral cells of *Eukarya*.

Q Describe the evidence that supports the idea that the major organelles of eukaryotes were once *Bacteria*.

2.15 Endoplasmic reticula are membranous structures in eukaryotes that either contain attached ribosomes (rough ER) or do not (smooth ER). Flagella and cilia are means of motility and in eukaryotic cells move by a whiplike mechanism instead of by rotation. Lysosomes specialize in degrading proteins and other macromolecules no longer needed by the cell. Microtubules, microfilaments, and intermediate filaments function as internal cell structures that combine to form the cell cytoskeleton.

Q Compare and contrast the structure and function of eukaryotic flagella with those of prokaryotic cells.

APPLICATION QUESTIONS

1. Assume you are given two cultures, one of a species of gram-negative *Bacteria* and one of a species of *Archaea*. Discuss at least four different ways you could analyze these two cultures and tell which culture was which.
2. Gram-negative and gram-positive bacteria have different susceptibility to antibiotics because of differences in cell envelope structure. Assume that you are given an antibiotic that is effective against gram-positive cells but ineffective against gram-negative cells because it cannot cross the outer membrane. How might you chemically modify this antibiotic to increase its efficacy against gram-negative organisms?

CHAPTER GLOSSARY

ABC transport system a membrane transport system consisting of three proteins, one of which hydrolyzes ATP; the system transports specific nutrients into the cell

Archaellum a long, thin cellular appendage present in some *Archaea* that rotates and is responsible for swimming motility

Basal body the "motor" portion of the bacterial flagellum, embedded in the cytoplasmic membrane and cell wall

Capsule a polysaccharide or protein outermost layer, usually rather slimy, present on some bacteria

Cell envelope the system of layered structures that surround the cytoplasm and define the outer boundary of the cell

Chemotaxis directed movement of an organism toward (positive chemotaxis) or away from (negative chemotaxis) a chemical gradient

Chloroplast the photosynthetic organelle of phototrophic eukaryotes

Cristae the internal membranes of a mitochondrion

Cytoplasmic membrane a semipermeable barrier that separates the cell interior (cytoplasm) from the environment

Cytoskeleton the cellular scaffolding typical of eukaryotic cells, made of microtubules, microfilaments, and intermediate filaments

Dipicolinic acid a substance unique to endospores which, when complexed with Ca^{2+}, confers heat resistance on these structures

Endospore a highly heat-resistant, thick-walled, differentiated structure produced by certain gram-positive *Bacteria*

Endosymbiotic theory the idea that mitochondria and chloroplasts originated from *Bacteria*

Flagellum a long, thin cellular appendage that rotates in *Bacteria* or has a whiplike motion in *Eukarya* and is responsible for swimming motility

Gas vesicles gas-filled cytoplasmic structures bounded by a single layered protein membrane that confer buoyancy on cells

Gliding motility a form of surface motility characterized by smooth continuous movement

Group translocation an energy-dependent transport system in which the substance transported is chemically modified during the process of being transported by a series of proteins

Histones highly basic proteins that compact and wind DNA in the nucleus of eukaryotic cells

Intermediate filament a filamentous polymer of fibrous keratin proteins, supercoiled into thicker fibers, that functions in maintaining cell shape and the positioning of certain organelles in the eukaryotic cell

Lipopolysaccharide (LPS) a combination of lipid with polysaccharide and protein that forms the major portion of the outer membrane in gram-negative *Bacteria*

Lysosome an organelle containing digestive enzymes for hydrolysis of proteins, fats, and polysaccharides

Magnetosome a particle of magnetite (Fe_3O_4) or greigite (Fe_3S_4) enclosed by a single membrane in the cytoplasm of magnetotactic *Bacteria*

Meiosis the nuclear division that halves the diploid number of chromosomes to the haploid

Microfilament a filamentous polymer of the protein actin that helps maintain the shape of a eukaryotic cell

Microtubule a filamentous polymer of the proteins α-tubulin and β-tubulin that functions in eukaryotic cell shape and motility

Mitochondrion the respiratory organelle of eukaryotic organisms

Mitosis nuclear division in eukaryotic cells in which chromosomes are replicated and partitioned into two daughter cells during cell division

Nucleus a membrane-enclosed structure in eukaryotic cells that contains the cell's DNA genome

Outer membrane a phospholipid- and polysaccharide-containing unit membrane that lies external to the peptidoglycan layer in cells of gram-negative *Bacteria* and is a component of the cell envelope in these organisms

Peptidoglycan a polysaccharide composed of alternating repeats of *N*-acetylglucosamine and *N*-acetylmuramic acid arranged in adjacent layers cross-linked by short peptides; a component of the cell envelope of virtually all *Bacteria*

Periplasm a region between the outer surface of the cytoplasmic membrane and the inner surface of the lipopolysaccharide layer of gram-negative *Bacteria*

Peritrichous flagellation having flagella located in many places around the surface of the cell

Phototaxis movement of an organism toward light

Pili thin, filamentous structures that extend from the surface of a cell and, depending on type, facilitate cell attachment, genetic exchange, or twitching motility

Polar flagellation having flagella emanating from one or both poles of the cell

Poly-β-hydroxybutyric acid (PHB) a common storage material of prokaryotic cells consisting of a polymer of β-hydroxybutyrate or another β-alkanoic acid or mixtures of β-alkanoic acids

S-layer an outermost cell surface layer composed of protein or glycoprotein present on some *Bacteria* and many *Archaea*

Simple transport system a transporter that consists of only a membrane-spanning protein and is typically driven by energy from the proton motive force

Stroma the lumen of the chloroplast, surrounded by the inner membrane

Teichoic acid a phosphorylated polyalcohol found in the cell wall of some gram-positive *Bacteria*

Thylakoids membrane stacks containing the photosynthetic pigments in cyanobacteria or in the chloroplast of eukaryotic phototrophs

Twitching motility a form of surface motility caused by extension and retraction of type IV pili

Microbial Metabolism 3

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MICROBIOLOGY**NOW**

Life Begins with Metabolism

Metabolism is the foundation upon which life is formed. Microorganisms have evolved tremendous metabolic diversity, but all microbes in their vast diversity have a common set of requirements. Life requires liquid water, a source of energy to do work, a source of electrons to perform biochemical reactions, and nutrients required to build macromolecules. Once we understand these requirements, we can begin to make predictions about how life behaves in any environment, whether a caustic hypersaline pond, inside a rock from the cold deserts of Antarctica, in a hydrothermal vent chimney from the ocean's depths, or even on another world. In short, the search for life begins with an understanding of metabolism.

Water is common in our solar system. There is water on Mars and on Earth's moon, on asteroids and comets, and there is evidence for water on several other planets and moons. Liquid water, however, is harder to find. One place that has liquid water is the moon Enceladus, which orbits Saturn. Enceladus has an ice-covered ocean with cracks in its surface through which erupt geysers that spew materials into space (see photo). The Cassini spacecraft photographed and flew through these plumes, detecting silicate minerals that form only in the presence of liquid water.

The gravity from Saturn squeezes Enceladus, resulting in volcanic activity and liquid water beneath its icy crust. These plumes also contain diverse organic and inorganic compounds and the gases H_2, CO_2, and methane (CH_4). Taken as a whole, these molecules are sufficient to provide free energy, a source of electrons, and nutrients required to support life. Indeed, microbes could likely survive at the hydrothermal vents thought to exist on Enceladus. We now know that all of the fundamental requirements for life are present on other worlds, and the search for extraterrestrial life is a search for microbial life.

Source: Waite, J.H., et al. 2017. Cassini finds molecular hydrogen in the Enceladus plume: Evidence for hydrothermal processes. *Science* 356: 155.

Metabolism is the series of biochemical reactions needed to sustain life. Metabolism includes **catabolism**—reactions used to obtain energy and break down complex molecules—and **anabolism**—reactions used to synthesize cellular material (**Figure 3.1**). Microorganisms have evolved tremendous metabolic diversity through which they influence all aspects of our biosphere. In this chapter, we will learn the unifying concepts of metabolism that underlie all living systems. In particular, we will examine the manner in which cells obtain energy and how they use this energy to synthesize the building blocks of macromolecules and cells (Figure 3.1*a*). We will also see that metabolism relies on the directed movement of electrons from an *electron donor* to an *electron acceptor* (Figure 3.1*b*). We will revisit these principles of metabolism in Chapter 6, where we explore the biosynthesis of macromolecules—the nucleic acids and proteins—and in Chapter 14, where the enormous metabolic diversity of the microbial world unfolds.

I • Fundamentals of Metabolism

Bioenergetics describes the transformation of energy during metabolism characterized by electron flow within the cell. Metabolism uses the energy currency of ATP to couple the exergonic reactions of catabolism to the endergonic reactions of anabolism.

According to the first law of thermodynamics, energy is neither created nor destroyed. Hence, in order to grow, cells must *conserve energy* by converting energy available from their surroundings into a form that can do work. Cells accomplish this by generating energy-rich compounds such as *adenosine triphosphate* (ATP)—a molecule capable of storing energy and releasing it to fuel cellular processes (Figure 3.1).

3.1 Defining the Requirements for Life

All living cells share certain fundamental metabolic requirements. All cells require *water* in which to perform metabolic reactions, as well as sources of *carbon* and other *nutrients* with which to synthesize cellular materials. All cells also require **free energy**—*the energy available to do work*—and **reducing power**—a source of electrons (e^-) that can be used to both generate free energy and perform certain biosynthetic reactions (Figure 3.1). We will review the nutrient requirements of cells in Chapter 4. Here we will learn to categorize and understand all forms of metabolism in terms of free energy and reducing power.

Free Energy

All chemical reactions can be described in terms of their relationship to energy. Some chemical reactions *release energy* as they proceed, while others *require an input of energy* to proceed. Consider the reaction for the aerobic respiration of glucose:

$$C_6H_{12}O_6 + 6\,O_2 \rightleftharpoons 6\,CO_2 + 6\,H_2O$$

In this reaction the reactants are on the left ($C_6H_{12}O_6 + 6\,O_2$) and the products are on the right ($6\,CO_2 + 6\,H_2O$). The *change* in free energy during a reaction is expressed as $\Delta G^{0\prime}$, where the symbol Δ is read as "change in." The "0" and "prime" in $\Delta G^{0\prime}$ indicate that the free-energy value is for *standard conditions*: pH 7 (approximate cytoplasmic conditions), 25°C, 1 atmosphere of pressure, and all reactants and products at molar concentrations. The term $\Delta G^{0\prime}$ is expressed in units of kilojoules/mole (abbreviated as kJ/mol), a unit of heat energy. If the $\Delta G^{0\prime}$ for a reaction is *negative* in arithmetic sign, then the reaction will proceed with the *release* of free energy and such reactions are **exergonic**. However, if $\Delta G^{0\prime}$ for the reaction is *positive*, the reaction *requires* energy in order to proceed and such reactions are **endergonic**. Thus, exergonic reactions *release* free energy whereas endergonic reactions *require* free energy. For example, the aerobic respiration of glucose provides a free-energy change of $\Delta G^{0\prime} = -2895$ kJ/mol of glucose. Hence, this reaction is exergonic and it provides a substantial amount of free energy that can be used by the cell to do work. We will learn how to calculate the free-energy change of a reaction in Section 3.3.

Catabolic pathways are exergonic processes in which cells generate free energy by transforming reactants into products (Figure 3.1*a*). Free energy is energy available to do work. The free energy produced in catabolism is conserved by synthesizing energy-rich molecules such as ATP (Figure 3.1*a*). The formation of ATP requires at least $\Delta G^{0\prime} = -31.8$ kJ/mol. Hence, the aerobic respiration of a mole of glucose could produce up to 91 moles of ATP under standard conditions, though under natural cellular conditions this reaction actually produces closer to 38 moles of ATP. This difference in ATP yield occurs because reactions in the cell do not occur under standard

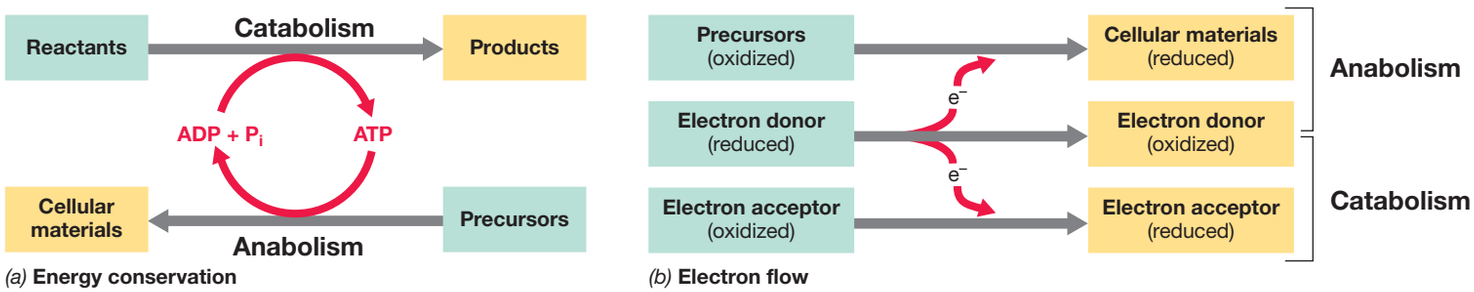


Figure 3.1 Metabolic coupling with respect to energy conservation and electron flow. *(a)* Catabolism employs exergonic reactions to drive the synthesis of ATP. Anabolism employs endergonic reactions, which consume ATP, to drive the biosynthesis of cellular material. Some energy would be lost as heat and cannot be conserved in the formation of ATP (not shown). *(b)* Cells require reducing power, in the form of a reduced electron donor, as a source of electrons (e^-) needed to carry out anabolic and catabolic reactions. Inputs to metabolism are labeled in {green} and outputs of metabolism are labeled in {yellow}.

conditions. We will see in Section 3.3 that the free energy available under natural conditions differs from the free energy calculated at standard conditions. In addition, chemical reactions release some portion of energy in the form of heat, which cells cannot conserve in the formation of ATP. The heat lost during metabolic reactions is what makes your body warm and what makes a decomposing compost pile become steaming hot.

Anabolic pathways are endergonic processes in which the synthesis of cellular material from simple precursors requires an input of energy (Figure 3.1*a*). The energy required to fuel anabolic reactions, and to biosynthesize cellular materials, comes from the hydrolysis of ATP. In this way, catabolic and anabolic reactions are fundamentally linked (Figure 3.1*a*).

Reducing Power

Reducing power is the ability to donate electrons during electron transfer reactions. Electron transfer reactions, called **redox reactions**, are comprised of two half reactions. During redox reactions, electrons are transferred from an **electron donor** in one half reaction to an **electron acceptor** in a second half reaction. When an electron is added to a substance, we say that the substance has been *reduced* because the addition of an electron reduces the oxidation state of the substance. In contrast, when an electron is removed from a substance we say it has been *oxidized* (Figure 3.1*b*). Consider again the aerobic respiration of glucose (**Figure 3.2**). In this example, which is a redox reaction, glucose is the electron donor and O_2 is the electron acceptor. Electrons are transferred from glucose to O_2, resulting in the oxidation of glucose to CO_2 and the reduction of O_2 to H_2O (Figure 3.2). Redox reactions are an essential component of catabolism, and we will see that they are integral to energy conservation within the cell.

Reducing power is also required by anabolic reactions (Figure 3.1*b*). The biosynthesis of cellular materials from simple precursors requires both free energy, in the form of ATP, and reducing power, in the form of electron carriers that transfer electrons to anabolic reactions. We will learn more about the metabolic significance of electron carriers and redox reactions in Section 3.2. First, we consider how different organisms obtain the free energy they need to drive ATP synthesis.

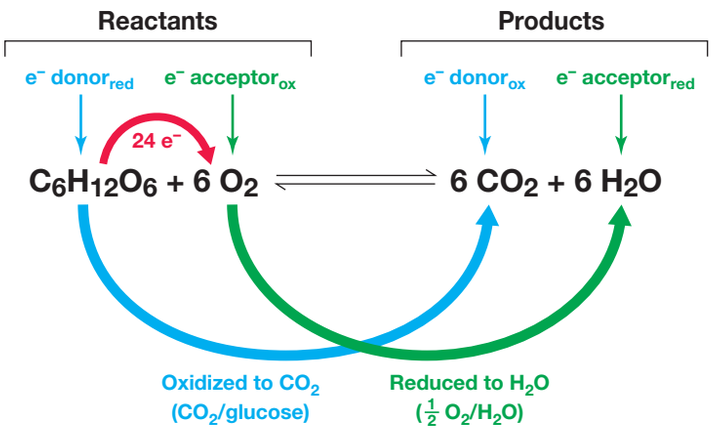


Figure 3.2 Example of an oxidation–reduction reaction. The oxidation of glucose to CO_2 is coupled with the reduction of O_2 to H_2O in aerobic chemoorganotrophic organisms. This redox reaction is composed of two half reactions: CO_2/glucose and $\frac{1}{2}\ O_2/H_2O$ (see Figure 3.4). The overall reaction is balanced, which means that equal amounts of each element (C, H, O) are present in reactants and products. The complete oxidation of one glucose to 6 CO_2 donates 24 electrons, which are accepted by 6 O_2.

Metabolic Classes of Microorganisms

All types of metabolism can be classified based on their source of energy (**Figure 3.3**). **Phototrophs** obtain energy for metabolism from light. Plants are one type of phototroph, but we will learn that many different types of phototrophic metabolism exist in the microbial world (see Section 3.11 and Chapters 14 and 15). **Chemotrophs** obtain energy for metabolism from chemical reactions (Figure 3.3). The aerobic respiration of glucose is an example of chemotrophic metabolism in that free energy comes from the chemical oxidation of glucose to CO_2 (Figures 3.2 and 3.3). Chemotrophic reactions are classified as *aerobic* if they require O_2 as an electron acceptor, but they are classified as anaerobic if their electron acceptor is anything other than O_2 (see Section 3.10). Chemotrophs can conserve energy from either *respiration* reactions or *fermentation* reactions (see Sections 3.7–3.9). For example, in the presence of O_2, yeast can perform aerobic respiration of glucose to CO_2, but when O_2 is limiting they alter their metabolism to perform anaerobic fermentation of glucose to ethanol and CO_2.

The ultimate source of energy for chemotrophic organisms can be *organic* or *inorganic*. Organic molecules are those molecules that contain carbon, excluding certain carbon-containing gases and minerals considered inorganic (for example, compounds such as CO_2, CO, carbonates, cyanides, diamond, and graphite are considered inorganic). Organisms that obtain their energy and reducing power from organic molecules are called **chemoorganotrophs** while those that obtain their energy and reducing power from inorganic molecules

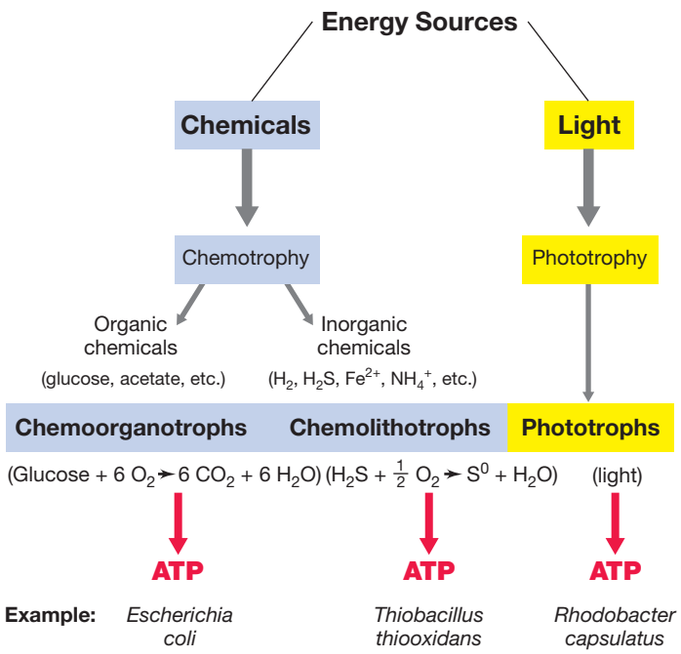


Figure 3.3 Classification of metabolic types based on energy sources. Microorganisms can obtain energy from organic chemicals, inorganic chemicals, or light. Most organisms employ only one type of metabolism, but some microbes can use different energy sources depending on environmental conditions.

are called **chemolithotrophs** (Figure 3.3). Most microorganisms in laboratory culture, and most pathogenic bacteria, use carbohydrates or proteins as their energy source and are thus chemoorganotrophs. Chemolithotrophs, by contrast, are important in many biogeochemical cycles. Electron donors for chemolithotrophs include gaseous hydrogen (H_2), hydrogen sulfide (H_2S), ammonia (NH_3), and ferrous iron (Fe^{2+}). Related groups of chemolithotrophs typically specialize in the oxidation of a group of similar inorganic compounds, and thus we have the "sulfur" bacteria, the "iron" bacteria, the "nitrifying" bacteria, and so on (see Chapters 14 and 15).

Phototrophs contain chlorophylls and other pigments that convert light energy into ATP and thus, unlike chemotrophs, do not require chemicals as a source of energy (Section 3.11). Two forms of phototrophy exist: *oxygenic photosynthesis* and *anoxygenic photosynthesis*. Oxygenic photosynthesis, in which O_2 is produced, is characteristic of cyanobacteria (▶ Section 15.3) and is also carried out by plants and algae. Anoxygenic photosynthesis (▶ Section 14.5), in which O_2 is not produced, predates oxygenic photosynthesis, and it occurs in diverse lineages of *Bacteria*. We explore the diversity of phototrophs further in Chapters 14 and 15.

Regardless of how a microorganism conserves energy, be it from chemicals or from light, all cells require large amounts of carbon in one form or another to make new cell materials. A **heterotroph** obtains carbon for biosynthesis from an organic compound; nearly all chemoorganotrophs are also heterotrophs. An **autotroph**, by contrast, uses carbon dioxide (CO_2) as its carbon source, reducing it to cell material at the expense of ATP (Section 3.12). Most chemolithotrophs and phototrophs are autotrophs. Autotrophs are also called *primary producers* because they synthesize new organic matter from inorganic carbon (CO_2). Virtually all organic matter on Earth has been synthesized by primary producers, in particular, by phototrophs.

We now expand on this introduction to metabolism and energy by emphasizing electron transfer reactions, energy-rich compounds, and enzymes in the biochemical workings of a cell.

Check Your Understanding

- What is free energy and how is it used by the cell?
- What is reducing power and what is its relationship to electron donors and acceptors?
- How does a chemoorganotroph differ from a chemolithotroph? A chemotroph from a phototroph?

3.2 Electron Transfer Reactions

Catabolism, and indeed life itself, depends on the directed flow of electrons from an *electron donor* to an *electron acceptor* during redox reactions (Figure 3.1*b*). Redox reactions are also required in many biosynthetic reactions that occur during anabolism (Sections 3.12–3.15). Redox reactions can be understood in terms of *reduction potential* (**Figure 3.4**), which measures the affinity of a substance for electrons.

Redox Reactions and Reduction Potentials

Although life requires the transfer of electrons within the cell, electrons cannot exist stably in solution. Therefore, electrons must be transferred directly from one atom or molecule to another during redox reactions. Redox reactions occur in pairs, called *half reactions* (also called *redox couples*). A half reaction depicts the conversion of one substance into another caused by adding or removing electrons. By convention, in writing a half reaction, the *oxidized* form of a substance is always placed on the left (before the forward slash) followed by the *reduced* form after the forward slash (Figure 3.4). For example, the addition of an electron to nitrate (NO_3^-) causes it to be reduced to nitrite (NO_2^-) and so we write this redox couple as NO_3^-/NO_2^- (Figure 3.4).

In a redox reaction the first half reaction produces one or more electrons that are then consumed by the second half reaction. The reactant in the first half reaction donates electrons and is *oxidized*, whereas the reactant in the second half reaction accepts these electrons and is *reduced*. In redox reactions of this type, we refer to the reactant that is *oxidized* as the *electron donor*, and the reactant that is *reduced* is the *electron acceptor*. Consider the redox reaction between glucose and O_2 (Figure 3.2). The two half reactions that contribute to this overall reaction are CO_2/glucose and $\frac{1}{2}\,O_2/H_2O$ (Figure 3.4). In this reaction, glucose is oxidized to CO_2, causing the reduction of O_2 to H_2O. Both glucose and O_2 are reactants in the overall reaction with glucose serving as electron donor and O_2 serving as electron acceptor. The products of this reaction are CO_2 and H_2O. Many

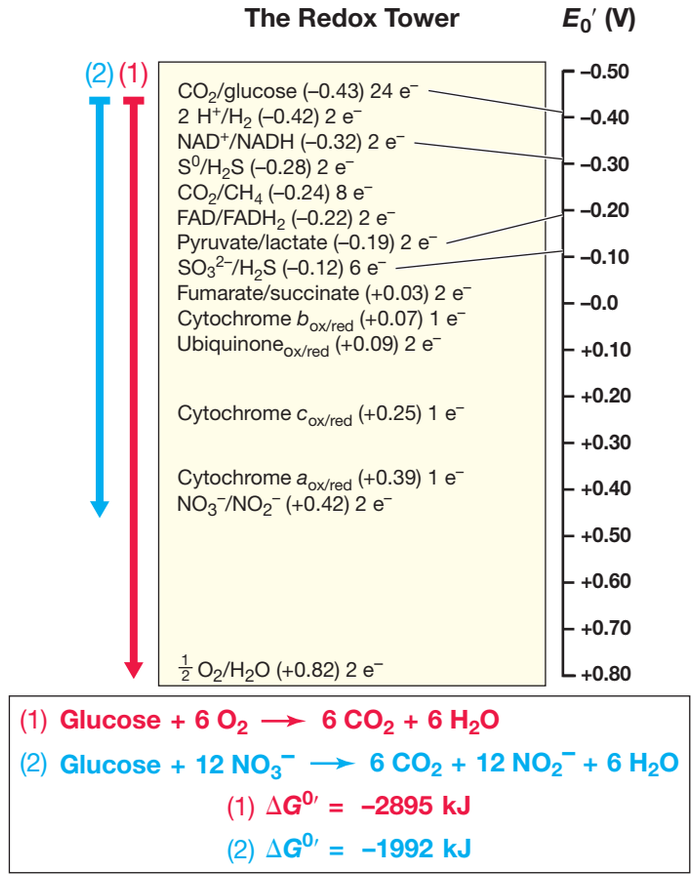


Figure 3.4 The redox tower. Redox couples are arranged from the strongest electron donors at the top to the strongest electron acceptors at the bottom. The greater the difference in reduction potential between electron donor and electron acceptor, the greater the free energy released. Note the differences in free-energy yield when glucose reacts with either O_2 (1) or nitrate (2).

TABLE 3.1 Reduction potentials, E_0' (volts), of some redox half reactions commonly encountered in microbiology[a]

Pyruvate/glucose (−0.70) 4 e^-	Flavodoxin$_{ox/red}$ (−0.12) 2 e^-	NO_3^-/NH_4^+ (+0.34) 8 e^-
Acetate/pyruvate (−0.68) 4 e^-	SO_3^{2-}/H_2S (−0.12) 6 e^-	NO_2^-/NO (+0.36) 2 e^-
Acetate/acetaldehyde (−0.60) 2 e^-	Menaquinone$_{ox/red}$ (−0.07) 2 e^-	SeO_4^{2-}/SeO_3^{2-} (+0.48) 2 e^-
SO_4^{2-}/HSO_3^- (−0.52) 2 e^-	APS/AMP + HSO_3^- (−0.06) 2 e^-	Tetrachloroethene/trichloroethylene + Cl^- (+0.58) 2 e^-
Ferredoxin$_{ox/red}$ (−0.42) 2 e^-	Rubredoxin ox/red (−0.06) 1 e^-	$NO_3^-/\frac{1}{2}N_2$ (+0.74) 5 e^-
CO_2/methanol (−0.38) 6 e^-	Acrylyl-CoA/proprionyl-CoA (−0.02) 2 e^-	Fe^{3+}/Fe^{2+} (+0.77) 1 e^- (at pH 2)
Coenzyme F420$_{ox/red}$ (−0.36) 2 e^-	Fe^{3+}/Fe^{2+} (+0.20) 1 e^- (at pH 7)	Mn^{4+}/Mn^{2+} (+0.80) 2 e^-
CO_2/acetate (−0.28) 8 e^-	TMAO/TMA (+0.13) 2 e^-	ClO_3^-/Cl^- (+1.03) 4 e^-
Methanophenazine$_{ox/red}$ (−0.26) 2 e^-	AsO_4^{3-}/AsO_3^{3-} (+0.14) 2 e^-	NO/N_2O (+1.18) 1 e^-
FMN/$FMNH_2$ (−0.22) 2 e^-	DMSO/DMS (+0.23) 2 e^-	N_2O/N_2 (+1.36) 1 e^-
Acetaldehyde/ethanol (−0.20) 2 e^-	Chlorobenzoate/benzoate + Cl^- (+0.30) 2 e^-	

[a]Entries are read as: oxidized form/reduced form (E_0' in volts) and number of e^- transferred per half reaction. Values are organized from lowest (most electronegative) to highest (most electropositive) reduction potential.

different redox couples exist in nature (Figure 3.4 and Table 3.1) and can participate in diverse redox reactions within the cell.

All redox couples have the ability to either donate or accept electrons to another redox couple. Within each redox couple, the reduced substance (which occurs after the forward slash) is the chemical form that donates electrons, and the oxidized substance (which occurs before the forward slash) is the form that accepts electrons. Whether a substance is an electron donor or an electron acceptor in a given redox reaction is determined by the **reduction potential** (E_0' at standard conditions) of the participating half reactions (Figure 3.4 and Table 3.1). Reduction potentials are measured in volts (V) compared to a reference substance (typically, the reference is H_2). Electrons are negatively charged and so the *reduced* substance in a redox couple will have a strong tendency to *donate* electrons when the redox couple has a negative reduction potential (for example, glucose in the CO_2/glucose couple, $E_0' = -0.43$ V). In contrast, the *oxidized* substance in a redox couple will have a strong tendency to *accept* electrons when the redox couple has positive reduction potential (for example, O_2 in the $\frac{1}{2}O_2/H_2O$ couple, $E_0' = +0.82$ V). Ultimately, the tendency of a substance to donate or accept electrons in a redox reaction is determined by the difference in reduction potential between the two participating redox couples. In a given redox couple the substance on the right is the chemical form that serves as an electron donor in a redox reaction, while the substance on the left is the chemical form that serves as an electron acceptor.

By convention, half reactions are written as reductions (as in Figure 3.4 and Table 3.1). However, when two half reactions are combined into a single redox reaction the half reaction that donates electrons (that is, the half reaction with the more negative E_0') proceeds as an oxidation, and its orientation is therefore written in reverse (as an oxidation) in the overall redox reaction. For example, the CO_2/glucose couple has a far more negative reduction potential than the $\frac{1}{2}O_2/H_2O$ couple, a difference of 1.25 V (Figure 3.4). Hence, in the redox reaction that includes these two couples, glucose is the electron donor and its half reaction is written as an oxidation, with the net reaction having the reactants glucose and O_2 and the products CO_2 and H_2O (Figures 3.2 and 3.4).

When aerobic respiration is prevented by the absence of O_2, some organisms can carry out anaerobic respiration using an alternative electron acceptor, such as nitrate (see Section 3.10). Nitrate (NO_3^-) is the oxidized substance in the NO_3^-/NO_2^- redox couple ($E_0' = 0.42$ V). The CO_2/glucose couple has a more negative reduction potential than the NO_3^-/NO_2^- couple, a difference of 0.85 V (Figure 3.4). Hence, glucose will serve as a favorable electron donor with nitrate as the electron acceptor (see Figure 3.4 for the balanced reaction). The positive difference in reduction potential of the two half reactions indicates that this will be a favorable reaction, though because there is less difference in reduction potential (0.85 V relative to 1.25 V), the anaerobic respiration of glucose using NO_3^- as electron acceptor yields less energy than aerobic respiration (Figure 3.4).

In this way, reduction potentials can be used to determine whether any given substance in a half reaction will serve as electron donor or electron acceptor in an overall redox reaction. In addition, *the greater the difference in the reduction potentials of the two half reactions in a redox couple, the more energy will be available to the cell.* A vast diversity of redox couples exist in nature, allowing for a tremendous diversity of redox reactions (Figure 3.4 and Table 3.1). We will see that microbes have evolved to harness energy from a large number of redox reactions, giving rise to the astounding metabolic diversity present in the microbial world (Chapters 14 and 15). We will learn in Section 3.3 how to use the difference in reduction potential between two half reactions to calculate the overall free-energy yield of a reaction.

Electron Carriers and NAD^+/NADH Cycling

Cells require a net movement of electrons from an electron donor to an electron acceptor, but such reactions are rarely performed in a single step. More often, the movement of electrons from electron donor to electron acceptor proceeds through a series of consecutive reactions at different locations within the cell. Hence, the cell needs soluble electron carriers such as **nicotinamide adenine dinucleotide (NAD^+/NADH)** to carry electrons from one place to another within the cell (Figure 3.5). NAD^+/NADH is a redox couple with a reduction potential of −0.32 V, which makes NADH a good electron donor and NAD^+ a weak electron acceptor (Figure 3.4). The reduction of NAD^+ to NADH requires 2 e^- and 1 H^+, but the

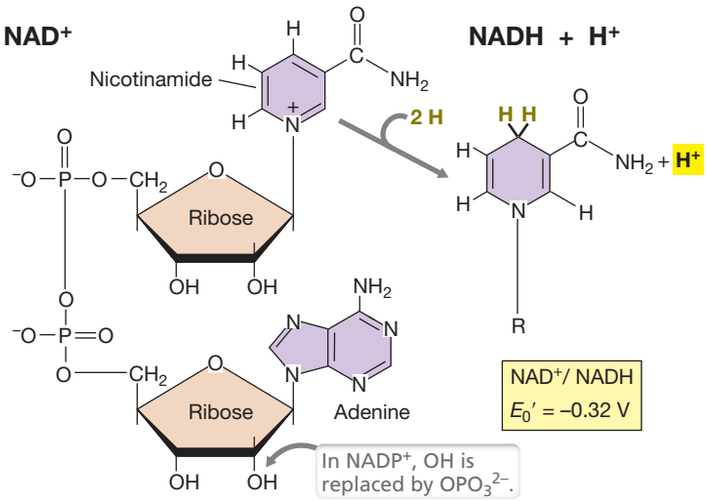


Figure 3.5 The redox coenzymes nicotinamide adenine dinucleotide (NAD^+) and $NADP^+$. NAD^+ undergoes oxidation–reduction as shown and is freely diffusible. "R" is the adenine dinucleotide portion of NAD^+; H is hydrogen (consisting of $H^+ + e^-$).

oxidation of electron donors typically results in the production of $2\ e^-$ and $2\ H^+$. Therefore, the reduction of NAD^+ typically results in the production of NADH + H^+ with the extra proton released into solution (Figure 3.5).

Mastering Microbiology
Art Activity: Figure 3.12 NAD^+/NADH cycling

Electron carriers such as NAD^+/NADH are common *coenzymes* (see Section 3.5) in cells. The use of electron carriers as coenzymes increases the diversity of redox reactions that are possible in a cell by allowing many different electron donors and acceptors to interact. For example, an enzyme can remove electrons from an electron donor and use them to reduce NAD^+ to NADH (**Figure 3.6**). The NADH then diffuses away from the enzyme and attaches to a different enzyme that oxidizes NADH back to NAD^+ when it reduces an electron acceptor (Figure 3.6).

Electron shuttling mediated by NAD^+/NADH is common in microbial catabolism. However, in addition to NAD^+/NADH, many other molecules may participate as electron shuttles. For example, nicotinamide adenine dinucleotide phosphate ($NADP^+$) is made from NAD^+ by adding a phosphate molecule (Figure 3.5). We will see that $NADP^+$/NADPH participates in many anabolic biosynthetic reactions, whereas NAD^+/NADH typically participates in catabolic redox reactions.

Check Your Understanding

- In the reaction $2\ H_2 + O_2 \rightleftharpoons 2\ H_2O$, what is the electron donor and what is the electron acceptor?
- Which of the following can accept electrons from NADH: CO_2, lactate, or pyruvate? Explain your reasoning.
- Can both glucose and H_2S be used to reduce NAD^+? Why or why not?

3.3 Calculating Changes in Free Energy

We have seen that cells conserve energy from chemical reactions or from light, and they transfer electrons from electron donors to electron acceptors by using electron carriers such as NADH. Ultimately, in order to conserve energy, cells need to synthesize energy-rich molecules such as ATP. We will see that numerous (but not all) electron transfer reactions are sufficiently exergonic to produce ATP and thus can support a vast diversity of microbial metabolisms. However, to better understand this diversity in metabolism, we once again return

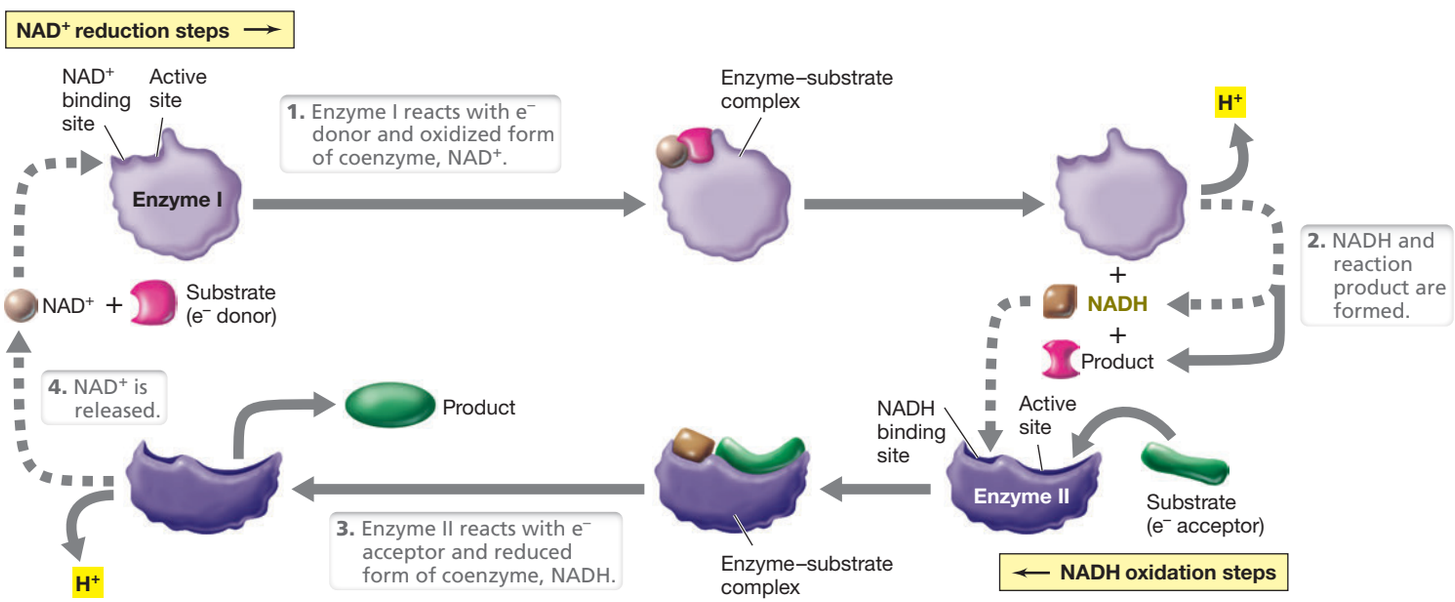


Figure 3.6 NAD^+/NADH cycling. A schematic example of redox reactions in which two different enzymes are linked by their requirement for either NAD^+ or NADH. One enzyme reduces NAD^+ in its reaction while the other enzyme oxidizes NADH in its reaction.

to the concept of reduction potential and this time place it in the context of free-energy change.

The Redox Tower and Its Relationship to $\Delta G^{0\prime}$

A convenient way of viewing the energy available in electron transfer reactions is to imagine a vertical tower that represents the entire range of reduction potentials possible for redox couples in nature, from those with the most negative E_0' on the top to those with the most positive E_0' at the bottom; this is a *redox tower* (Figure 3.4). Now imagine electrons from an electron donor near the top of the tower falling and being "caught" by electron acceptors at lower levels. The difference in reduction potential between the donor and acceptor redox couples is expressed as $\Delta E_0'$, the change in reduction potential. For example, we have seen that the oxidation of glucose with O_2 as electron acceptor yields far more energy than when nitrate (NO_3^-) is the electron acceptor (Section 3.2 and Figure 3.4).

The further an electron drops before it is caught by an acceptor, the greater is the $\Delta E_0'$ between the two redox couples and the greater is the amount of energy released in the net reaction. That is, $\Delta E_0'$ is proportional to $\Delta G^{0\prime}$ (Figure 3.4). This relationship is expressed more precisely in the equation $\Delta G^{0\prime} = -nF\Delta E_0'$, where n is the number of electrons transferred and F is the Faraday constant (96.5 kJ/V). Hence, we can see that redox reactions provide a source of energy to the cell and that the greater the difference in reduction potential between electron donor and electron acceptor, the greater the energy available to the cell. This is illustrated for an example reaction, the oxidation of acetate to CO_2, in equation 1 of **Table 3.2**.

Calculating $\Delta G^{0\prime}$ from the Free Energy of Formation

There is a second way to calculate $\Delta G^{0\prime}$ if one knows the *free energy of formation* (G_f^0) of the reactants and products in the reaction. The free energy of formation is equal to the energy released or required during the formation of a given molecule from its constituent elements. **Table 3.3** lists the G_f^0 for a few common substances. By convention, the free energy of formation of the *elements* in their elemental and electrically neutral form (for instance, C, H_2, N_2) is zero. For most compounds, G_f^0 is negative. This reflects the fact that compounds tend to form spontaneously (that is, with a free-energy release) from their elements. However, the positive G_f^0 for nitrous oxide (N_2O) (Table 3.3) indicates that this compound does not form spontaneously. Instead, over time it decomposes spontaneously to yield N_2 and O_2. The compounds listed in Table 3.3 are only a small subset of free energy of formation values available from physical chemistry reference sources.

Using free energies of formation, it is possible to calculate $\Delta G^{0\prime}$ of a reaction. For the reaction $A + B \rightleftharpoons C + D$, $\Delta G^{0\prime}$ is calculated by subtracting the sum of the free energies of formation of the reactants (A + B) from that of the products (C + D). Thus

$$\Delta G^{0\prime} = G_f^0[C + D] - G_f^0[A + B]$$

The value obtained for $\Delta G^{0\prime}$ tells us whether the reaction is exergonic (and can be a potential energy source for the cell) or endergonic (and requires an energy input to proceed). The phrase "products minus reactants" is a simple way to recall how to calculate changes in free energy during chemical reactions. This is illustrated for an example reaction, the oxidation of acetate to CO_2, in Table 3.2, equation 2.

TABLE 3.2 Example of free-energy-change calculations using electrochemical potentials or G_f^0 values

For the reaction in which acetate is oxidized completely to CO_2:[a]

$CH_3COO^- + H^+ + 2\,O_2 \rightarrow 2\,CO_2 + 2\,H_2O$

1. Calculation from E_0' values:[b]
   $\Delta G^{0\prime} = -nF\Delta E_0'$
   $= [-8(96.5)(1.1)]$
   $= -849$ kJ/reaction
2. Calculation from G_f^0 values:
   $\Delta G^{0\prime} = [G_f^0\text{ (products)} - G_f^0\text{ (reactants)}]$
   $= [G_f^0(2\,CO_2 + 2\,H_2O) - G_f^0(CH_3COO^- + H^+ + 2\,O_2)]$
   $= -852$ kJ/reaction

[a]The reaction is balanced and is an 8-electron oxidation ($n = 8$ in equation 2). G_f^0 values were taken from Table 3.3.
[b]F is the Faraday constant (96.5 kJ/V) and $\Delta E_0'$ is calculated from the E_0' values in Figure 3.4 and Table 3.1.

TABLE 3.3 Free energy of formation (G_f^0, kJ/mol) for some common substances[a]

Sugars	Organic and fatty acids		Amino acids and alcohols	Gases and inorganic compounds
Fructose (−951.4)	Acetate (−369.4)	Ketoglutarate (−797.5)	Alanine (−371.5)	O_2, N_2, H_2, S^0, Fe^0 (0)
Glucose (−917.2)	Benzoate (−245.6)	Lactate (−517.8)	Aspartate (−700.4)	CH_4 (−50.8)
Lactose (−1515.2)	Butyrate (−352.6)	Malate (−845.1)	*n*-Butanol (−171.8)	CO_2 (−394.4); CO (−137.4)
Ribose (−369.4)	Caproate (−335.9)	Propionate (−361.1)	Ethanol (−181.7)	H_2O (−237.2); H^+ (−39.8); OH^- (−198.7)
Sucrose (−757.3)	Citrate (−1168.3)	Pyruvate (−474.6)	Glutamate (−699.6)	N_2O (+104.2); NO (+86.6)
	Formate (−351.1)	Succinate (−690.2)	Glutamine (−529.7)	NO_2^- (−37.2); NO_3^- (−111.3)
	Fumarate (−604.2)	Valerate (−344.3)	Glycerol (−488.5)	NH_3 (−26.57); NH_4^+ (−79.4)
	Glyoxylate (−468.6)		Mannitol (−942.6)	H_2S (−27.87); HS^- (+12.1)
			Methanol (−175.4)	SO_4^{2-} (−744.6); $S_2O_3^{2-}$ (−513.4)
			n-Propanol (−175.8)	Fe^{2+} (−78.8); Fe^{3+} (−4.6); FeS (−100.4)

[a]Values for free energy of formation taken from Speight, J. 2005. *Lange's Handbook of Chemistry*, 16th edition, and Thauer, R.K., K. Jungermann, and H. Decker. 1977. Energy conservation in anaerobic chemotrophic bacteria. *Bacteriol. Rev. 41:* 100–180.

Before free-energy calculations can be made, it is first necessary to balance the reaction. That is, (1) the total number of each kind of atom and ionic charges must be identical on both sides of the reaction, and (2) the oxidation–reduction state must balance such that all of the electrons removed from one substance are transferred to another substance. Once a reaction is balanced, its $\Delta G^{0\prime}$ can be calculated, and from this, the potential of the reaction as a means of energy conservation for a cell can be assessed.

Calculating Free-Energy Change in Natural Conditions

Although calculations of $\Delta G^{0\prime}$ are often very accurate estimates of actual free-energy changes, in some cases they are not. Consider the oxidation of propionate to H_2 and CO_2 (**Figure 3.7**). This reaction is important in wetlands because microorganisms called *methanogens* in these habitats ultimately consume H_2 and CO_2 and turn them into methane (CH_4), a powerful greenhouse gas (Chapters 14, 17, and 21). Propionate oxidation is quite unfavorable at standard conditions when reactants and products are all present at molar concentration ($\Delta G^{0\prime} = +304$ kJ/mol). In wetlands, however, methanogens consume H_2, lowering its concentration dramatically, and low H_2 concentrations allow propionate oxidation to become exergonic (Figure 3.7). We will see when we pick up the bioenergetics theme again in Chapter 14 that the actual concentrations of products and reactants are almost never present at molar concentrations in a microbe's natural habitat. Hence, the calculation of $\Delta G^{0\prime}$, while a useful approximation, can misrepresent the actual energy available to cells as they occur in the environment. In this regard, what is most relevant to a bioenergetic calculation is not $\Delta G^{0\prime}$, but ΔG, the free-energy change that occurs *under the actual conditions* in which the organism is growing. The equation for ΔG takes into account the actual concentrations of reactants and products in the organism's habitat and is expressed as

$$\Delta G = \Delta G^{0\prime} + RT \ln K_{eq}$$

where R and T are physical constants and K_{eq} is the equilibrium constant for the reaction. For the reaction $aA + bB \rightleftharpoons cC + dD$, $K_{eq} = [C]^c[D]^d/[A]^a[B]^b$, A and B are reactants and C and D are products; a, b, c, and d are the number of molecules of each, and the brackets indicate concentrations.

Returning to the example of propionate oxidation (Figure 3.7), we can see that the reaction becomes increasingly exergonic when propionate (the reactant) increases in concentration and when H_2 (the product) decreases in concentration. Hence, propionate oxidation to CO_2 and H_2, though unfavorable at standard conditions, becomes more favorable as reactant concentrations go up and as product concentrations go down. The H_2 concentration has a greater effect on ΔG than the propionate concentration because 7 H_2 are formed for every propionate molecule oxidized, and hence H_2 has a far greater impact on the value of the equilibrium constant for the reaction. While propionate oxidation is endergonic under standard conditions, propionate-oxidizing microbes can thrive in the environment as long as H_2-consuming microbes (such as methanogens) coexist with them. Such interdependency is common in the microbial world and we will learn more about metabolic interactions between microbial partners in Chapter 14.

While $\Delta G^{0\prime}$ and ΔG are rarely identical, at this point in our understanding of bioenergetics the expression $\Delta G^{0\prime}$ is sufficient to appreciate the general principles of energy flow in microbial systems. The main point to keep in mind is that *only exergonic reactions* yield energy that can be conserved by the cell, and this will be our focus in the next few sections.

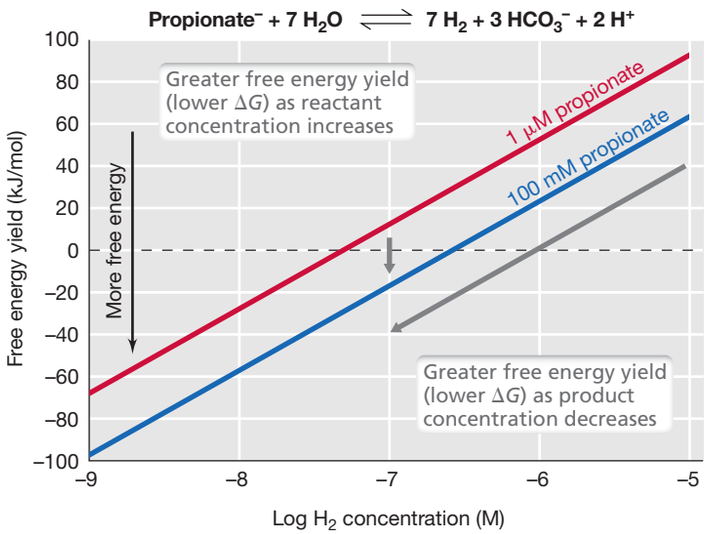


Figure 3.7 Calculating free energy under natural conditions (ΔG). The example is the oxidation of propionate to H_2 and CO_2 (as shown at the top of the figure, with CO_2 written as HCO_3^- [bicarbonate] because this is the form in which it naturally occurs in solution at neutral pH). This reaction is endergonic under standard conditions ($\Delta G^{0\prime} = +304$ kJ/mol) but can be exergonic under natural conditions in an environment where the H_2 produced in the reaction is quickly consumed by other microbes. The ΔG was calculated at different concentrations of reactants (propionate) and products (H_2).

Check Your Understanding

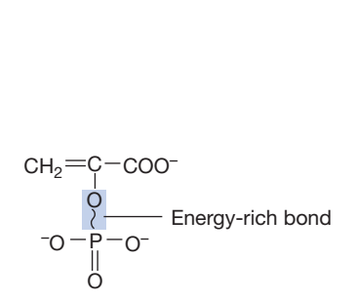
- Using Figure 3.4, calculate $\Delta G^{0\prime}$ for the reaction $CH_4 + 2\,O_2 \rightleftharpoons CO_2 + 2\,H_2O$.
- Does glucose formation from the elements release or require energy?
- Using Table 3.3, calculate $\Delta G^{0\prime}$ for the reaction $2\,CH_4 + O_2 \rightleftharpoons 2\,CH_3OH$.

3.4 Cellular Energy Conservation

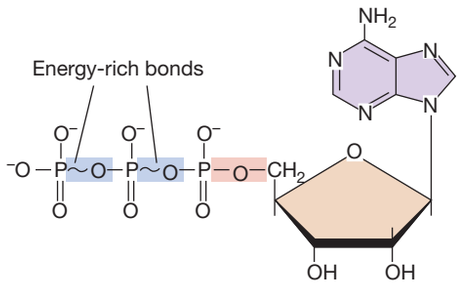
Chemotrophs conserve energy from chemical reactions while phototrophs conserve energy from light. We have just reviewed how to calculate the free-energy change of chemical reactions. Now we introduce mechanisms of energy conservation. Ultimately, cells conserve energy by synthesizing energy-rich compounds. We begin by reviewing the energy-rich compounds that cells commonly use to conserve energy.

Adenosine Triphosphate

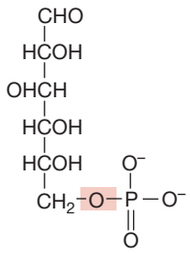
The most important energy-rich phosphate compound in cells is **adenosine triphosphate (ATP)**. ATP consists of the ribonucleoside



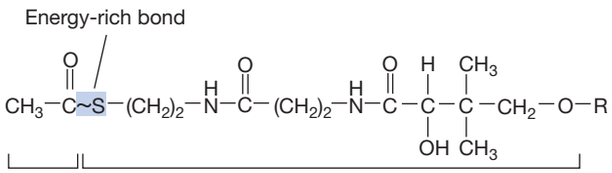
Phosphoenolpyruvate



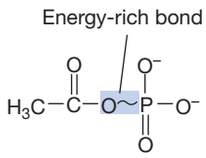
Adenosine triphosphate (ATP)



Glucose 6-phosphate



Acetyl-CoA



Acetyl phosphate

Compound	$G^{0'}$ kJ/mol
$\Delta G^{0'}$ > 30 kJ	
Phosphoenolpyruvate	−61.9
1,3-Bisphosphoglycerate	−49.4
Acetyl phosphate	−44.8
ATP	−31.8
ADP	−31.8
Acetyl-CoA	−35.7
$\Delta G^{0'}$ < 30 kJ	
AMP	−14.2
Glucose 6-phosphate	−13.8

Figure 3.8 Energy-rich bonds in compounds that conserve energy in microbial metabolism. The range in free energy of hydrolysis values for the phosphate or sulfur bonds highlighted in the compounds is listed in the table. The "R" group of acetyl-CoA is a 3′-phospho ADP group.

adenosine to which three phosphate molecules are bonded in series. From the structure of ATP (**Figure 3.8**), it can be seen that only two of the phosphate bonds (ATP ⇌ ADP + P_i and ADP ⇌ AMP + P_i) have free energies of hydrolysis more exergonic than −30 kJ/mol. By contrast, the phosphate bond in AMP contains only about half as much energy as those in ADP or ATP (Figure 3.8).

Although the energy released in ATP hydrolysis is −31.8 kJ/mol, a caveat must be mentioned here to more precisely state the energy requirements for the synthesis of ATP. In an actively growing *Escherichia coli* cell, the ratio of ATP to ADP is maintained at about 7:1, and this increases the actual free-energy requirements for ATP synthesis. Thus, in an actively growing cell, the actual energy that must be released (that is, the ΔG, Section 3.3) to drive the synthesis of ATP is closer to −55 to −60 kJ/mol. Nevertheless, for the purposes of learning and applying the basic principles of bioenergetics, we will assume that reactions conform to "standard conditions" ($\Delta G^{0\prime}$), and therefore we will assume that the energy needed to drive ATP synthesis (from ADP + P_i) is −31.8 kJ/mol. This unit is the fundamental currency of energy conservation within the cell and we will see that a major goal of energy conservation in catabolism is to obtain sufficient energy to form ATP.

Energy-Rich Compounds

ATP is not the only energy-rich compound in the cell; several others exist that, like ATP, have energy-rich phosphate bonds, and still others have energy-rich sulfur bonds. The biosynthesis of these compounds functions to trap free energy, and their hydrolysis releases this energy to drive endergonic reactions such as ATP synthesis.

A range of molecules have bonds consisting of a phosphate linked to an organic compound (Figure 3.8). However, not all phosphate bonds are energy-rich bonds. As seen in this figure, the $\Delta G^{0\prime}$ of hydrolysis of the phosphate bond in glucose 6-phosphate is −13.8 kJ/mol. By contrast, the $\Delta G^{0\prime}$ of hydrolysis of the phosphate bond in phosphoenolpyruvate is −61.9 kJ/mol, almost four times that of glucose 6-phosphate. Although the phosphate in either compound could be hydrolyzed to release energy, cells need compounds whose $\Delta G^{0\prime}$ of phosphate hydrolysis exceeds −31.8 kJ/mol to synthesize ATP.

Some energy-rich compounds have carbon–sulfur bonds. These include, in particular, derivatives of *coenzyme A* (for example, acetyl-CoA in Figure 3.8). Coenzyme A derivatives contain energy-rich *thioester* bonds, and hydrolysis of these bonds yields sufficient free energy to couple to the synthesis of an energy-rich phosphate bond. For example, in the coupled reaction

$$\text{Acetyl-S-CoA} + H_2O + \text{ADP} + P_i \rightleftharpoons \text{acetate}^- + \text{HS-CoA} + \text{ATP} + H^+$$

the energy released in the hydrolysis of coenzyme A is conserved in the synthesis of ATP. We will return to the importance of coenzyme A derivatives in microbial bioenergetics many times in Chapter 14.

Mechanisms of Energy Conservation

Cells conserve energy by generating ATP through one of three fundamental mechanisms. The first mechanism is **substrate-level phosphorylation**. In substrate-level phosphorylation the energy-rich bond of a substrate is hydrolyzed to directly drive the formation of ATP. For example, hydrolysis of the phosphate bond in

phosphoenolpyruvate is sufficiently exergonic to drive ATP formation (see Figures 3.8 and 3.11). We will see that substrate-level phosphorylation is the dominant mechanism of energy conservation in fermentative organisms (see Section 3.7 and Figure 3.21).

The second mechanism of energy conservation is **oxidative phosphorylation**. In oxidative phosphorylation the movement of electrons from an electron donor to an electron acceptor (Figure 3.1*b*) generates a *proton motive force*. The **proton motive force** is an electrochemical gradient formed by energy-conserving reactions that transport protons outside the cytoplasmic membrane. This electrochemical gradient creates a force that is ultimately used to synthesize ATP (◀ Section 2.1 and Figure 2.4). Oxidative phosphorylation is the defining feature of respiration reactions and it is performed by diverse chemotrophic organisms (Sections 3.9–3.11).

The third mechanism of energy conservation is **photophosphorylation**. In photophosphorylation light energy is used to form the proton motive force that powers ATP synthesis and is the dominant mechanism of energy conservation in phototrophic organisms. Both photophosphorylation and oxidative phosphorylation ultimately rely on electron transfer reactions to drive the formation of the proton motive force (Section 3.11).

We now turn our attention to enzymes—the cell's premier catalysts—and the importance of catalysis in cellular metabolism.

Check Your Understanding

- What are the three fundamental mechanisms of conserving cellular energy and in what types of organisms would these mechanisms be most important?
- Explain whether 1,3-bisphosphoglycerate or glucose 6-phosphate (Figure 3.8) could be used to generate ATP through substrate-level phosphorylation.
- Which mechanisms of energy conservation rely on the proton motive force?

3.5 Catalysis and Enzymes

Free-energy calculations reveal only whether energy is released or required in a given reaction; they say nothing about the *rate* of the reaction. If the rate of a reaction is very slow, it may be of no value to a cell. For example, consider the formation of water from O_2 and H_2. The energetics of this reaction are quite favorable: $H_2 + \frac{1}{2}O_2 \rightarrow H_2O$, $\Delta G^{0\prime} = -237$ kJ. However, if O_2 and H_2 were mixed in a sealed bottle, no measurable amount of water would form, even after years. This is because the bonding of O_2 and H_2 to form H_2O requires that these two gases become reactive. This requires that their bonds be broken, which requires a small amount of energy. This energy is called **activation energy**.

Activation energy can be viewed as the minimum energy required for a chemical reaction to begin. For an exergonic reaction, the situation is as shown in **Figure 3.9**. Although the activation energy barrier is virtually insurmountable in the absence of a *catalyst*—a substance that facilitates a reaction but is not consumed by it—in the presence of a proper catalyst, this barrier is reduced, allowing the reaction to proceed.

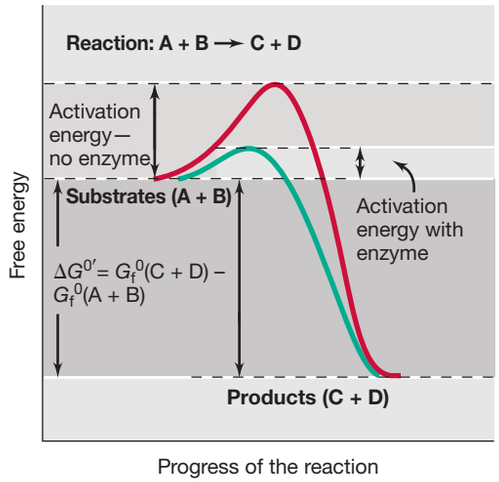


Figure 3.9 Activation energy and catalysis. Even chemical reactions that release energy may not proceed spontaneously if not activated. Once the reactants are activated, the reaction proceeds spontaneously. Catalysts such as enzymes lower the required activation energy.

Enzymes

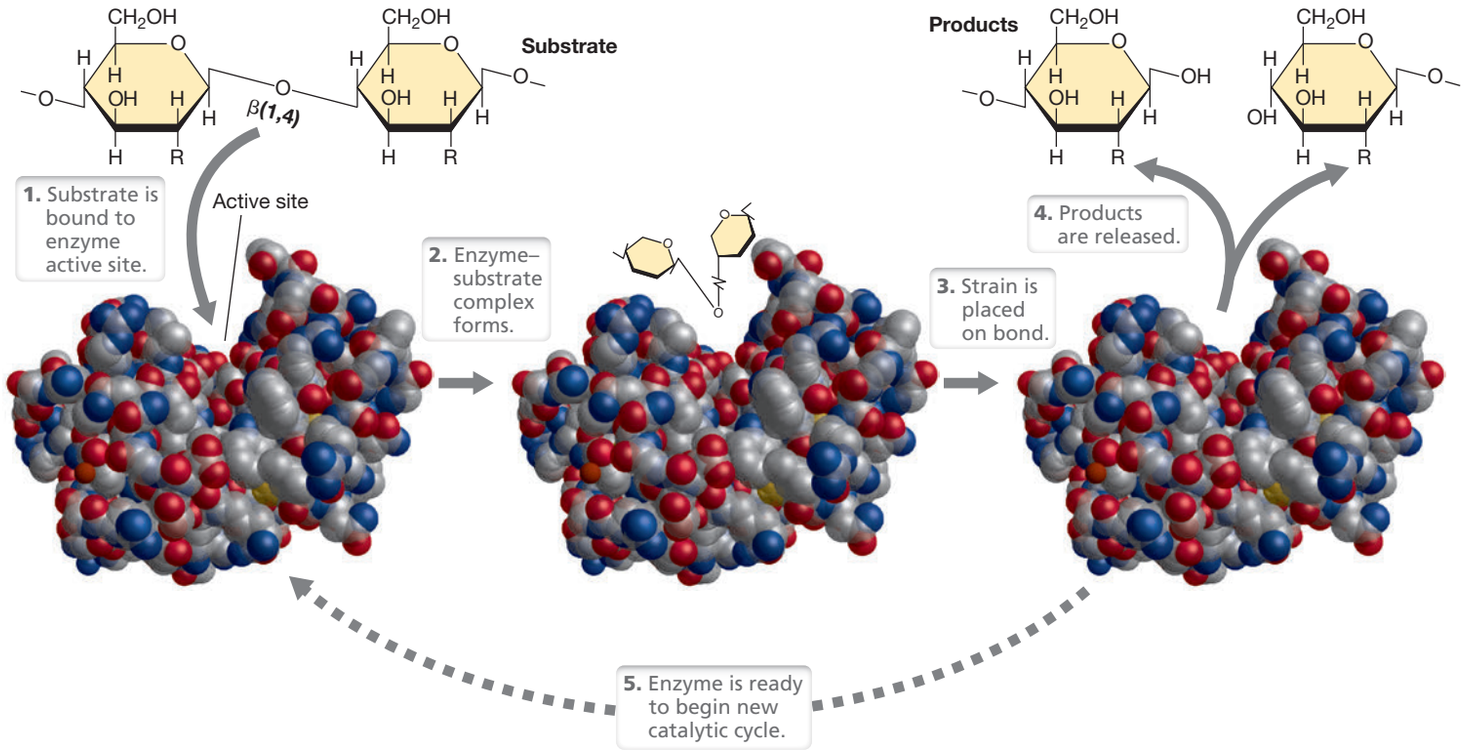
Catalysts function by lowering the activation energy of a reaction (Figure 3.9), thereby increasing the reaction rate. Catalysts have no effect on the energetics or the equilibrium of a reaction but only affect the *rate* at which a reaction proceeds. Most cellular reactions will not proceed at significant rates without catalysis.

The major catalysts in cells are **enzymes**, which are proteins (or in a few cases, RNAs) that are highly specific for the reactions they catalyze. This specificity is a function of the precise three-dimensional structure of the enzyme. In an enzyme-catalyzed reaction, the enzyme combines with the reactant, called a *substrate*, forming an *enzyme–substrate complex*. Then, as the reaction proceeds, the *product* is released and the enzyme is returned to its original state, ready to catalyze a new round of the reaction (**Figure 3.10**). The enzyme is generally much larger than the substrate(s), and the portion of the enzyme to which substrate binds is the enzyme's *active site*; the entire enzymatic reaction, from substrate binding to product release, may take only a few milliseconds.

Many enzymes contain small nonprotein molecules that participate in catalysis but are not themselves substrates. These small molecules can be divided into two classes based on the way they associate with the enzyme: *prosthetic groups* and *coenzymes*. Prosthetic groups bind tightly to their enzymes, usually covalently and permanently. The heme group present in cytochromes such as cytochrome *c* (Section 3.8) is an example of a prosthetic group. By contrast, **coenzymes**, with a few exceptions, are loosely and often transiently bound to enzymes; thus, a single coenzyme molecule may associate with a number of different enzymes. NADH is an example of a coenzyme (Figures 3.5 and 3.6), as is ATP. Most coenzymes are derivatives of vitamins (▶ Section 4.1 and Table 4.1).

Enzyme Catalysis

To catalyze a reaction, an enzyme must bind its substrate and position it properly in its active site. The enzyme–substrate complex



Mastering Microbiology
Art Activity: Figure 3.8 The catalytic cycle of an enzyme

Figure 3.10 The catalytic cycle of an enzyme. The enzyme depicted here, lysozyme, catalyzes the cleavage of the β-1,4-glycosidic bond in the polysaccharide backbone of peptidoglycan (◀ Section 2.3). Following substrate binding in the enzyme's active site, strain is placed on the bond, and this favors breakage. Space-filling model of lysozyme courtesy of Richard Feldmann.

(Figure 3.10) aligns reactive groups in the substrate(s) and places strain on specific bonds. This reduces the activation energy required to make the reaction proceed (Figure 3.9). This is shown in Figure 3.10 for the enzyme lysozyme, an enzyme whose substrate is the polysaccharide backbone of peptidoglycan, the bacterial cell wall polymer (◀ Section 2.3).

The reaction depicted in Figure 3.9 is exergonic. By contrast, some enzymes catalyze endergonic reactions where they convert energy-poor substrates into energy-rich products. In these cases not only must an activation energy barrier (Figure 3.9) be overcome, but sufficient free energy must also be put into the reaction in order to raise the energy level of the substrates to that of the products. This is done by *coupling* the energy-requiring reaction to an energy-yielding one, such as the hydrolysis of ATP or dissipation of the proton motive force, so that the overall reaction proceeds with a free-energy change that is either negative in arithmetic sign or zero.

Theoretically, all enzymes are reversible in their activity. However, enzymes that catalyze highly exergonic or highly endergonic reactions typically function in only one direction. If a particularly exergonic or endergonic reaction needs to be reversed, a different enzyme usually catalyzes the reverse reaction.

Check Your Understanding

- What is the function of a catalyst? What are the building blocks of an enzyme?
- Where on an enzyme does the substrate bind?
- What is activation energy?

II • Catabolism: Chemoorganotrophs

Chemoorganotrophs use organic carbon molecules to fuel metabolism, and glycolysis and the citric acid cycle are central metabolic pathways used in the metabolism of organic carbon molecules.

We have learned in the previous sections that cells harvest energy from redox reactions and they conserve this energy in the synthesis of ATP and other high-energy compounds. We have also learned that there are many different metabolic types of organisms, each defined by its source of energy (Figure 3.3).

Here we examine the metabolic pathways that cells use to transfer electrons and conserve energy. We begin by examining chemoorganotrophs that perform fermentation and respiration. **Fermentation** is a form of anaerobic catabolism in which organic compounds both donate electrons and accept electrons, and redox balance is achieved without the need for external electron acceptors. By contrast, **respiration** is a form of aerobic or anaerobic catabolism in which an electron donor, which can be either organic or inorganic, is oxidized using an external electron acceptor such as O_2 (in aerobic respiration) or some other compound (in anaerobic respiration). We will revisit the concepts of fermentation and respiration in detail in Chapter 14 and so focus here only on the essentials that define these processes.

3.6 Glycolysis, the Citric Acid Cycle, and the Glyoxylate Cycle

Chemoorganotrophs obtain the electrons they need to conserve energy from the oxidation of organic compounds, such as glucose. A nearly universal pathway for the catabolism of glucose is the *Embden–Meyerhof–Parnas* pathway, better known as **glycolysis**, a series of reactions in which glucose is oxidized to pyruvate. Many metabolic reactions are modular and glycolysis is a prime example of such a module. Glycolysis can participate in several forms of catabolism including fermentation, aerobic respiration, and anaerobic respiration.

Glycolysis

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Microbiology Animation: Glycolysis: Steps

Glycolysis can be divided into two stages, each consisting of one or more enzymatic reactions. Stage I consists of "preparatory" reactions; these are not redox reactions and do not release energy but instead form a key intermediate of the pathway. In stage II, redox reactions occur, energy is conserved, and two molecules of pyruvate are formed (**Figure 3.11**).

To begin glycolysis, glucose is phosphorylated to form glucose 6-phosphate. The latter is then isomerized to fructose 6-phosphate, and a second phosphorylation leads to the production of fructose 1,6-bisphosphate. These steps consume, rather than produce, ATP. The enzyme aldolase then splits fructose 1,6-bisphosphate into two 3-carbon molecules, *glyceraldehyde 3-phosphate* and its isomer, *dihydroxyacetone phosphate*, which is converted into glyceraldehyde 3-phosphate. To this point, all of the reactions, including the consumption of ATP, have proceeded without any redox changes (Figure 3.11).

The first redox reaction of glycolysis occurs when glyceraldehyde 3-phosphate is oxidized to 1,3-bisphosphoglycerate. In this reaction (which occurs twice, once for each of the two glyceraldehyde 3-phosphates), the enzyme glyceraldehyde-3-phosphate dehydrogenase reduces NAD^+ to NADH. Simultaneously, each glyceraldehyde 3-phosphate molecule is phosphorylated by the addition of a molecule of inorganic phosphate. This reaction, in which *inorganic* phosphate is converted to *organic* form, sets the stage for energy conservation, since 1,3-bisphosphoglycerate is an energy-rich compound (Figure 3.11; see also Figure 3.8). ATP is then synthesized by substrate-level phosphorylation when (1) each molecule of 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate, and (2) each molecule of phosphoenolpyruvate is converted to pyruvate (Figure 3.11). As a result, glycolysis consumes *two* ATP molecules in stage I and produces *four* ATP molecules in stage II. The result is that glycolysis produces *a net yield of 2 molecules of ATP, 2 molecules of NADH, and 2 molecules of pyruvate per molecule of glucose*.

The glycolytic pathway on its own, while sufficient to make ATP, is insufficient to sustain life because it lacks redox balance. That is, glycolysis produces NADH but it lacks an electron acceptor and so

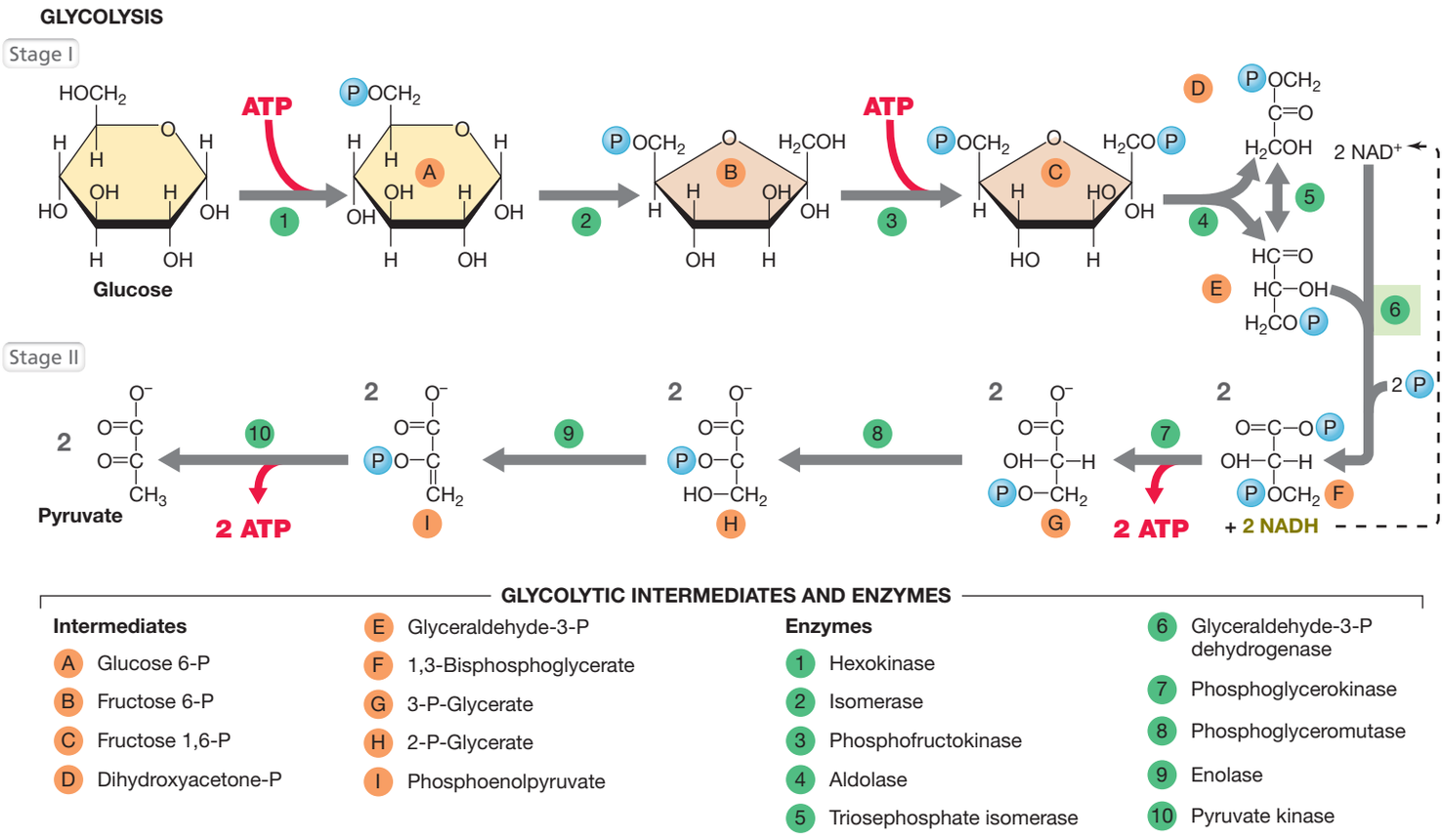

Figure 3.11 Embden–Meyerhof–Parnas pathway (glycolysis). The sequence of reactions in the catabolism of glucose to pyruvate. Glycolysis results in the formation of 2 each of pyruvate, ATP, and NADH per glucose molecule.

lacks the ability to regenerate the NAD^+ needed to oxidize glyceraldehyde-3-P to 1,3-bisphosphoglycerate. Cells performing glycolysis can achieve redox balance by using one of several different mechanisms including fermentation, where NADH oxidation is linked to the reduction of pyruvate (Section 3.7), or respiration, where glycolysis is coupled to the citric acid cycle and respiration (Section 3.8).

The Citric Acid Cycle

The pathway by which pyruvate is oxidized to CO_2 is called the **citric acid cycle (CAC)** (Figure 3.12). Pyruvate from glycolysis is decarboxylated, leading to the production of CO_2, NADH, and the energy-rich compound *acetyl-CoA*, which enters the CAC (Figure 3.12 and see Figure 3.8). The acetyl group of acetyl-CoA combines with the four-carbon CAC intermediate oxaloacetate, forming the six-carbon compound citric acid, for which the CAC is named. A sequence of reactions follows, and two additional CO_2 molecules, three more NADH, and one $FADH_2$ are formed per pyruvate oxidized. Ultimately, oxaloacetate is regenerated as the next acetyl acceptor, thus completing the cycle (Figure 3.12). The result is that the citric acid cycle produces *a net yield of 1 molecule of ATP (or GTP), 4 molecules of NADH (or NADPH), 1 molecule of $FADH_2$, and 3 molecules of CO_2 per molecule of pyruvate*.

Linkage of glycolysis with the citric acid cycle allows one molecule of glucose to be completely oxidized to six molecules of CO_2, while producing 4 molecules of ATP (2 in glycolysis and 2 in the CAC). However, redox is still out of balance because these pathways have combined to produce 10 molecules of NADH and 2 of $FADH_2$ per molecule of glucose. Redox balance is ultimately achieved in respiration reactions (Section 3.9). However, before we turn our attention to respiration, we consider another important aspect of the citric acid cycle.

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Microbiology Animation: Kreb's Cycle: Steps

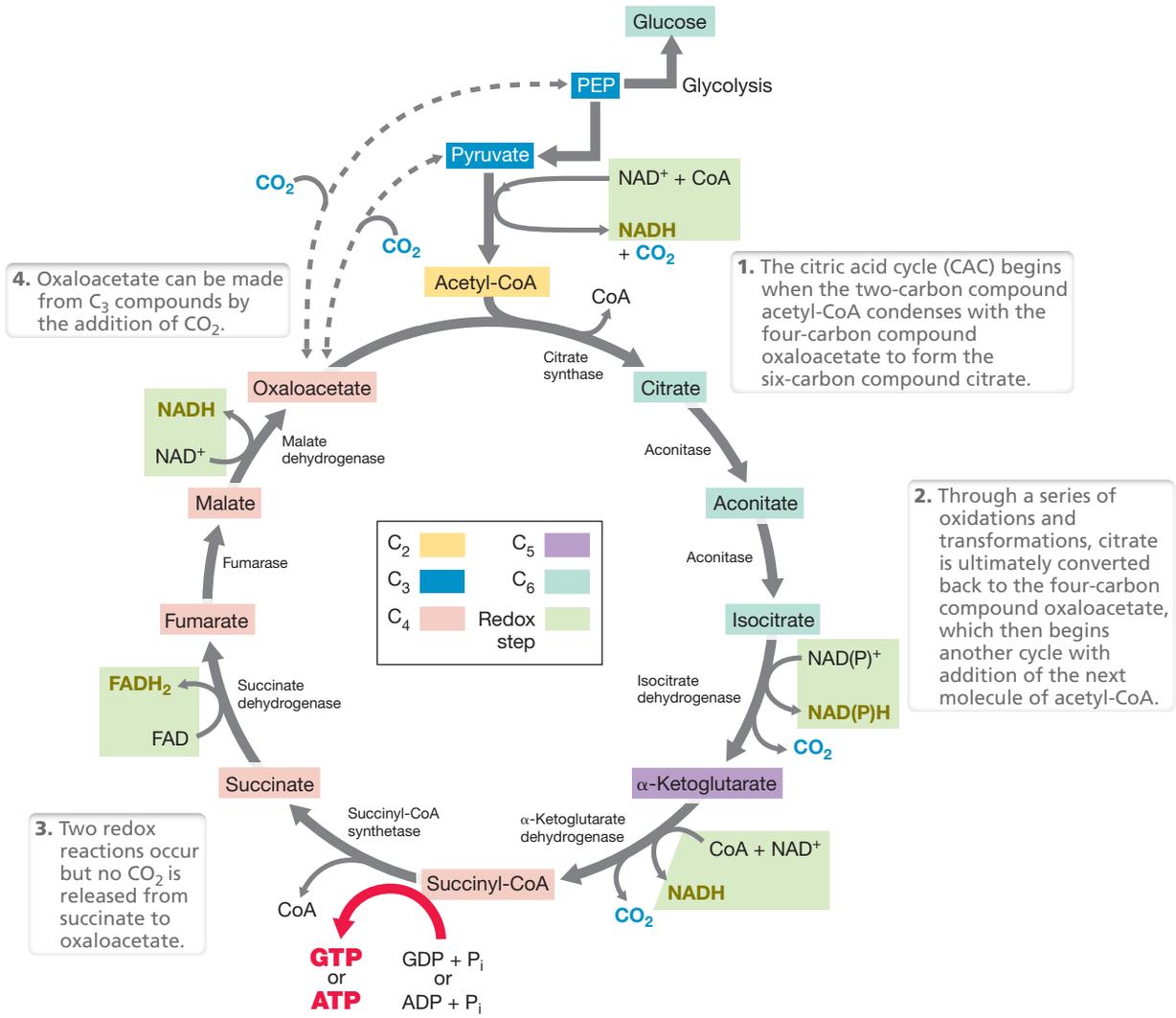


Figure 3.12 The citric acid cycle. The citric acid cycle begins when the two-carbon compound acetyl-CoA (derived from pyruvate) condenses with the four-carbon compound oxaloacetate to form the six-carbon compound citrate. Through a series of oxidations and transformations, citrate is converted to two CO_2 and the acetyl acceptor molecule, oxaloacetate. For every pyruvate that enters the cycle, 3 CO_2, 1 ATP, 4 NADH, and 1 $FADH_2$ are produced.

Biosynthesis and the Citric Acid Cycle

Besides its role in oxidizing pyruvate to CO_2, the citric acid cycle also plays a major role in biosynthesis. The cycle is composed of several key organic compounds, small amounts of which are drawn off during growth to produce new cell material. Particularly important in this regard are α-ketoglutarate and oxaloacetate, which are precursors of several amino acids (Section 3.14), and succinyl-CoA, needed to form cytochromes, chlorophyll, and related molecules. Any shortage of oxaloacetate is corrected by the addition of CO_2 (carboxylation) to pyruvate or phosphoenolpyruvate (Figure 3.12).

Oxaloacetate is also an important intermediate because it can be converted to phosphoenolpyruvate (a precursor of glucose) if necessary (Section 3.13). In addition, acetate is important because it provides the raw material for fatty acid biosynthesis (Section 3.15). The CAC thus plays two major roles in the cell: *oxidation of organic compounds* and *the biosynthesis of key metabolites.* The same can be said about the glycolytic pathway, as certain intermediates from this pathway are also drawn off for biosynthetic needs (Sections 3.13 and 3.14) and then replenished from glucose in the next round of glycolysis.

Other Pathways for Chemoorganotrophy

Chemoorganotrophs can use glycolysis and the CAC to oxidize several compounds including glucose, citrate, malate, fumarate, and succinate (all C_4 or C_6 compounds). In addition, many unrelated catabolic pathways can be linked to glycolysis and the CAC to enable the oxidation of an even wider diversity of organic compounds. For example, many sugars can be converted to glucose by *isomerase* enzymes and then metabolized by glycolysis. Likewise, isomerases that yield CAC intermediates can be used to metabolize a wide range of organic compounds.

Some chemoorganotrophs can also grow on two-carbon (C_2) compounds by using the **glyoxylate cycle** (Figure 3.13). Microbes cannot grow on C_2 compounds such as acetate by using the CAC only. This is because some oxaloacetate must be drawn off from the CAC to biosynthesize glucose and amino acid precursors (Sections 3.13 and 3.14). It is possible to readily synthesize oxaloacetate from a range of C_4 and C_6 compounds but not from C_2 compounds. This inability to regenerate oxaloacetate during growth on acetate means that the CAC would starve for the oxaloacetate needed to accept the acetyl group from acetyl-CoA (Figure 3.12). Hence, cells use the glyoxylate cycle (so named because the C_2 compound *glyoxylate* is a key intermediate) to grow using C_2 compounds.

The glyoxylate cycle is composed of several citric acid cycle enzymatic reactions plus two additional enzymes: *isocitrate lyase*, which cleaves isocitrate into succinate and glyoxylate, and *malate synthase*, which converts glyoxylate and acetyl-CoA to malate (Figure 3.13). The succinate formed can be used for biosynthesis, while the glyoxylate combines with acetyl-CoA (C_2) to yield malate (C_4). From malate, the acceptor molecule oxaloacetate is produced and can enter a new round of acetyl-CoA oxidation in the citric acid cycle (Figure 3.13).

Another example of this principle comes from chemoorganotrophs that grow on C_3 compounds. Cells using standard CAC reactions to grow on C_3 compounds, such as pyruvate, are also unable to regenerate oxaloacetate. However, carboxylation reactions catalyzed by the enzymes *pyruvate carboxylase* or *phosphoenolpyruvate carboxylase*, which convert pyruvate or phosphoenolpyruvate into oxaloacetate (respectively), allow cells growing on C_3 compounds to regenerate oxaloacetate (Figure 3.12).

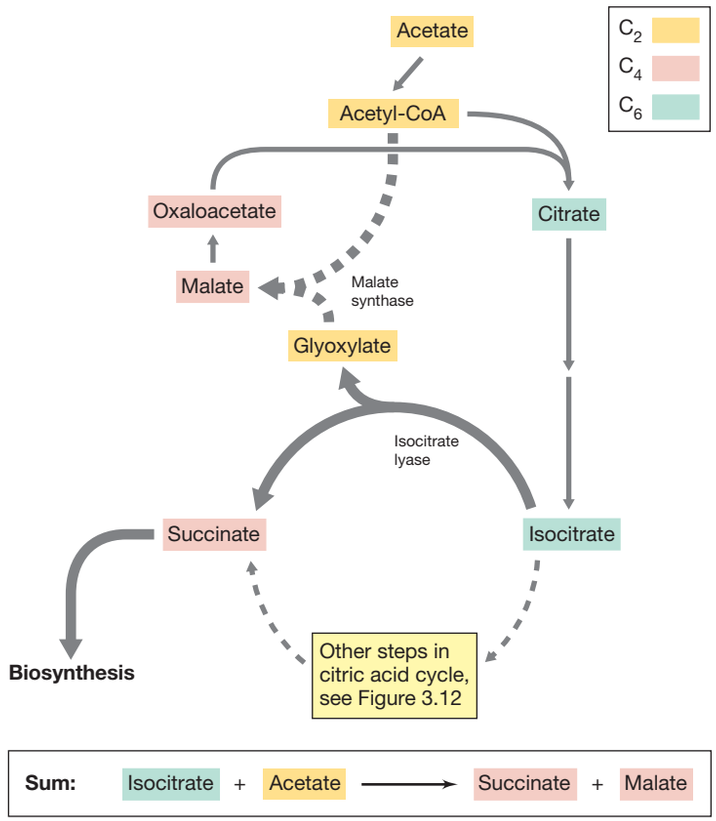


Figure 3.13 The glyoxylate cycle. These reactions occur in conjunction with the citric acid cycle when cells grow on two-carbon electron donors, such as acetate. The glyoxylate cycle regenerates oxaloacetate (from malate) to maintain an acetyl acceptor for the citric acid cycle.

It should thus be clear that metabolic reactions are modular entities and that the reactions of glycolysis, the citric acid cycle, and the glyoxylate cycle allow for a wide diversity of organic electron donors to fuel the energy needs of chemoorganotrophs.

Check Your Understanding

- Which reactions in glycolysis are redox reactions?
- Why does an organism need the enzyme *pyruvate carboxylase* to enable growth if pyruvate is its sole source of energy and reducing power?
- Why is it not possible for an organism to grow by using only glycolysis?

3.7 Principles of Fermentation

Microbes that ferment glucose achieve energy conservation by substrate-level phosphorylation and achieve redox balance by reducing the pyruvate produced in glycolysis (Section 3.6) and then excreting the reduced molecule to the environment as a waste product (Figure 3.14). These fermentation products are often highly reduced substances such as organic acids or alcohols. However,

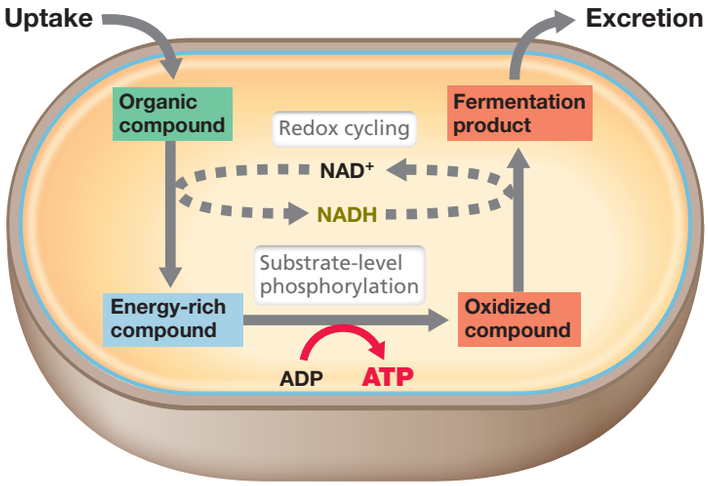


Figure 3.14 The essentials of fermentation. An organic compound is oxidized, and the electrons are ultimately recycled back to one of the oxidized organic products because an external electron acceptor is lacking. The fermentation product so formed is excreted from the cell and ATP is produced by substrate-level phosphorylation. Most of the fermentable substrate is used for energy generation and only a relatively small amount is used for biosynthesis.

fermentation products are not waste products to humans. Instead, they are the foundation of the baking and fermented beverage industries, including beer and wine production, and are key ingredients in many other fermented foods, such as the lactic and other acids in fermented dairy products (yogurt, sour cream, buttermilk, and the like), cheeses, pickles, and certain sausages and fish products (◀ Section 1.6). In addition, fermentation products produced by the human microbiome can exert a powerful influence on human health (Chapter 24).

Energy Conservation and Redox Balance in Fermentation

There are a tremendous diversity of fermentation reactions and many employ glycolysis, but in the final analysis, all fermentations must achieve two major results: first, the cell must conserve energy, and second, the cell must achieve a final redox balance. Since fermentative organisms rely on substrate-level phosphorylation to conserve energy, they need to produce compounds that contain energy-rich bonds with which to synthesize ATP (Section 3.4). In addition, since fermentative organisms lack an external electron acceptor they need to find a way to oxidize NADH back to NAD^+. Fermentative organisms solve the latter problem by creating an electron acceptor from the organic electron donor they consumed. For example, yeast ferments one molecule of glucose to two molecules of ethanol and two molecules of CO_2. During this fermentation, yeast gets all of its ATP from glycolysis and then replenishes NAD^+ by donating electrons from NADH to pyruvate, which is the end product of glycolysis (Figure 3.11). In this way, pyruvate is the electron acceptor, and the reduction of pyruvate produces the fermentation products ethanol and CO_2, which are excreted from the cell.

Lactic acid bacteria provide an alternative example of this same principle. These bacteria convert one molecule of glucose to two molecules of lactic acid (see Figure 3.21). Like yeast, lactic acid bacteria produce all of their ATP in glycolysis and achieve redox balance by reducing pyruvate. However, unlike yeast, lactic acid bacteria employ different enzymes to reduce pyruvate and as a result, produce lactic acid as their fermentation product instead of ethanol plus CO_2. Lactic acid bacteria are a tremendously important group of microorganisms. They are responsible for the production of many important fermented foods (◀ Figure 1.16). Lactic acid bacteria are also the cause of dental caries, and the lactic acid they produce is essential to maintaining the health of the human large intestine and vagina (Chapter 24).

Fermentative Diversity

While sugars can be readily fermented using glycolytic reactions, there is a tremendous diversity of fermentative pathways. Many different organic substrates can be fermented, including amino acids, fatty acids, purines, pyrimidines, and even aromatic compounds. Many fermentative organisms that use these substrates produce acetate or other volatile fatty acids (for example, propionate, butyrate) as fermentation products. Volatile fatty acid fermentation products contribute to energy conservation because they provide an opportunity to make ATP by substrate-level phosphorylation. The key intermediate in such reactions is the coenzyme-A derivative of each fatty acid, since these are energy-rich compounds. For example, the final step of the butyric acid fermentation performed by the anaerobic bacterium *Clostridium butyricum* is

$$\text{Butyryl-CoA} + \text{ADP} + P_i \rightarrow \text{butyric acid} + \text{ATP} + \text{CoA}$$

The formation of butyryl-CoA, which has an energy-rich bond, allows *C. butyricum* to make ATP by substrate-level phosphorylation. In addition, redox balance in many fermentative organisms can be improved by the production of molecular hydrogen (H_2). The $2\,H^+/H_2$ redox couple has a very low reduction potential ($\Delta E_0' = -0.42$) and so only organisms that produce very low reduction potentials in their metabolisms can produce H_2 (for example, NADH cannot reduce H^+ to H_2, Figure 3.4). Often the production of H_2 is associated with the activity of *ferredoxin*, a very low-potential electron carrier (Section 3.8) and catalyzed by the enzyme *hydrogenase*.

Fermentation reactions support the growth of an enormous diversity of microorganisms, but substrate-level phosphorylation can only conserve a fraction of the energy available in organic substrates. We will see in the next two sections that respiration can conserve far more energy than fermentation and is the favored form of metabolism in oxic environments.

Check Your Understanding

- Why do lactic acid bacteria produce lactic acid?
- Why is acetate formation energetically beneficial during fermentative metabolism?
- What two metabolic events result from any fermentation?

3.8 Principles of Respiration: Electron Carriers

Mastering Microbiology
Microbiology Animation: Electron Transport Chain: The Process

In *respiration*, electrons are transferred from reduced electron donors such as glucose to external electron acceptors such as O_2. During glycolysis and the citric acid cycle, the complete oxidation of glucose

to 6 CO_2 produces a considerable amount of NADH and $FADH_2$ (Figure 3.12). These molecules must be reoxidized back to NAD^+ and FAD in order to achieve redox balance. In glucose respiration, NADH and $FADH_2$ are reoxidized when they donate electrons to electron transport reactions. *Electron transport* takes place within the cytoplasmic membrane, culminating in the reduction of an external electron acceptor and the formation of an electrochemical gradient across the membrane. The electrochemical gradient is formed when protons (or in some cases, other ions) are pumped across the cytoplasmic membrane, and this electrochemical gradient—the proton motive force—is ultimately used to conserve energy through ATP synthesis. Hence, at a fundamental level, respiration is a process in which chemical energy from redox reactions is conserved by using electron transport reactions to pump protons (or other ions) across a membrane. We now focus on the enzymes, cofactors, and coenzymes that participate in electron transfer reactions.

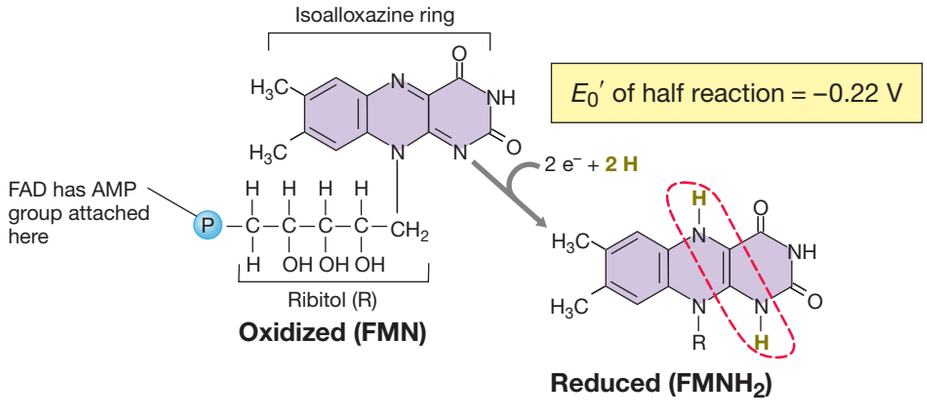


Figure 3.15 Flavin mononucleotide (FMN), a hydrogen atom carrier. The site of oxidation–reduction (dashed red circle) is the same in FMN and the related coenzyme flavin adenine dinucleotide (FAD, not shown). FAD contains an adenosine monophosphate (AMP) group bonded through the phosphate group on FMN.

NADH Dehydrogenases and Flavoproteins

Electron transport reactions occur within membranes, typically in the cytoplasmic membrane of prokaryotic cells or the mitochondrial membrane of eukaryotic cells. Several types of oxidation–reduction enzymes participate in electron transport. These include *NADH dehydrogenases, flavoproteins, iron–sulfur proteins*, and *cytochromes*. Also participating are small nonprotein electron carriers called *quinones*. The carriers are arranged in the membrane in order of *increasingly more positive* reduction potential, with NADH dehydrogenase first and the cytochromes last (see Figures 3.16 and 3.19).

NADH dehydrogenases contain an active site that binds NADH. The 2 e^- and the H^+ from NADH, and one H^+ from the cytoplasm, are transferred by the NADH dehydrogenase to a flavoprotein, the next carrier in the chain. This regenerates NAD^+, which can return to participate in glycolysis, the citric acid cycle, or other cellular processes (Sections 3.6 and 3.7). Flavoproteins contain a derivative of the vitamin riboflavin (**Figure 3.15**). The flavin portion, which is bound to its protein as a prosthetic group, is reduced as it accepts 2 e^- + 2 H^+ and oxidized when 2 e^- are passed to the next carrier in the chain (note that flavoproteins *accept* 2 e^- + 2 H^+ but *donate* only electrons, the protons are ultimately released to the cytoplasm). Two types of flavins are commonly found in cells, flavin mononucleotide (FMN, Figure 3.15) and flavin adenine dinucleotide (FAD).

Cytochromes, Other Iron Proteins, and Quinones

Cytochromes are proteins that contain heme prosthetic groups (**Figure 3.16**). Cytochromes undergo oxidation and reduction through loss or gain of an electron by the iron atom that exists as either Fe^{2+} or Fe^{3+}. Several classes of cytochromes exist, differing widely in their reduction potentials (Figure 3.4). Different classes of cytochromes are designated by letters, such as cytochrome *a*, cytochrome *b*, cytochrome *c*, and so on, depending on the type of heme they contain. The redox potential of a cytochrome is ultimately governed by its protein structure and the type of heme present. Occasionally, cytochromes form into complexes with other cytochromes or with iron–sulfur proteins. An important example is the cytochrome bc_1 complex, which is a multiprotein enzyme complex that contains two different *b*-type cytochromes and one *c*-type cytochrome. The cytochrome bc_1 complex plays an important role in energy metabolism, as we will see in the next section.

In addition to the cytochromes, in which iron is bound to heme, one or more proteins with nonheme iron are also components of electron transport chains. These proteins contain prosthetic groups made up of clusters of iron and sulfur atoms, with Fe_2S_2 and Fe_4S_4 clusters being the most common (**Figure 3.17**). For example, bacterial **ferredoxin**, a nonheme iron–sulfur protein of low reduction potential (about −0.4 V) (Table 3.1) and an important component in H_2 production (Section 3.7), contains an Fe_4S_4 cluster. The reduction potentials of iron–sulfur proteins vary from −0.2 to about −0.45 V,

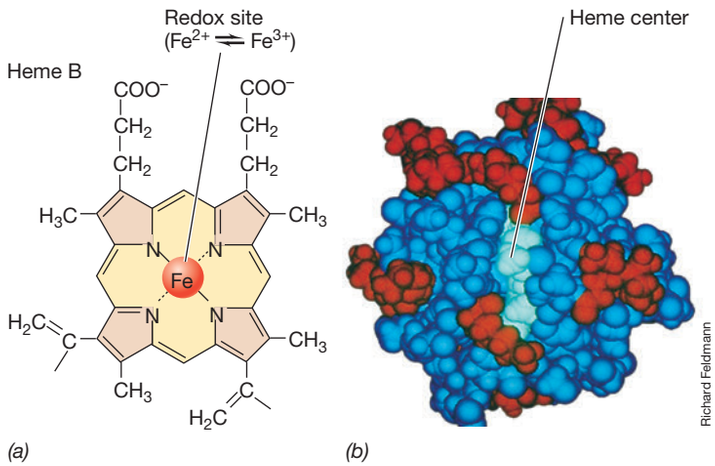


Figure 3.16 Cytochrome and its structure. *(a)* Structure of heme, the iron-containing portion of cytochromes. Hemes are tetrapyrroles, composed of four pyrrole rings. Cytochromes carry electrons only, and the redox site is the iron atom, which can alternate between the Fe^{2+} and Fe^{3+} oxidation states. *(b)* Space-filling model of cytochrome *c*; heme (light blue) is covalently linked via disulfide bridges to cysteine residues in the protein (dark blue).

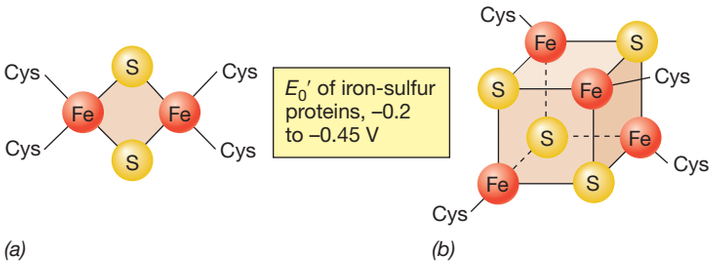


Figure 3.17 Arrangement of the iron–sulfur centers of nonheme iron–sulfur proteins. *(a)* Fe_2S_2 center. *(b)* Fe_4S_4 center. The cysteines (Cys) link the protein to its Fe/S cluster.

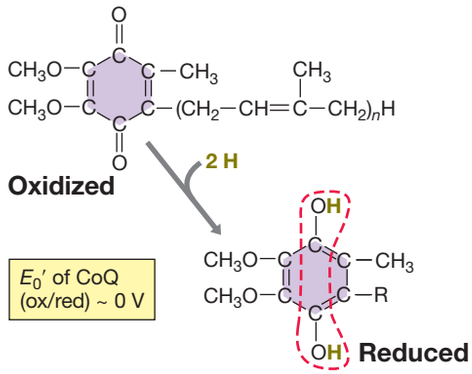


Figure 3.18 Structure of oxidized and reduced forms of ubiquinone (coenzyme Q, or CoQ). The five-carbon unit in the side chain (an isoprenoid) occurs in multiples, typically 6–10. Oxidized ubiquinone requires 2 e^- and 2 H^+ to become fully reduced (dashed red outline).

depending on the iron–sulfur cluster present, the structure of the protein that contains it, and how the cluster is embedded in the protein. Thus, different iron–sulfur proteins can function at different locations in the electron transport chain. Like cytochromes, iron–sulfur proteins carry only electrons.

Quinones (**Figure 3.18**) are small hydrophobic redox molecules that lack a protein component. Because they are small and hydrophobic, quinones can move about within the membrane. Like the flavins (Figure 3.15), quinones accept 2 e^- + 2 H^+ but transfer only 2 e^- to the next carrier in the chain. Quinones typically function to link iron–sulfur proteins (Figure 3.17) and the initial cytochrome (Figure 3.16) in the electron transport chain. Several types of quinones exist, but ubiquinone (also called coenzyme Q) and menaquinone are the most common quinones and are widely distributed in species of *Bacteria* and *Archaea*.

Check Your Understanding

- In what major way do quinones differ from other electron carriers in the membrane?
- Which electron carriers described in this section accept 2 e^- + 2 H^+? Which accept electrons only?
- Consider an electron transport chain that transports electrons from NADH to O_2. In what order would cytochromes *a*, *b*, and *c* need to occur in the electron transport chain? (*Hint*: Refer to Figure 3.4.)

3.9 Principles of Respiration: Generating a Proton Motive Force

As we will see later (Section 3.10), respiration can be either aerobic or anaerobic. However, energy conservation in all forms of respiration is linked to an energized state of the membrane—the proton motive force. In addition, we will see that electron transfer reactions and the generation of a proton motive force are also required for energy conservation in phototrophy (Section 3.11). Hence, electron transport and the generation of a proton motive force is a unifying concept that underlies energy conservation in all forms of metabolism except fermentation.

Electron Transport

Understanding the linkage between electron transport and ATP synthesis requires an appreciation for the organization of the electron transport chain in the cytoplasmic membrane (**Figure 3.19**). While the exact composition of the electron transport chain varies between organisms, and multiple electron transport chains can sometimes function simultaneously in a single organism, the principles of electron transport are nearly universal.

Electron transport begins when an electron donor adds electrons to the electron transport chain. In chemoorganotrophic organisms, electrons enter electron transport from either NADH or $FADH_2$. By contrast, many different inorganic electron donors can feed into electron transport in various chemolithotrophic organisms (see Section 3.11). However, regardless of the source of electrons, electron carriers within the transport chain are embedded in the membrane in order of their *increasingly positive* reduction potentials (Figure 3.19; see also Figure 3.4). During electron transport, electrons are passed on from lower-potential (more negative $\Delta E_0'$) carriers to higher-potential (more positive $\Delta E_0'$) carriers until at the end of the chain they ultimately reduce a *terminal electron acceptor*, such as O_2.

The movements of electrons through the electron transport chain are exergonic reactions that provide the free energy to pump protons to the *outer surface* of the membrane, thereby generating the proton motive force. Recall that H_2O naturally dissociates to form H^+ and OH^-. Hence, removal of H^+ from the cytoplasm results in the accumulation of OH^- on the cytoplasmic side of the membrane. Though small, H^+ are not free to diffuse across the membrane because they are charged and polar; thus, they must be extruded and this occurs during electron transport reactions. This separation of H^+ from OH^- creates a difference in pH and an electrochemical potential across the membrane, and this electrochemical potential energizes the membrane (Figure 3.19) much like the potential energy that exists from charge separation in a battery. This electrochemical gradient establishes the proton motive force, which can be used by the cell to do work in the form of ATP synthesis, active transport, motility, and a few other energy-requiring reactions.

The mechanisms of electron transport in a model chemoorganotrophic bacterium widely used for studies of respiration, *Paracoccus denitrificans*, is shown in Figure 3.19. This bacterium can grow by aerobic respiration of glucose. In so doing, it uses glycolysis and the citric acid cycle to produce NADH and $FADH_2$ destined for the electron transport chain, and it uses O_2 as its terminal electron acceptor. We know the electron transport chain of *P. denitrificans* in detail because it is used as a model system to study mitochondrial

Mastering Microbiology
Art Activity: Figure 3.22 Generation of the proton motive force during aerobic respiration

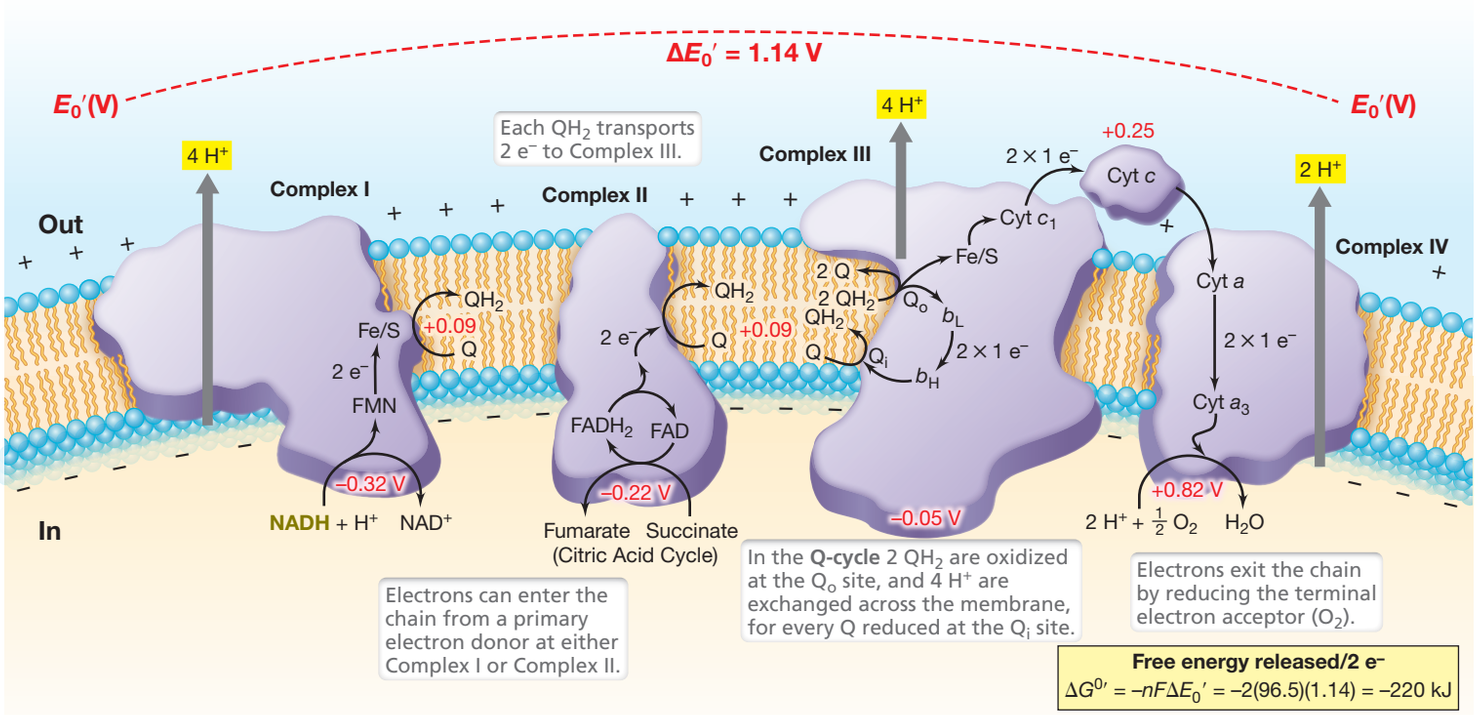


Figure 3.19 Generation of the proton motive force during aerobic respiration. The orientation of electron carriers in the cytoplasmic membrane of the bacterium *Paracoccus denitrificans*. The + and − charges at the inner and outer membrane surfaces represent H^+ and OH^-, respectively. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Q, quinone; Fe/S, iron–sulfur protein; cyt *a, b, c,* cytochromes (b_L and b_H, low- and high-potential *b*-type cytochromes, respectively). The Q-cycle occurs at Complex III (cytochrome bc_1). During the Q-cycle, two molecules of QH_2 are oxidized and a total of 4 H^+ exchanged across the membrane for every 2 e^- that pass through the complex. Complex II, the succinate dehydrogenase complex, bypasses Complex I and feeds electrons directly into the quinone pool at a more positive E_0' than NADH (see the redox tower in Figure 3.4).

function. This resemblance occurs because the mitochondrion is the result of an ancient symbiosis that took place between a bacterial cell and the ancestor of all *Eukarya* (▶ Section 13.4). Electron transport takes place in the cytoplasmic membrane of *P. denitrificans* and in the mitochondrial membrane of eukaryotic cells.

Generation of the Proton Motive Force: Complexes I and II

In *P. denitrificans* and many aerobic bacteria, electron transport begins at *Complex I* (Figure 3.19). The term *complex* refers to the fact that this system is composed of many proteins that function as a unit (for example, in *Escherichia coli*, Complex I contains 14 different proteins). Complex I is also called *NADH:quinone oxidoreductase* (or simply *NADH dehydrogenase*) because the overall reaction is one in which NADH is oxidized and a quinone (Figure 3.18) is reduced. Complex I oxidizes NADH to NAD^+ and transfers the resulting 2 e^- to a protein containing an FMN coenzyme and from there to a series of Fe/S proteins within the complex, before ultimately reducing ubiquinone (Q) to ubiquinol (QH_2). This reduction causes ubiquinone to pick up 2 H^+ from the inside of the cell. The QH_2 is able to diffuse within the membrane to Complex III, the next step in the electron transport chain. In addition, the electron transfer reactions within Complex I cause a conformational change within the complex resulting in transport of 4 H^+ across the membrane (Figure 3.19).

As an alternative entry point to the electron transport chain, electrons from $FADH_2$ enter at *Complex II*. Complex II is also called the *succinate dehydrogenase complex* because the oxidation of succinate to fumarate in the citric acid cycle (Figure 3.12) reduces FAD^+ to $FADH_2$, ultimately resulting in the reduction of Complex II (Figure 3.19). The 2 e^- from $FADH_2$ are transferred through Complex II to ubiquinone (Q), which accepts these 2 e^- and 2 H^+ from the cytoplasm to become ubiquinol (QH_2). In this way Complexes I, II, and III are all linked by the *quinone pool*. Quinones are reduced by either Complex I or Complex II, and their reduction requires 2 H^+, which are obtained from the cytoplasmic side of the membrane; QH_2 is then free to diffuse within the membrane to Complex III. However, electrons entering at Complex I result in greater energy conservation than those entering at Complex II because the former translocates 4 additional H^+ for every 2 e^- transported (Figure 3.19).

Complexes III and IV: bc_1 and *c*- and *a*-Type *Cytochromes*

Complex III consists of the cytochrome bc_1 complex (Figure 3.19). Electrons enter Complex III from QH_2 at the Q_o site and they leave when donated to cytochrome *c*. When QH_2 donates its 2 e^- to Complex III, it releases its two H^+ outside of the cytoplasmic membrane, contributing to the proton motive force. However, QH_2 carries 2 e^- and cytochrome *c* only carries 1 e^-. This difference creates an opportunity for conserving additional energy through a mechanism known as the *Q-cycle*. During the Q-cycle, cytochrome bc_1 carries out an *electron bifurcation* in which the two 2 e^- from QH_2 ($E_0' = +0.09$ V)

are sent in different directions. The first e^- is transported to cytochrome *c* ($E_0' = +0.25$ V) in an exergonic reaction whose free-energy release helps drive transfer of the second e^- in the slightly endergonic reaction to subunit b_L ($E_0' = -0.06$ V). The reduction potential of the b_L subunit is sufficient to transfer e^- to the b_H subunit and then ultimately back to a second molecule of ubiquinone (Q) that is docked at the Q_i site of Complex III. It takes *two* such cycles to reduce fully the ubiquinone at the Q_i site to QH_2, and the net result of the Q-cycle is that 4 H^+ are transferred to the cytoplasmic side of the membrane for every 2 e^- that move through Complex III to cytochrome *c* (Figure 3.19).

Cytochrome *c* functions as a periplasmic shuttle to transfer e^- from Complex III to the high-redox-potential cytochromes *a* and a_3 of *Complex IV* (Figure 3.19). Complex IV functions as the terminal oxidase and reduces O_2 to 2 H_2O in the final step of the electron transport chain. It takes 4 e^- to reduce O_2 to 2 H_2O a reaction that also consumes 4 H^+ from the cytoplasm. This transfer of electrons causes a conformational change in Complex IV that allows 1 H^+ to be pumped to the outer surface of the membrane for every e^- transferred through Complex IV (Figure 3.19).

The net result for an organism like *P. denitrificans* is that for every 2 e^- transported from NADH to O_2, a total of 10 H^+ are transferred to the outside of the membrane (4 at Complex I, 4 at Complex III, and 2 at Complex IV), and another 2 are consumed in the cytoplasm during the formation of water. In contrast, for every 2 e^- transported from $FADH_2$ to O_2 a total of 6 H^+ are transferred to the outside of the membrane (4 at Complex III and 2 at Complex IV), and another 2 are consumed in the cytoplasm during the formation of water.

Thus far in respiration we have disposed of electrons generated in various catabolic reactions and simultaneously generated a proton motive force. The latter drives ATP synthesis through the activity of a remarkable and extremely small cellular motor, and we consider this now.

ATP Synthase

How does the proton motive force (pmf), generated from electron transport, actually drive ATP synthesis? Interestingly, a strong parallel exists between the mechanism of ATP synthesis and the mechanism that drives rotation of the flagellar motor in swimming bacteria (◀ Section 2.9). A large protein complex called **ATP synthase (ATPase)** uses energy from the pmf to catalyze formation of ATP (**Figure 3.20**). As it does for the flagellar motor, the pmf generates torque within the ATP synthase enzyme complex, and this torque is translated into rotary motion, creating mechanical energy that is used to catalyze the chemical synthesis of ATP from ADP and P_i. ATP formation by ATP synthase is called *oxidative phosphorylation* when the pmf is generated by respiratory electron flow. In contrast, ATP formation by ATP synthase is called *photophosphorylation* when the pmf is generated from light energy during phototrophy (Section 3.11).

ATPases consist of two components, a multiprotein complex called F_1 that sticks into the cytoplasm and actually catalyzes ATP synthesis, and a membrane-integrated multiprotein complex called F_o that carries out proton translocation across the membrane (Figure 3.20). ATP synthase is found in nearly all cellular organisms, and this enzyme complex is highly conserved, indicating that it is of ancient origin and was likely present in the common ancestor of all cells (Chapter 13).

ATPase catalyzes a reversible reaction between ATP and ADP + P_i, and F_1 and F_o are actually two rotary motors. The movement of protons through the ATPase drives the rotation of its c protein subunits, which are organized in a ring within the F_o subunit. Each c protein subunit picks up one H^+ from outside the membrane and deposits it in the cytoplasm for every complete revolution. This generates a torque that is transmitted to F_1 via the coupled rotation of the γϵ subunits (Figure 3.20). The rotation causes conformational changes in the β subunits of F_1 that allows them to bind ADP + P_i.

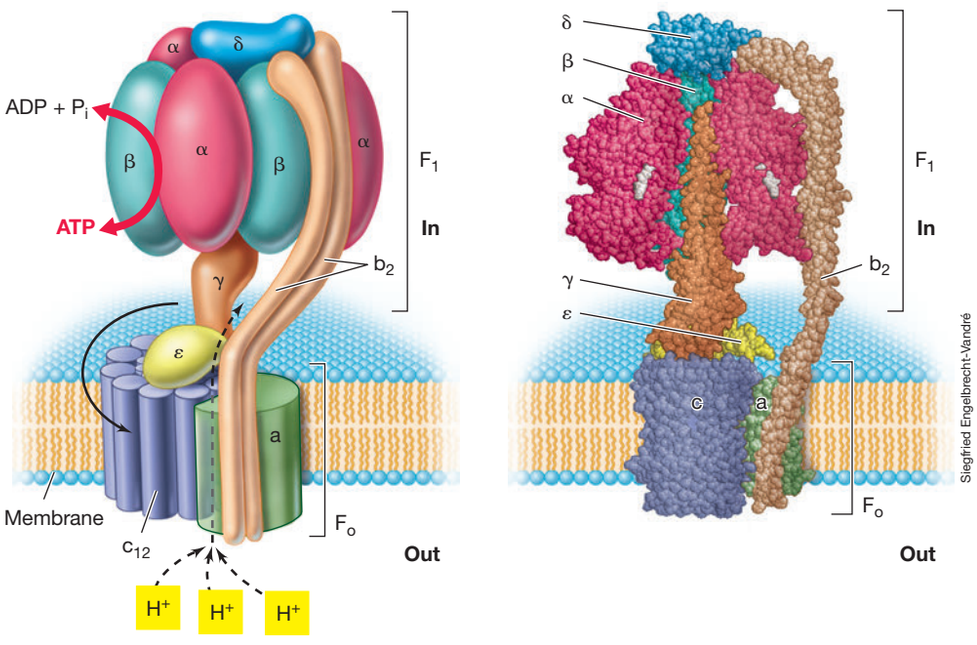


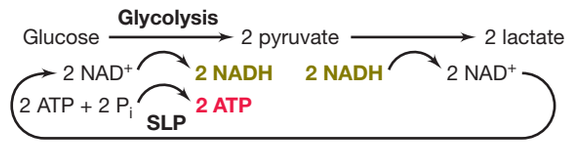
Figure 3.20 Structure and function of the reversible ATP synthase (ATPase) in *Escherichia coli*. *(a)* Schematic. F_1, the stator, consists of five different polypeptides forming an $\alpha_3\beta_3\gamma\epsilon\delta$ complex. F_1 is the catalytic complex responsible for the interconversion of ADP + P_i and ATP. F_o, the rotor, is integrated in the membrane and consists of three polypeptides in an ab_2c_{12} complex. As protons enter, the dissipation of the proton motive force drives ATP synthesis (3–5 H^+/ATP). *(b)* Space-filling model. The color-coding corresponds to the art in part *a*. Since proton translocation from outside the cell to inside the cell leads to ATP synthesis by ATPase, it follows that proton translocation from inside to outside in the electron transport chain (Figure 3.19) represents work done on the system and a source of potential energy. This energy can be used to drive a number of energy-requiring processes in the cell, including in particular motility and some forms of nutrient transport. Nearly all cells of all domains contain ATPases. Also, some bacterial and archaeal ATPases are linked to a sodium (Na^+) rather than a proton (H^+) gradient.

ATP is synthesized when the β subunits return to their original conformation and the free energy captured in the rotated β subunits is released. For every full rotation of the *c* ring within the F_o subunit, three ATP are formed by the F_1 subunit. The ATPase of *E. coli* has 10 c protein subunits and as a result, approximately 3.3 H^+ are required to synthesize one ATP. However, the number of c protein subunits within an ATPase varies from 8 to 15 in different organisms and thus the number of protons needed to synthesize one ATP also varies.

Oxidative phosphorylation allows the cell to conserve far more energy than is possible in fermentation because the substrate, for example glucose, is completely oxidized (**Figure 3.21**). A total of 38 ATP are synthesized during the aerobic respiration of glucose to CO_2, with 34 of these formed as a result of oxidative phosphorylation (Figure 3.21). By contrast, in lactic acid fermentation, only 2 ATP are produced by substrate-level phosphorylation during the oxidation of glucose in glycolysis. Lactic acid bacteria excrete lactate, which still contains considerable free energy that has not been conserved by the cell.

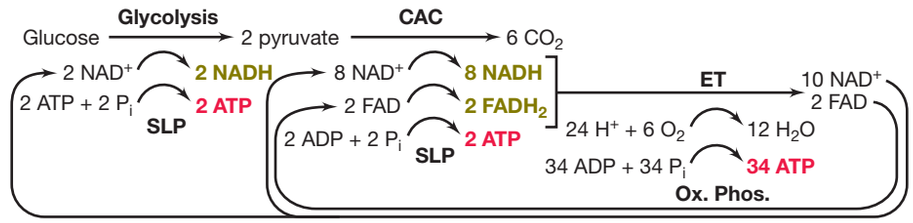
ATPases are reversible motors. Hence, ATP hydrolysis can reverse the activity of ATPase and cause protons to be transported out of the cytoplasm. The net result in this case is *generation of* rather than *dissipation of* the proton motive force. Reversibility of the ATPase explains why strictly fermentative bacteria still contain ATPases even though they lack electron transport chains and are unable to carry out oxidative phosphorylation. Many important reactions in the cell, such as flagellar rotation and some forms of transport, are coupled to energy from the proton motive force. Thus, in these strictly fermentative organisms, ATPase hydrolyzes ATP formed from substrate-level phosphorylation (Section 3.7) to generate the pmf needed to carry out cellular functions such as motility and transport.

Lactic acid fermentation



Net: Glucose + 2 ADP + 2 P_i ⟶ 2 lactate + **2 ATP**

Aerobic respiration



Net: Glucose + 6 O_2 + 38 ADP + 38 P_i ⟶ 6 CO_2 + 6 H_2O + **38 ATP**

Figure 3.21 Energetics in fermentation and aerobic respiration. Fermentation and respiration both oxidize NADH and allow for the regeneration of NAD^+. However, respiration conserves far more energy than fermentation as a result of oxidative phosphorylation. Note that reduction of 6 O_2 with 24 e^- produces 12 H_2O while only 6 H_2O are produced in the net reaction. The difference between the gross and net production of water occurs because H_2O is in equilibrium with H^+ and OH^- and only the net production of water is written in the balanced reaction. SLP, substrate-level phosphorylation; Ox. Phos., oxidative phosphorylation; CAC, citric acid cycle; ET, electron transport.

Check Your Understanding

- How do electron transport reactions generate the proton motive force?
- How much energy is released per NADH oxidized through the electron transport chain of *Paracoccus* shown in Figure 3.19?
- What structure in the cell links the proton motive force to ATP synthesis? How does it function?

III • Catabolism: Electron Transport and Metabolic Diversity

The respiration of organic or inorganic electron donors and the light-driven reactions of photosynthesis all generate ATP by harvesting energy from the proton motive force that forms from electron transport reactions in the cytoplasmic membrane.

With the exception of fermentation, in which energy is conserved by substrate-level phosphorylation (Section 3.7), all other mechanisms of energy conservation are linked to the proton motive force (or in a few organisms, a gradient of sodium ions, Na^+, instead of protons, as we will see in Chapter 14). Whether energy comes from chemical reactions or light, and whether electrons come from the oxidation of organic or inorganic chemicals, nonfermentative energy conservation is always the result of electron transport reactions and the formation of a proton motive force (see Figure 3.22). The proton motive force is then used by ATPase to form ATP (Figure 3.20).

In earlier parts of this chapter we learned about the fundamental metabolic principles that govern aerobic and fermentative chemoorganotrophs. Now we turn our attention to how these same fundamental principles apply to the vast diversity of metabolic types found in the microbial world. We return to this theme of catabolic diversity and explore its many facets in Chapter 14.

3.10 Anaerobic Respiration and Metabolic Modularity

Respiration can occur under both oxic (O_2 is present) and anoxic (O_2 is absent) conditions. The distinguishing feature between aerobic and anaerobic respiration is that aerobic respiration requires O_2 as the terminal electron acceptor, whereas **anaerobic respiration** *uses electron acceptors other than* O_2 (**Figure 3.22**). Recall that when life began there was no O_2 in the atmosphere, and O_2 did not reach current levels until about 600 million years ago (◀ Section 1.5). This means that anaerobic microbes dominated much of the history of life, and these organisms still occur widely today in any environment that lacks O_2, such as wetlands, sediments, and in the guts of animals.

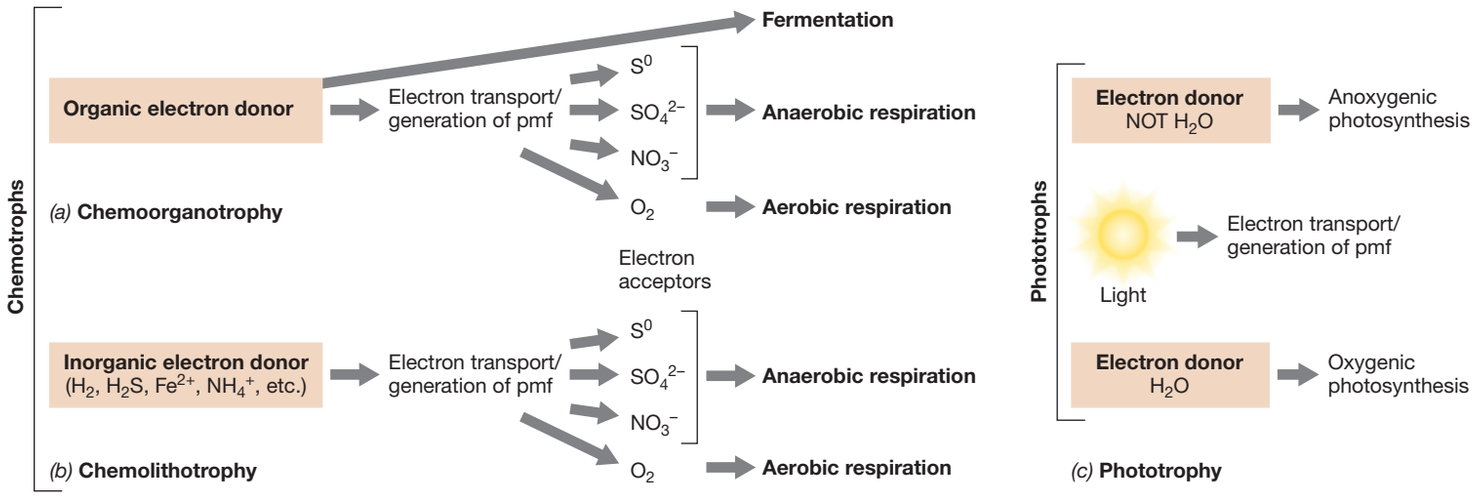


Figure 3.22 Metabolic diversity and its relationship to oxygen. *(a)* Chemoorganotrophs can be fermentative or engage in aerobic or anaerobic respiration, whereas *(b)* chemolithotrophs are typically respiratory. *(c)* Phototrophs are oxygenic if they generate O_2 by oxidizing H_2O and anoxygenic if they do not. Pmf, proton motive force.

Fermentative organisms and microbes that perform anaerobic respiration can both grow under anoxic conditions, and in this sense they are both anaerobes. However, these two forms of metabolism are distinct because all types of *respiration require an external electron acceptor and generate ATP primarily by oxidative phosphorylation*, whereas all types of *fermentation do not require an external electron acceptor and generate ATP by substrate-level phosphorylation*. We examine respiratory options in *Escherichia coli* now.

Respiration in *Escherichia coli*

E. coli is a metabolically versatile chemoorganotroph whose electron transport chain is similar to but not identical with that of *Paracoccus denitrificans* (Figure 3.19). *E. coli* can grow by aerobic respiration in the presence of O_2, it can carry out fermentation in the absence of external electron acceptors, and it can grow by anaerobic respiration when nitrate is present in the absence of O_2. As a result, *E. coli* can optimize its metabolism to grow under a range of conditions. When using an organic carbon source, *E. coli* will grow most rapidly by aerobic respiration, but under anaerobic conditions, as in an animal gut or in sediments, it can grow more rapidly by nitrate respiration than by fermentation, provided that nitrate is available. The energy *E. coli* can conserve during respiration is a function of the organization of its electron transport chain. *E. coli* can insert many different proteins in its electron transport chain, and this versatility allows it to conserve energy through respiration with a range of different electron donors and acceptors.

The basic organization of the *E. coli* respiratory chain (**Figure 3.23**) is Complex I followed by Complex II followed by *quinones* (for example, ubiquinone) and a *terminal reductase* that contains *b*-type and *o*-type cytochromes (in contrast to the *a*-type cytochromes of Complex IV). Depending on environmental conditions *E. coli* can swap out these components, employing alternative quinones (for example, ubiquinone or menaquinone), or employing alternative dehydrogenases or terminal reductases (*E. coli* has at least five sets of each). Note, however, that Complex III is missing from *E. coli* (Figure 3.23); this has energetic consequences.

The lack of Complex III means that during aerobic respiration, *E. coli* conserves less energy than *P. denitrificans*. For every 2 electrons that pass through its electron transport chain to O_2, *E. coli* exchanges only 8 H^+ across the membrane while *P. denitrificans* exchanges 10 H^+ (compare Figures 3.19 and 3.23*a*). The free energy available to *P. denitrificans* and *E. coli* during the aerobic respiration of glucose is identical, but the organization of the electron transport chain in *P. denitrificans* allows it to conserve more energy than *E. coli*. This means that *P. denitrificans* will typically be able to outcompete *E. coli* for glucose if they are both growing aerobically. Hence, we can see that the amount of energy conserved by a respiratory organism is influenced by both bioenergetics and by the organization of its electron transport chain.

If nitrate is present in the absence of O_2, *E. coli* will use *nitrate reductase* as its terminal reductase (Figure 3.23*b*). Nitrate reductase allows *E. coli* to perform anaerobic respiration with nitrate. However, the NO_3^-/NO_2^- couple is less electropositive than is the O_2/H_2O couple (Figure 3.4), and this is why anaerobic respiration provides less energy than aerobic respiration. This bioenergetic difference is reflected in the energy that can be conserved in electron transport, because only 6 H^+ are exchanged across the membrane for every 2 electrons passing to nitrate (rather than the 8 H^+ extruded aerobically, Figure 3.23). Likewise, for any given electron donor, aerobic organisms will always be able to conserve more energy—and will therefore outcompete—anaerobic organisms. However, oxygen is consumed very rapidly in poorly mixed aqueous environments because it is such a good electron acceptor and because it is poorly soluble in water. Hence, anoxic habitats and anaerobic microbes are widespread in nature and crucial to global biogeochemical cycles (Chapter 21).

Check Your Understanding

- If three bacteria were competing for glucose and one was capable only of aerobic respiration, one anaerobic respiration with nitrate (NO_3^-) as electron acceptor, and one anaerobic respiration with sulfate (SO_4^{2-}) as electron acceptor, which do

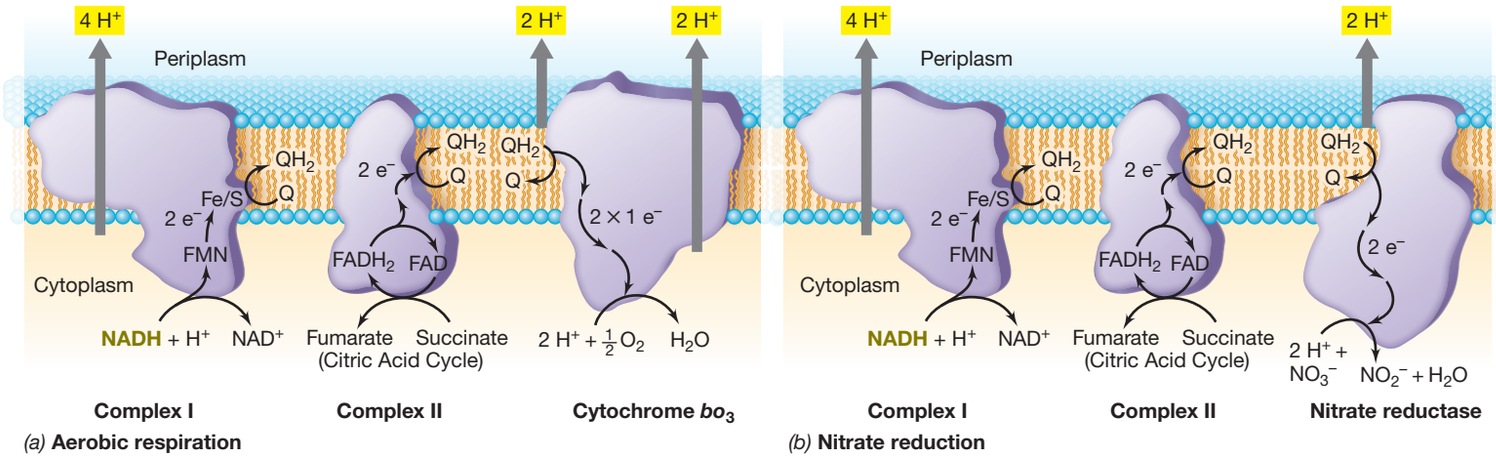


Figure 3.23 Respiration and nitrate-based anaerobic respiration in *Escherichia coli*. Electron transport processes in the membrane of *E. coli* when *(a)* O_2 or *(b)* NO_3^- is used as terminal electron acceptor. Note the difference in the number of protons translocated with the different terminal electron acceptors and compare these to the difference in the energetics of these reactions when glucose is the electron donor (see Figure 3.4). Q, ubiquinone.

you think would be most competitive in an oxic environment? In an anoxic environment? (*Hint:* Review Figure 3.4 and Table 3.1.)

- Explain why *Paracoccus denitrificans* would outcompete *Escherichia coli* if both organisms were competing for glucose in the presence of O_2.
- If respiratory metabolism always conserves more energy than fermentative metabolism, then why are fermentative organisms so common?

3.11 Chemolithotrophy and Phototrophy

Thus far in this chapter we have focused on chemoorganotrophs, chemotrophic organisms that rely on organic electron donors. Now we will focus on *chemolithotrophs*, which use inorganic electron donors, and on *phototrophs*, which get their energy from light (Figure 3.3). Chemolithotrophs can be aerobes or anaerobes and they are found in a wide range of environments, most anywhere that reduced inorganic compounds are present.

Chemolithotrophy

Examples of common inorganic electron donors that fuel chemolithotrophic metabolisms include H_2S, H_2, Fe^{2+}, and NH_4^+ (Figure 3.22). Chemolithotrophic metabolisms begin with the oxidation of an inorganic electron donor and with the electrons entering an electron transport chain. For example, the hydrogen bacterium *Ralstonia eutropha* grows as an aerobic chemolithotroph with H_2 as its electron donor ($2\,H^+/H_2$, -0.42 V). *R. eutropha* has a membrane-bound hydrogenase that splits H_2 in the periplasm and reduces quinones in the electron transport chain (**Figure 3.24**). Electron transport then generates a proton motive force, as we have already seen for the oxidation of organic electron donors by chemoorganotrophs (Figures 3.19 and 3.23). Cells of *R. eutropha* actually have two hydrogenases (Figure 3.24). The membrane-bound hydrogenase contributes to energy conservation, as we have described, whereas the soluble hydrogenase, which is present in the cytoplasm, is used to reduce NAD^+ to NADH needed for autotrophic growth (Figure 3.24). This reduction is possible because the E_0' of the $2\,H^+/H_2$ couple is more electronegative than the E_0' of the NAD^+/NADH couple (Figure 3.4).

Respiration by chemoorganotrophs and chemolithotrophs are united by their dependence on oxidative phosphorylation for energy conservation. Just as we saw for anaerobic respiration, the energy available to chemolithotrophs depends on the $\Delta E_0'$ between the electron-donating and electron-accepting reactions (Figure 3.4). Like many chemoorganotrophs, many chemolithotrophs can have either aerobic or anaerobic metabolisms (Figure 3.22). Indeed, almost any combination of electron donor and electron acceptor can sustain life if these reactions are coupled to an electron transport chain used in oxidative phosphorylation and if the $\Delta E_0'$ of the redox reaction releases sufficient free energy to form ATP.

One major difference between chemoorganotrophs and chemolithotrophs is in their source of cellular carbon. Chemoorganotrophs are heterotrophs and therefore use organic compounds (glucose, acetate, etc.) as carbon sources. These organic compounds are readily converted into cellular materials during biosynthetic reactions. By contrast, chemolithotrophs typically use carbon dioxide (CO_2) as a carbon source and are therefore autotrophs (we describe a major autotrophic pathway—the Calvin cycle—in Section 3.12). Autotrophy requires a significant amount of reducing power (Figure 3.1) such as NADH, but the electron donors used by chemolithotrophs often have a more electropositive E_0' than does NADH/NAD^+ (Figure 3.4). How do such chemolithotrophs form NADH? In the case of *R. eutropha*, we saw that a cytoplasmic hydrogenase is used to form NADH directly from NAD^+ and H_2 (Figure 3.24). However, most chemolithotrophs lack hydrogenases and solve this problem by employing *reverse electron transport*, an energy-consuming process that we will learn about in the context of phototrophy.

Phototrophy

In the process of photosynthesis, carried out by *phototrophs*, light energy is used instead of chemical reactions to drive electron flow and generate a proton motive force. During these events, ATP

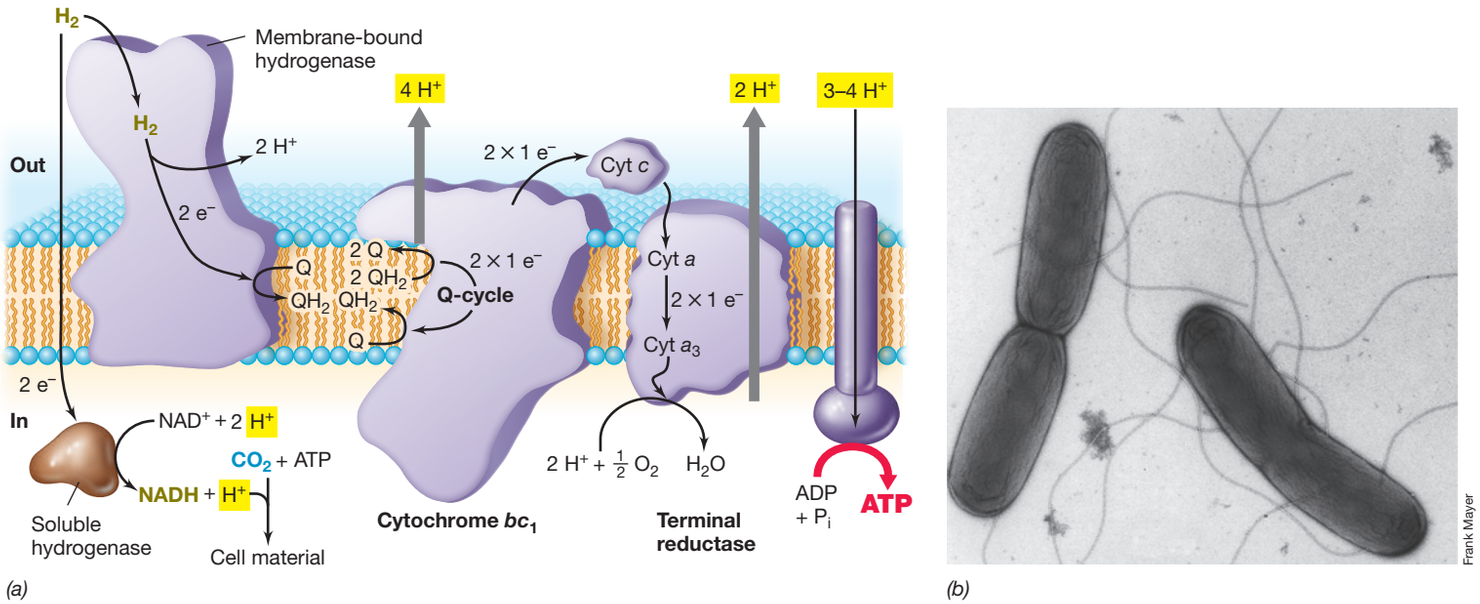


Figure 3.24 **Energy conservation in *Ralstonia eutropha*, an autotrophic chemolithotroph that oxidizes H_2.** *(a)* In *R. eutropha*, two hydrogenases are present; the membrane-bound hydrogenase participates in electron transport, whereas the soluble hydrogenase makes NADH for the Calvin cycle (see Section 3.12). Cyt, cytochrome; Q, quinone. For details of the Q-cycle, see Figure 3.19. *(b)* Transmission electron micrograph of negatively stained cells of *R. eutropha*. A cell is about 0.6 μm in diameter and contains several flagella.

synthase generates ATP by *photophosphorylation* (**Figure 3.25*a***), the light-driven analog of oxidative phosphorylation. Phototrophs can be either *oxygenic* (for example cyanobacteria, algae, and plants) or *anoxygenic* (diverse types of *Bacteria*), depending on whether or not the process generates O_2 as a waste product from the oxidation of H_2O (Figure 3.22). Anoxygenic phototrophs evolved long before oxygenic phototrophs (◀ Section 1.5), and there is far more metabolic diversity among the anoxygenic phototrophs than among oxygenic phototrophs. However, light-driven photoreactions in both oxygenic and anoxygenic phototrophs operate on many of the same principles.

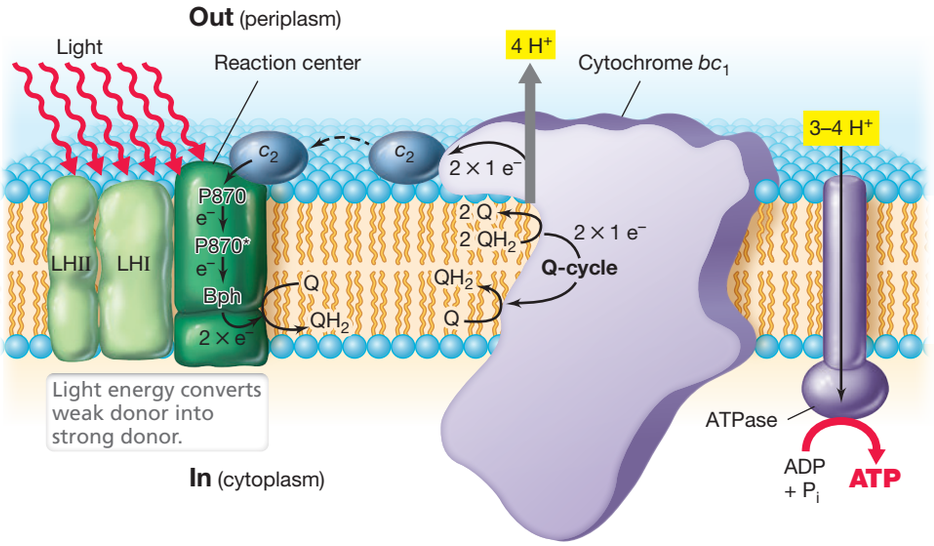
Purple bacteria are one type of anoxygenic phototrophs, which are common in anoxic aquatic environments (Figure 3.25*b*). Like all phototrophic organisms, purple bacteria produce a **photosynthetic reaction center** that converts light energy into chemical energy (ATP, Figure 3.25*a*).While we focus here primarily on energy conservation during photophosphorylation, we will learn more about the operation of photosynthetic reaction centers in Chapter 14. All reaction centers contain photopigments such as chlorophylls or bacteriochlorophylls, which are structurally similar to heme molecules (▶ Section 14.3).

Phototrophs use photopigments to absorb light energy and transfer this energy to the photosynthetic reaction center (Figure 3.25*a*). The reaction center in purple bacteria uses the energy from light to convert a form of bacteriochlorophyll *a* called *P870*, which is a relatively weak electron donor, into a very strong electron donor (E_0' near −1.0 V) called *P870**. The excited P870* then donates two electrons to a quinone within the cytoplasmic membrane. The reduced quinone in turn donates its electrons to the *cytochrome bc_1* complex, which is similar in structure and function to the Complex III we saw in oxidative phosphorylation (Figure 3.19). The net result is photophosphorylation, which results when light energy is used to generate a proton motive force that is harnessed by ATP synthase to make ATP (Figure 3.25*a*).

A major difference between respiratory electron transport and photosynthetic electron transport in purple bacteria is that in purple bacteria electrons from cytochrome bc_1 are not transferred to an external electron acceptor (such as O_2). Instead the electrons are passed to cytochrome c_2 and then back to P870, returning it to its original E_0'. Hence, purple bacteria reuse the same electrons over and over again to make ATP as long as light is present to fuel the initial P870 → P870* transition. This process is called *cyclic photophosphorylation* because electrons move within a closed loop. We consider other aspects of photosynthesis, including the differences between anoxygenic photosynthesis and oxygenic photosynthesis, in Chapter 14.

Generation of Reducing Power

For purple bacteria and chemolithotrophs to grow autotrophically, the formation of ATP is not enough. Reducing power (NADH) is also necessary to produce cellular material. Reducing power can come from many different electron donors. Many purple bacteria and chemolithotrophs use reduced sulfur compounds such as H_2S as their electron donors. However, the E_0' of S^0/H_2S (−0.28 V) is insufficiently electronegative to reduce NAD^+ (−0.32 V); hence, these bacteria cannot make NADH directly from H_2S. These organisms solve this problem by pushing electrons from the quinone pool backwards (against the electrochemical gradient) towards a dehydrogenase-like protein (similar to Complex I) so that they reduce NAD^+ to NADH. This process, called *reverse electron transport*, requires energy and is driven by dissipation of the proton motive force. We will see this pattern once more when we discuss biosynthetic reactions, in which the reversibility of reactions and pathways is often used to synthesize a great diversity of biomolecules.



(a) **Electron transport in purple bacteria**

Daniel H. Buckley

(b) **Bloom of purple bacteria in a salt marsh**

Figure 3.25 Electron flow in anoxygenic photosynthesis in a purple bacterium. *(a)* Energy conservation in purple bacteria results from cyclic photophosphorylation. The photosynthetic reaction center uses light energy to energize electrons for use in electron transport and the generation of proton motive force. Four protons are translocated for every two electrons that pass from the reaction center to cytochrome bc_1. P870 is a form of bacteriochlorophyll *a* in the ground state and is a fairly electropositive electron donor. P870* is formed after light excitation of P870 and is a strongly electronegative electron donor. LH, light-harvesting bacteriochlorophyll complexes; Bph, bacteriopheophytin; Q, quinone; c_2, cytochrome c_2. For details of the Q-cycle, see Figure 3.19. *(b)* A bloom of purple bacteria that formed in the Sippewissett Salt Marsh, Cape Cod, Massachusetts, USA. High rates of O_2 consumption, and its low solubility in water, can cause anoxic conditions in a water film less than 100 μm thick, and this allows anoxygenic phototrophs to grow on the sediment surface.

Check Your Understanding

- What traits are shared between oxidative phosphorylation and photophosphorylation?
- Which process do you think evolved first, photosynthesis or respiration? Use your knowledge of metabolism to defend your answer.
- What is reverse electron transport, and why is it unnecessary for chemoorganotrophs?

IV • Biosynthesis

Biosynthesis encompasses the anabolic reactions that produce cellular materials and requires ATP and building blocks composed of C, N, and other elements obtained from organic or inorganic sources.

We conclude this chapter with an overview of how the monomers that make up the four classes of cellular macromolecules are biosynthesized and also look at how polysaccharides and lipids are biosynthesized in a general way. The biosynthesis of *informational* macromolecules—proteins and nucleic acids—is the theme of Chapter 6. Collectively, these biosynthetic reactions are that aspect of metabolism we call *anabolism*.

All cells require a source of carbon and nitrogen to perform biosynthetic reactions. The atmosphere contains a large reservoir of inorganic carbon as CO_2, and nitrogen as N_2. However, these gases must be chemically reduced before they can be assimilated into cell material. The reductive processes associated with the assimilation of CO_2 and N_2 are called *CO_2 fixation* and *N_2 fixation*, respectively. Both of these pathways require substantial amounts of energy from the cell in the form of ATP and reducing power. These processes evolved very early in the history of life and they are widely distributed among species of *Bacteria* and *Archaea*.

3.12 Autotrophy and Nitrogen Fixation

Many phototrophic and chemolithotrophic microbes get the carbon they need, and in some cases the nitrogen they need, by reducing CO_2 and N_2, respectively, from air. Because CO_2 and N_2 are such highly oxidized molecules, their assimilation into cell material requires large amounts of ATP and reducing power (Figure 3.1). Here we consider the assimilation of CO_2—autotrophy—only in the context of the Calvin cycle. In Chapter 14 we will see that autotrophy shows considerable metabolic diversity beyond the Calvin cycle.

The Calvin Cycle

In many autotrophic organisms, including all oxygenic phototrophs, the carbon needed for biosynthesis is derived from the reduction of CO_2 by the **Calvin cycle** (Figure 3.26). The Calvin cycle is present in phototrophic purple bacteria (Figure 3.25*b*), cyanobacteria, algae, green plants, most chemolithotrophic *Bacteria*, and a few *Archaea*. The cycle requires CO_2, a CO_2-acceptor molecule, NADPH, ATP, and two key enzymes, *ribulose bisphosphate carboxylase* and *phosphoribulokinase*.

The first step in the Calvin cycle is catalyzed by the enzyme *ribulose bisphosphate carboxylase*, **RuBisCO** for short. RuBisCO catalyzes the

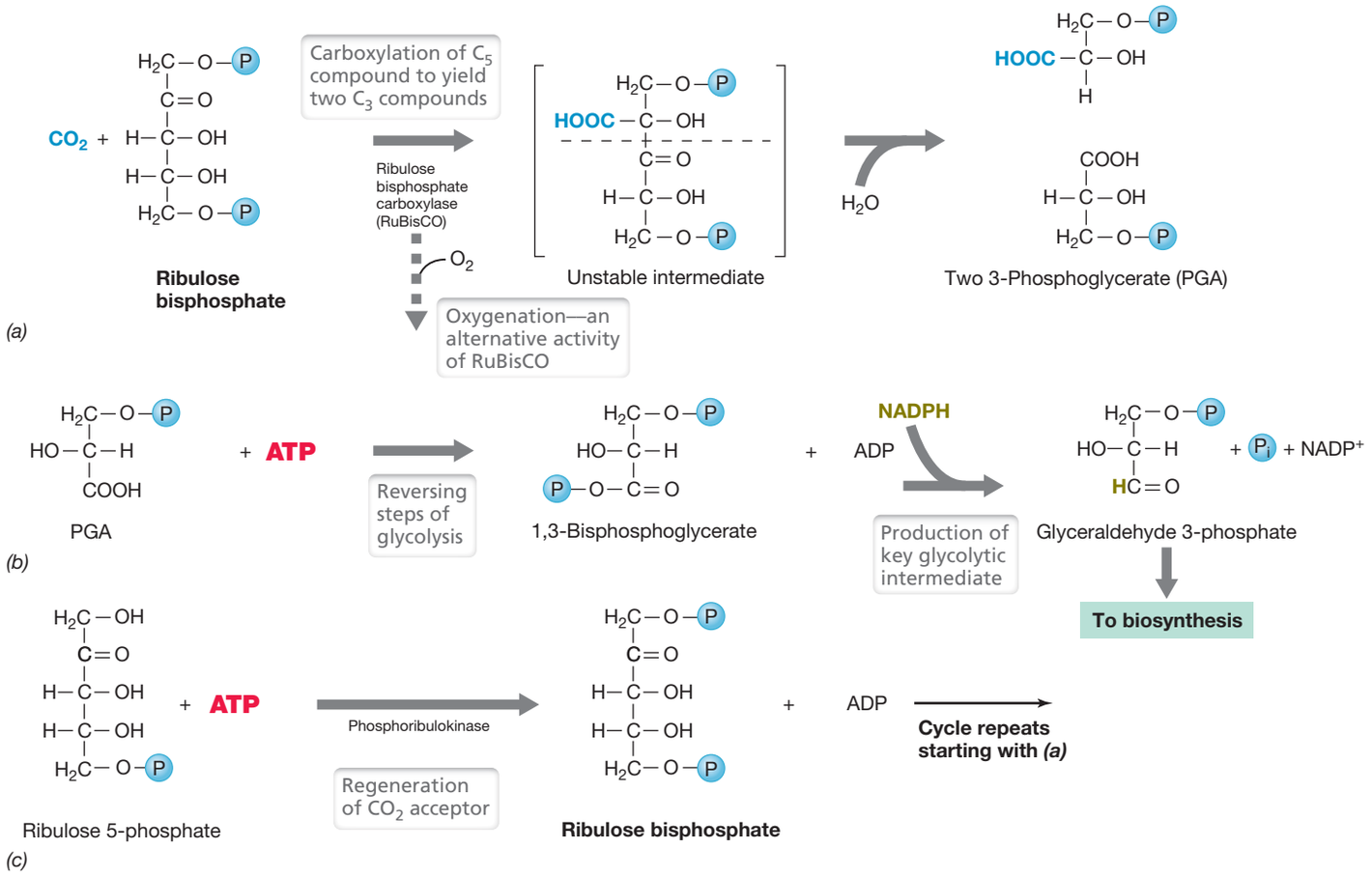


Figure 3.26 Key reactions of the Calvin cycle. *(a)* Reaction of the enzyme ribulose bisphosphate carboxylase. *(b)* Steps in the conversion of 3-phosphoglycerate (PGA) to glyceraldehyde 3-phosphate. Note that both ATP and NADPH are required. *(c)* Conversion of ribulose 5-phosphate to the CO_2 acceptor molecule ribulose 1,5-bisphosphate by the enzyme phosphoribulokinase.

formation of two molecules of 3-phosphoglycerate (PGA, a C_3 compound) from ribulose bisphosphate (a C_5 compound) and CO_2 as shown in Figure 3.26*a*. The PGA is then phosphorylated and reduced to a key intermediate of glycolysis, glyceraldehyde 3-phosphate. From this, glucose can be formed by reversal of the early steps in glycolysis (Figure 3.11).

Instead of focusing on the incorporation of a single molecule of CO_2, it is easiest to consider Calvin cycle reactions based on the incorporation of 6 molecules of CO_2, as this is what is required to make one hexose ($C_6H_{12}O_6$). For RuBisCO to incorporate 6 molecules of CO_2, 6 molecules of ribulose bisphosphate (total, 30 carbons) are required; carboxylation of these yields 12 molecules of PGA (total, 36 carbons) (**Figure 3.27**). A series of biochemical rearrangements between various sugars follows, resulting in 6 molecules of ribulose 5-phosphate (30 carbons) plus one hexose (6 carbons) for cell biosynthesis. The final step in the Calvin cycle is the phosphorylation of each ribulose 5-phosphate by the enzyme *phosphoribulokinase* (Figures 3.26*c* and 3.27) to regenerate 6 molecules of the acceptor molecule, ribulose bisphosphate. All totaled, *12 NADPH and 18 ATP* are required to synthesize one glucose from 6 CO_2 by the Calvin cycle.

Although the Calvin cycle is the most widespread and important pathway of CO_2 fixation in the biosphere, many autotrophic *Bacteria* and *Archaea* have evolved alternative pathways for fixing CO_2. These alternative autotrophic pathways typically reduce CO_2 to the level of acetyl-coenzyme A (acetyl-CoA), a central metabolite that feeds into all major biosynthetic pathways (Section 3.6).We will learn about these alternative pathways for CO_2 fixation in Chapter 14 when we once again consider metabolic diversity.

Nitrogen Fixation

In addition to carbon, cells need a significant amount of nitrogen to synthesize proteins, nucleic acids, and many other organic molecules. Most microbes obtain this nitrogen from "fixed" forms of nitrogen in their environment, such as ammonia (NH_3) or nitrate (NO_3^-). However, many *Bacteria* and *Archaea* can form ammonia from gaseous dinitrogen (N_2), a process called **nitrogen fixation** (**Figure 3.28**). The process of nitrogen fixation is of enormous ecological and agricultural importance (◀ Section 1.6) as it provides much of the fixed nitrogen that supports global ecosystems and is important to leguminous crops, such as soybeans, lentils, and alfalfa, which form symbiotic associations with nitrogen-fixing bacteria (▶ Section 23.4).

Nitrogen fixation is catalyzed by the enzyme complex **nitrogenase**. Nitrogenase consists of two proteins, *dinitrogenase* and *dinitrogenase reductase* (Figure 3.28). Both proteins contain iron, and

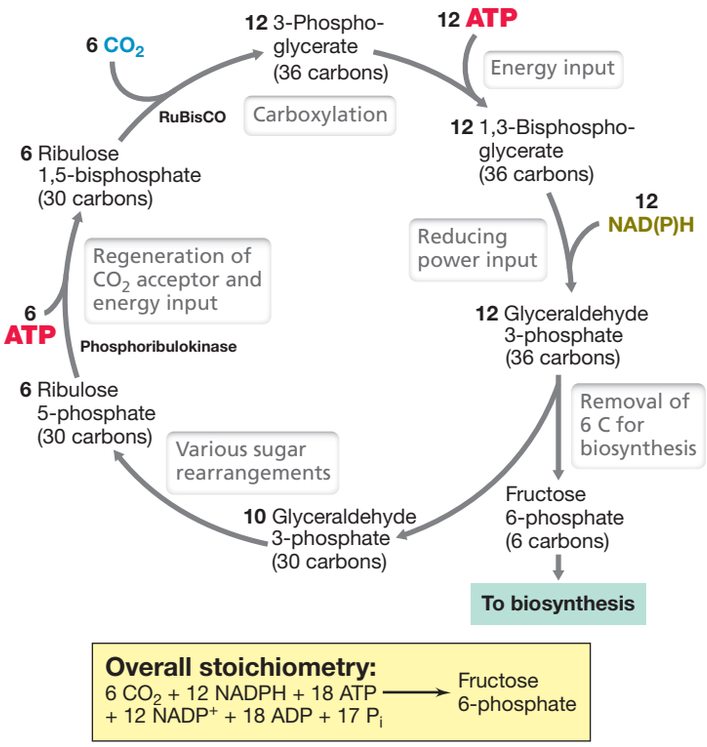


Figure 3.27 The Calvin cycle. Shown is the production of one hexose molecule from six CO_2. For each six molecules of CO_2 incorporated, one fructose 6-phosphate is produced that can be drawn off for biosynthesis. In phototrophs, the energy to produce ATP comes from light; in chemolithotrophs, the energy to produce ATP comes from the oxidation of inorganic electron donors. In oxygenic phototrophs, electrons for NAD(P)H come from water; in anoxygenic phototrophs, the electrons come from reduced substances such as H_2S. In chemolithotrophs, NAD(P)H is formed from inorganic electron donors.

dinitrogenase contains molybdenum as well. The iron and molybdenum in dinitrogenase are part of the enzyme cofactor called the *iron–molybdenum cofactor* (*FeMo-co*), and reduction of N_2 occurs at this site. Owing to the stability of the triple bond in N_2, its activation and reduction is very energy demanding. Six electrons are needed to reduce N_2 to NH_3, and the successive reduction steps occur directly on nitrogenase with no free intermediates accumulating. The electrons for N_2 reduction are transferred to dinitrogenase reductase from low-potential iron–sulfur proteins such as flavodoxin (or ferredoxin) (Table 3.1). The sequence of electron transfer in nitrogenase is: electron donor → dinitrogenase reductase → dinitrogenase → N_2 (Figure 3.28). Although only *six* electrons are necessary to reduce N_2 to two NH_3, *eight* electrons are actually consumed in the process, two electrons being lost as H_2 for each molecule of N_2 reduced. For unknown reasons, H_2 evolution is an obligatory step in nitrogen fixation and occurs in the first step of the nitrogenase reduction cycle.

In addition to electrons, ATP is required for nitrogen fixation. ATP binds to dinitrogenase reductase, and, following its hydrolysis to ADP, lowers the reduction potential of the protein. This allows dinitrogenase reductase to interact with and reduce dinitrogenase. Electrons are transferred from dinitrogenase reductase to dinitrogenase one at a time, and each cycle of reduction requires two ATP. Thus, a total of 16 ATP are required for the reduction of N_2 to 2 NH_3 (Figure 3.28).

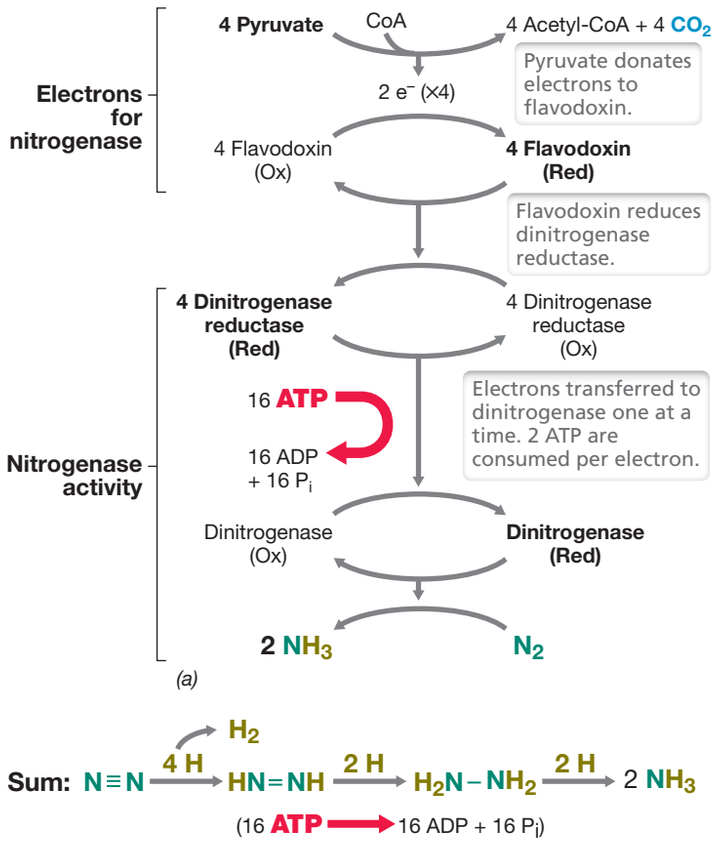


Figure 3.28 Biological nitrogen fixation by nitrogenase. The nitrogenase complex is composed of dinitrogenase and dinitrogenase reductase. Electrons from reduced flavodoxin (or ferredoxin) are used to reduce dinitrogenase reductase, and this enzyme in turn reduces dinitrogenase at the expense of ATP. Dinitrogenase ultimately donates electrons to N_2 at the active site of the enzyme, resulting in the formation of 2 NH_3. H is hydrogen (which consists of $H^+ + e^-$).

Nitrogenase is inhibited by oxygen (O_2), but many nitrogen-fixing bacteria are obligate aerobes. In these organisms, nitrogenase is protected from oxygen inactivation by a combination of the rapid removal of O_2 by respiration and the production of O_2-retarding slime layers on the outer cell surface (**Figure 3.29**). In certain cyanobacteria, the nitrogenase enzyme is protected from O_2 by its localization in a differentiated cell called a *heterocyst* (Figure 3.29*c*; ▶ Section 15.3). Inside the heterocyst, conditions are anoxic despite the fact that photosynthesis and O_2 production occur in neighboring vegetative cells. Oxygen is not produced in the heterocyst, and this protects nitrogenase so that it can fix N_2 into organic nitrogen compounds, which are then shared with adjacent photosynthetic cells (▶ Section 8.9).

Check Your Understanding

- How many electrons and how much ATP are required to make one hexose molecule by the Calvin cycle?
- How many electrons and how much ATP are required to fix one molecule of N_2 into two of NH_3?
- Describe two ways in which N_2-fixing organisms can protect their nitrogenase from O_2.

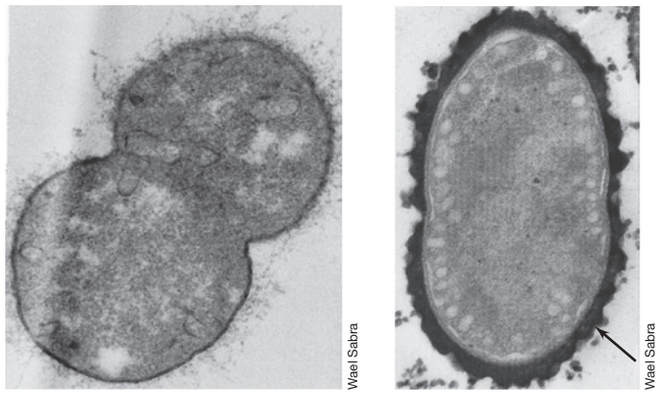


Figure 3.29 **Two ways of protecting nitrogenase from O_2.** Induction of slime formation by O_2 is demonstrated by comparing transmission electron micrographs of *(a)* nitrogen-fixing *Azotobacter vinelandii* cells grown with 2.5% O_2 and showing very little slime with *(b)* nitrogen-fixing *A. vinelandii* cells grown in air (21% O_2) and showing an extensive, darkly staining slime layer (arrow). The slime retards diffusion of O_2 into the cell, thus preventing nitrogenase inactivation by O_2. A single cell of *A. vinelandii* is about 2 μm in diameter. *(c)* Fluorescence photomicrograph of cells of the filamentous cyanobacterium *Anabaena* showing a single heterocyst (green). The heterocyst is a thick-walled differentiated cell that does not produce O_2 but instead specializes in nitrogen fixation and protects nitrogenase from O_2 inactivation.

3.13 Sugars and Polysaccharides

Polysaccharides are key components of microbial cell walls, and cells often store carbon and energy reserves in the form of the polysaccharides glycogen or starch (Chapter 2). How are such large molecules made?

Polysaccharide Biosyntheses and Gluconeogenesis

Polysaccharides are synthesized from *activated* forms of glucose, either uridine diphosphoglucose (*U*DPG; **Figure 3.30*a***) or adenosine diphosphoglucose (*A*DPG). UDPG is the precursor of several glucose derivatives used in the biosynthesis of important structural polysaccharides, such as *N*-acetylglucosamine and *N*-acetylmuramic acid in peptidoglycan or the lipopolysaccharide component of the gram-negative outer membrane (◀ Sections 2.3 and 2.4). Polysaccharides are biosynthesized by adding activated glucose to a preexisting polymer fragment. For example, glycogen is synthesized as ADPG + glycogen → ADP + glycogen-glucose.

When a cell is growing on a hexose such as glucose, obtaining glucose for polysaccharide synthesis is obviously not a problem. But when the cell is growing on other carbon compounds, glucose must be biosynthesized. This process, called *gluconeogenesis*, uses phosphoenolpyruvate, one of the intermediates of glycolysis, as a starting material and travels backwards through the glycolytic pathway (Figure 3.11) to form glucose. Phosphoenolpyruvate can also be synthesized from oxaloacetate, a citric acid cycle intermediate (Figure 3.12). An overview of gluconeogenesis is shown in Figure 3.30*b*.

Pentose Metabolism and the Pentose Phosphate Pathway

Pentoses (C_5 sugars) are formed by the removal of one carbon atom from a hexose, typically as CO_2. The pentoses needed for nucleic acid synthesis, ribose (in RNA) and deoxyribose (in DNA), are formed as shown in Figure 3.30*c*. The enzyme *ribonucleotide reductase*

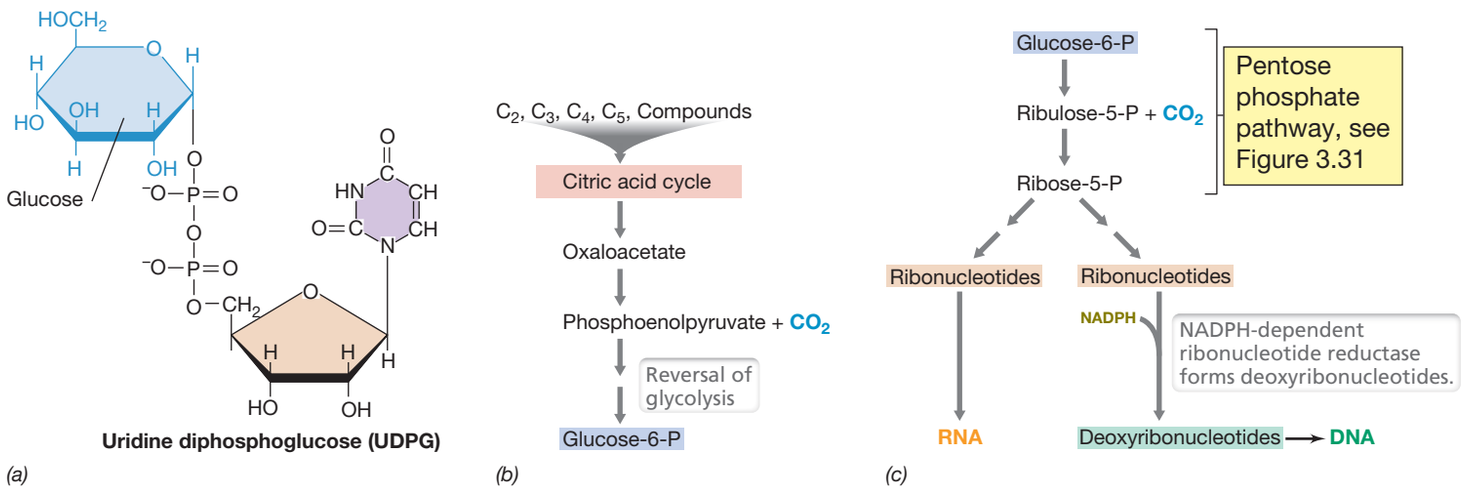
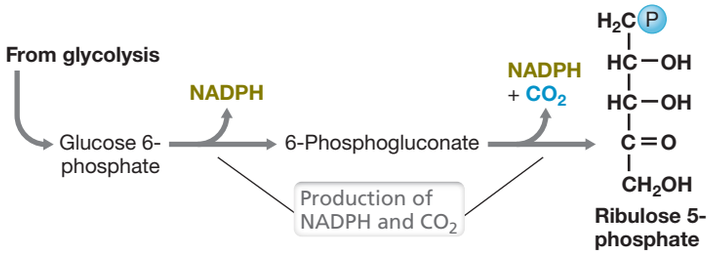
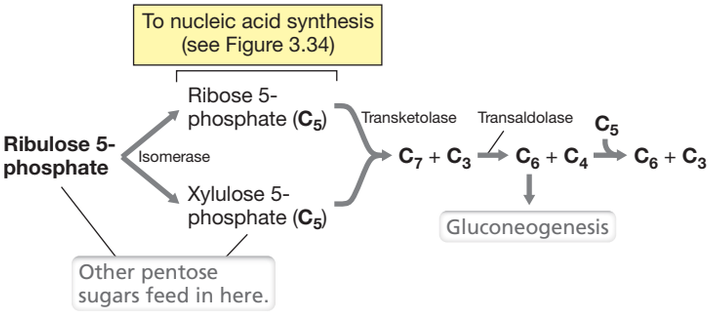


Figure 3.30 **Sugar metabolism.** *(a)* Polysaccharides are synthesized from activated forms of hexoses such as UDPG. *(b)* Gluconeogenesis. When glucose is needed, it can be biosynthesized from other carbon compounds by reversing the steps in glycolysis. *(c)* Pentoses for nucleic acid synthesis are formed by decarboxylation of hexoses such as glucose 6-phosphate. Note how the precursors of DNA are produced from the precursors of RNA by the enzyme ribonucleotide reductase.



(a)



(b)

Figure 3.31 Pentose phosphate pathway. This pathway generates pentoses from other sugars for biosynthesis and also functions to catabolize pentose sugars. *(a)* Production of the key intermediate, ribulose 5-phosphate. *(b)* Other reactions in the pentose phosphate pathway.

converts ribose into deoxyribose by reduction of the hydroxyl (−OH) group on the 2′ carbon of the 5-carbon pentose ring (▶ Section 6.1 and Figure 6.1*b*). This reaction occurs after, not before, synthesis of nucleotides. Thus, *ribo*nucleotides are biosynthesized, and some of them are later reduced to *deoxy*ribonucleotides for use as precursors of DNA.

The major pathway for pentose production is the **pentose phosphate pathway** (Figure 3.31). In this pathway, glucose, a hexose, is oxidized to CO_2, NADPH, and the key intermediate, *ribulose 5-phosphate*; from the latter, several different pentose derivatives can be formed. When pentoses are used as electron donors for energy conservation, they feed directly into the pentose phosphate pathway, typically becoming phosphorylated to form ribose phosphate or a related compound before being further catabolized (Figure 3.31*b*).

Besides its importance in pentose metabolism, the pentose phosphate pathway is also responsible for producing many other important sugars in the cell, including those containing 4 to 7 carbons. These sugars can eventually be converted to hexoses for either catabolic or biosynthetic purposes (Figures 3.30 and 3.31). A final important role of the pentose phosphate pathway is that it generates NADPH, a coenzyme used in many biosyntheses and in particular as a reductant for the production of deoxyribonucleotides (Figure 3.30*c*) and in the biosynthesis of fatty acids (see Figure 3.35). Although most cells have an exchange mechanism for converting NADH into NADPH, the pentose phosphate pathway is the major means by which NADPH is synthesized directly.

Check Your Understanding

- What form of activated glucose is used in the biosynthesis of glycogen by bacteria?
- What is gluconeogenesis?
- What functions does the pentose phosphate pathway play in the cell?

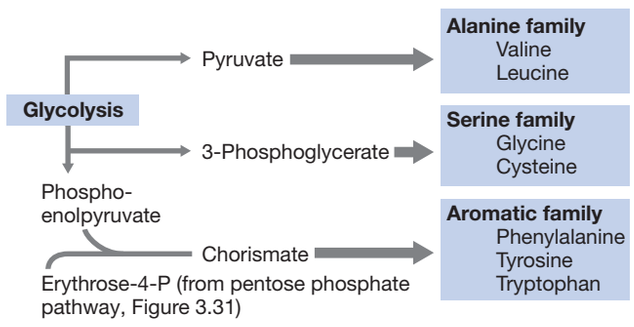
3.14 Amino Acids and Nucleotides

The monomers in proteins and nucleic acids are the amino acids and nucleotides, respectively. Their biosyntheses are typically multistep biochemical pathways that we need not consider in detail here. Instead, we identify the carbon skeletons needed for the biosynthesis of amino acids and nucleotides, look at their origins in pathways we have already considered, and summarize the mechanisms by which they are made.

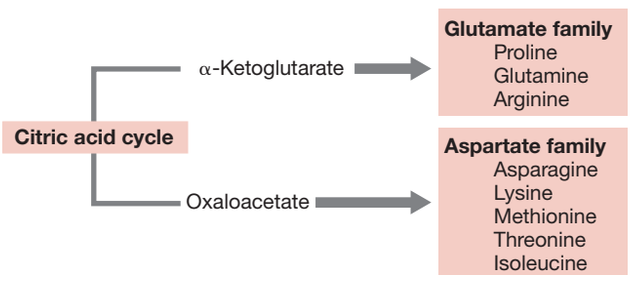
Monomers of Proteins: Amino Acids

Organisms that cannot obtain some or all of their amino acids preformed from the environment must synthesize them from glucose or other carbon sources. Amino acids are grouped into structurally related *families* that share several biosynthetic steps. The carbon skeletons for amino acids come almost exclusively from intermediates of glycolysis (Figure 3.11) or the citric acid cycle (Figure 3.12) (Figure 3.32).

The amino group ($-NH_2$) of amino acids is typically derived from an inorganic nitrogen source, such as ammonia (NH_3). Ammonia is



(a)



(b)

Figure 3.32 Amino acid families. Glycolysis *(a)* and the citric acid cycle *(b)* provide the carbon skeletons for most amino acids. Synthesis of the various amino acids in a family may require many steps starting with the parent amino acid (shown in bold as the name of the family).

most often incorporated during biosynthesis of the amino acids glutamate or glutamine by the enzymes *glutamate dehydrogenase* and *glutamine synthetase*, respectively (**Figure 3.33**). When NH_3 is present at high levels, glutamate dehydrogenase or other amino acid dehydrogenases are used. However, when NH_3 is present at low levels, glutamine synthetase, with its energy-consuming reaction mechanism (Figure 3.33*b*) and correspondingly high affinity for its substrates, is employed. The enzymes glutamate dehydrogenase and glutamine synthetase are present in most *Bacteria* and *Archaea*.

Once ammonia is incorporated into glutamate or glutamine, the amino group of these amino acids can be transferred to other carbon skeletons to form other nitrogenous compounds. For example, glutamate can donate its amino group to oxaloacetate in a transaminase reaction, producing α-ketoglutarate and aspartate (Figure 3.33*c*). Alternatively, glutamine can react with α-ketoglutarate to form two molecules of glutamate in an aminotransferase reaction (Figure 3.33*d*). The end result of these types of reactions is the shuttling of ammonia into various carbon skeletons from which further biosynthetic reactions occur to form all of the amino acids needed to make proteins and other nitrogen-containing biomolecules.

Monomers of Nucleic Acids: Nucleotides

The biochemistry behind purine and pyrimidine biosynthesis is quite complex and so only an outline of their biosyntheses is necessary here. Purines are constructed literally atom by atom from several carbon and nitrogen sources, including even CO_2 (**Figure 3.34**). The purine nucleotide skeleton, inosinic acid (Figure 3.34*b*), is the precursor of the purine nucleotides *adenine* and *guanine*. Once these are synthesized (in their triphosphate forms) and attached to ribose, they are ready to be incorporated into DNA (following ribonucleotide reductase activity, Figure 3.30*c*) or RNA.

Like the purine ring, the pyrimidine ring is also constructed from several sources (Figure 3.34*c*). From the pyrimidine nucleotide skeleton uridylate (Figure 3.34*d*), all of the pyrimidines—*thymine, cytosine,* and *uracil*—are derived.Structures of all the purines and pyrimidines are shown in Chapter 6 (▶ Figure 6.1*c*).

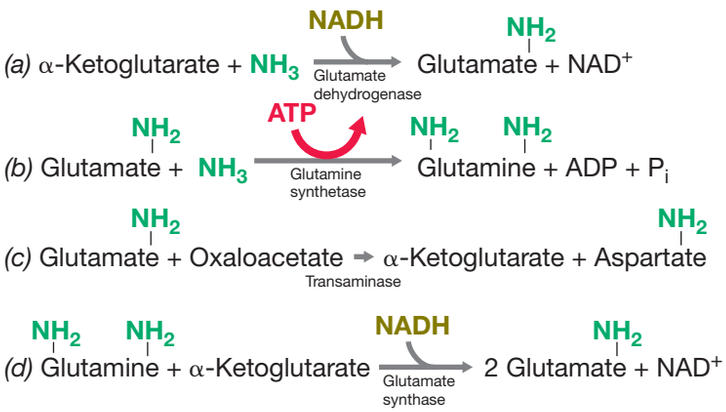


Figure 3.33 Ammonia incorporation in bacteria. Ammonia (NH_3) and the amino groups of all amino acids are shown in green. Two major pathways for NH_3 assimilation in bacteria are those catalyzed by the enzymes *(a)* glutamate dehydrogenase and *(b)* glutamine synthetase. *(c)* Transaminase reactions transfer an amino group from an amino acid to an organic acid. *(d)* The enzyme glutamate synthase forms two glutamates from one glutamine and one α-ketoglutarate.

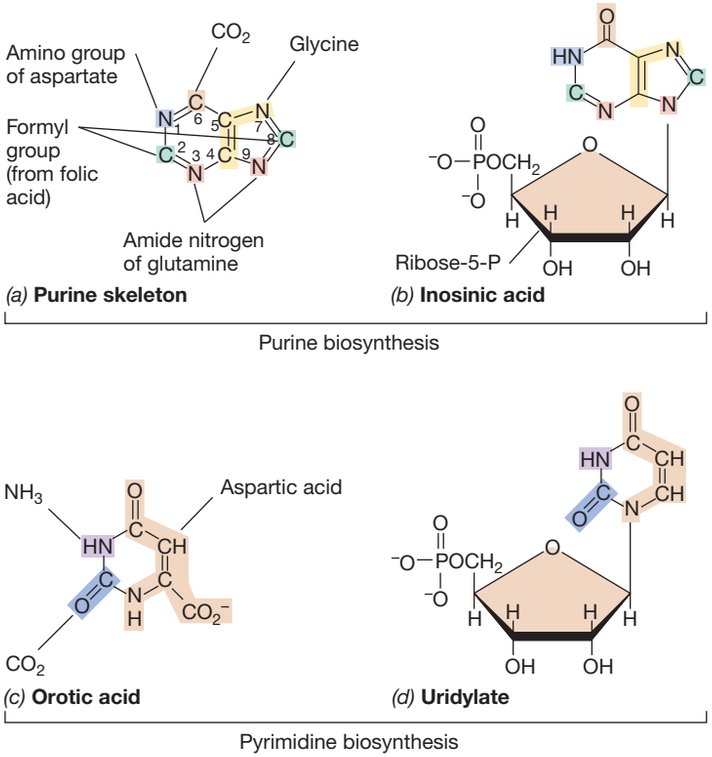


Figure 3.34 Biosynthesis of purines and pyrimidines. *(a)* Components of the purine skeleton, labeled with their sources. *(b)* Inosinic acid, the precursor of all purine nucleotides. *(c)* Components of the pyrimidine skeleton, orotic acid, labeled with their sources. *(d)* Uridylate, the precursor of all pyrimidine nucleotides. Uridylate is formed from orotate following a decarboxylation and the addition of ribose 5-phosphate.

Check Your Understanding

- What is an amino acid family?
- List the steps required for the cell to incorporate NH_3 into amino acids.
- Which nitrogen bases are purines and which are pyrimidines?

3.15 Fatty Acids and Lipids

Lipids are major components of the cytoplasmic membrane and of the outer membrane of gram-negative bacteria; lipids can also be carbon and energy reserves. Fatty acids are the backbone of microbial lipids. However, recall that fatty acids, per se, are found only in *Bacteria* and *Eukarya*. *Archaea* do not contain fatty acids in their lipids but instead have hydrophobic isoprenoid side chains that play a similar structural role (Chapter 2). Our focus here is on the biosynthesis of fatty acids in *Bacteria*.

Fatty Acid Biosynthesis

Fatty acids are biosynthesized two carbon atoms at a time by the activity of a protein called *acyl carrier protein* (ACP). ACP holds the growing fatty acid as it is being constructed and releases it once it has reached its final length (**Figure 3.35**). Although fatty acids are constructed *two* carbons at a time, each C_2 (acetyl) unit originates

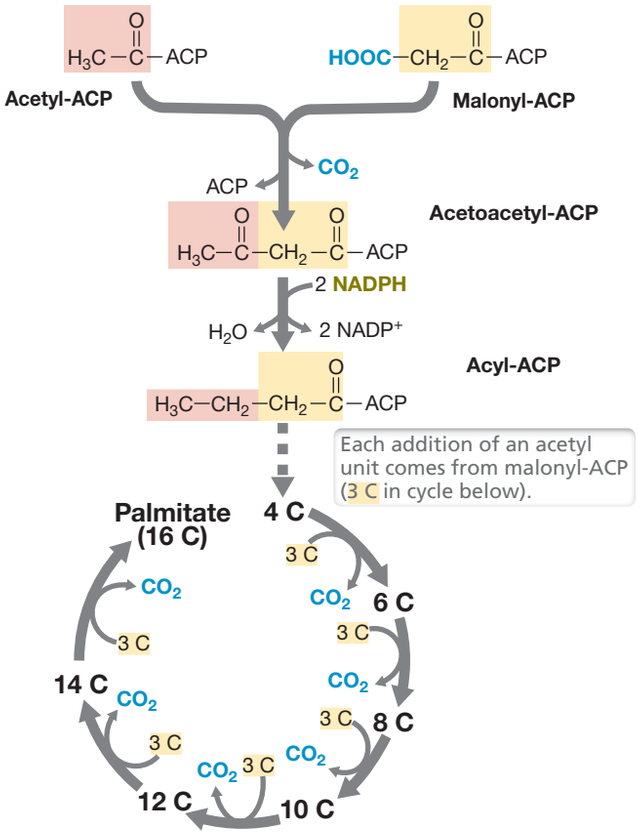


Figure 3.35 The biosynthesis of the C_{16} fatty acid palmitate. The condensation of acetyl-ACP (which is formed from acetyl-CoA) and malonyl-ACP forms acetoacetyl-ACP. ACP (acyl carrier protein) is a small protein used to add carbon subunits to a growing fatty acid chain. Each successive addition of an acetyl unit comes from malonyl-ACP.

from the *three*-carbon compound *malonate*, which is attached to the ACP to form malonyl-ACP. As each malonyl residue is donated, one molecule of CO_2 is released (Figure 3.35).

In addition to saturated, even-carbon-number fatty acids, fatty acids can also be unsaturated, branched, or have an odd number of carbon atoms. Unsaturated fatty acids contain one or more double bonds in the long hydrophobic portion of the molecule. The number and position of these double bonds is often species-specific or group-specific, and the double bonds are formed by desaturation of a saturated fatty acid. Branched-chain fatty acids are biosynthesized using a branched-chain initiating molecule, and odd-carbon-number fatty acids (for example, C_{13}, C_{15}, C_{17}) are biosynthesized using an initiating molecule that contains a propionyl (C_3) group instead of acetyl.

The fatty acid composition of cellular lipids varies from species to species and can also vary in a given organism with differences in growth temperature. Cells adjust their fatty acid composition primarily to keep the cytoplasmic membrane from getting either too stiff in the cold or too fluid as temperatures rise. Growth at low temperatures promotes the biosynthesis of shorter-chain and unsaturated fatty acids, whereas growth at higher temperatures promotes the biosynthesis of longer-chain and more saturated fatty acids. The most common fatty acids in lipids of *Bacteria* are those with chain lengths of C_{12}–C_{20}.

Lipid Biosynthesis

In the assembly of lipids in cells of *Bacteria* and *Eukarya*, fatty acids are first added to a molecule of glycerol. For simple triglycerides (fats), all three glycerol carbons are esterified with fatty acids. To form complex lipids, one of the carbon atoms in glycerol is embellished with a molecule of phosphate, ethanolamine, carbohydrate, or some other polar substance (◀ Figure 2.1*c*). In *Archaea*, although membrane lipids are constructed from isoprene to form the phytanyl (C_{15}) or biphytanyl (C_{30}) side chains, the glycerol backbone of archaeal membrane lipids contains a polar group (sugar, phosphate, sulfate, or polar organic compound) as for the lipids of *Bacteria* and *Eukarya*. Polar groups are important in lipids for forming the canonical membrane architecture: a hydrophobic interior with hydrophilic inner and outer surfaces (◀ Section 2.1 and Figures 2.1 and 2.2).

Check Your Understanding

- Explain how fatty acids are constructed two carbon atoms at a time while the immediate donor of these carbons is a three-carbon compound.
- What differences exist in lipids from the three domains of life?
- What is the result of adding a polar head group to lipids?

CHAPTER REVIEW

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I • Fundamentals of Metabolism

3.1 All cells require water, carbon and other nutrients, free energy, and reducing power. Microorganisms conserve energy either from the oxidation of chemicals or from light. Chemoorganotrophs use organic chemicals as their electron donors, while chemolithotrophs use inorganic chemicals. Phototrophic organisms convert light energy into chemical energy (ATP) and include both oxygenic and anoxygenic species.

Q When a microorganism is growing on glucose as both an energy and a carbon source, to which energy and carbon utilization classes does it belong? When a microorganism can synthesize its own organic compounds from carbon dioxide when growing on H_2, to which energy and carbon utilization classes does it belong?

3.2 Oxidation–reduction reactions require electron donors and electron acceptors. The tendency of a compound to accept or release electrons is expressed by its reduction potential (E_0'). Redox reactions in a cell often employ redox coenzymes such as NAD^+/NADH as electron shuttles.

Q The following is a series of coupled electron donors and electron acceptors (written as donor:acceptor). Using the data in Table 3.1 and Figure 3.4, order this series from most energy yielding to least energy yielding: H_2:Fe^{3+}, NO:Mn^{4+}, H_2S:O_2, methanol:NO_3^- (producing NO_2^-), H_2:O_2, Fe^{2+}:O_2, NO_2^-:Fe^{3+} (producing NO_3^-), and H_2S:NO_3^-.

3.3 Chemical reactions in the cell are accompanied by changes in energy, expressed in kilojoules per mole. Reactions either release or consume free energy. $\Delta G^{0\prime}$ is a measure of the energy released (if $\Delta G^{0\prime}$ is negative in arithmetic sign) or consumed (if $\Delta G^{0\prime}$ is positive in arithmetic sign) in a reaction under standard conditions and reveals which reactions can be used by an organism to conserve energy.

Q Calculate $\Delta G^{0\prime}$ for the following reaction: glucose + $6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$. Is this reaction exergonic or endergonic? Distinguish between $G^{0\prime}$, G, and G_f^0.

3.4 Energy is conserved in compounds that contain energy-rich phosphate or sulfur bonds. The most common of these compounds is ATP, the prime energy carrier in the cell. ATP can be formed by substrate-level phosphorylation, oxidative phosphorylation, or photophosphorylation.

Q Why is acetyl phosphate considered an energy-rich compound while glucose 6-phosphate is not? What are the primary mechanisms of ATP formation in fermentative metabolism and respiratory metabolism, respectively?

3.5 Enzymes are protein catalysts that increase the rate of biochemical reactions by activating the substrates that bind to their active site. Enzymes are highly specific in the reactions they catalyze, and this specificity resides in the three-dimensional structures of the polypeptide(s) that make up the protein(s).

Q What makes enzymes highly specific?

II • Catabolism: Chemoorganotrophs

3.6 The glycolytic pathway is used to break down glucose to pyruvate. The citric acid cycle oxidizes pyruvate to CO_2 and releases electrons for the electron transport chain and is also a source of biosynthetic intermediates. The glyoxylate cycle is necessary for the catabolism of two-carbon electron donors, such as acetate.

Q Why are glycolysis and the citric acid cycle, on their own, insufficient to sustain life? What else is needed?

3.7 In the absence of external electron acceptors, organic compounds can be catabolized anaerobically only by fermentation. Fermentations require that an energy-rich organic compound be formed that can yield ATP by substrate-level phosphorylation. Redox balance is achieved by the production of fermentation products.

Q Define the term substrate-level phosphorylation. How does it differ from oxidative phosphorylation? What compound(s) do fermentative bacteria need to synthesize in order to make ATP by substrate-level phosphorylation?

3.8 Electron transport chains are composed of membrane-associated redox proteins that are arranged in order of their increasing E_0' values. The electron transport chain functions in a concerted fashion to carry electrons from the primary electron donor to the terminal electron acceptor, which is O_2 in aerobic respiration.

Q What is the value to the electron transport chain of alternating between electron carriers that carry only electrons and those that carry electrons and protons?

3.9 During electron transport, protons are extruded to the outside of the membrane to form the proton motive force. Key electron carriers include flavins, quinones, the cytochrome bc_1 complex, and other cytochromes. The cell uses the proton motive force to make ATP through the activity of ATPase.

Q How is the proton motive force generated during electron transport? How do cells generate ATP from a proton motive force?

III • Catabolism: Electron Transport and Metabolic Diversity

3.10 When conditions are anoxic, several electron acceptors can substitute for O_2 in anaerobic respiration. During respiration, energy is conserved by the transport of electrons through the electron transport chain that generates a proton motive force; ATP is synthesized by ATP synthase by dissipating the proton motive force.

Q *E. coli* can oxidize glucose to 6 CO_2 and 6 H_2O by aerobic respiration or by anaerobic respiration with nitrate. How much ATP would it form per glucose oxidized in each circumstance?

3.11 Chemolithotrophs use inorganic compounds as electron donors to drive electron transport reactions and thereby generate a proton motive force. Phototrophs use light energy to drive electron transport reactions and thereby generate a proton motive force. The proton motive force underlies energy conservation in all forms of respiration and photosynthesis.

Q What is the role of electron transport in cyclic photophosphorylation? If a purple bacterium is performing cyclic photophosphorylation and growing autotrophically, why would it need an electron donor?

IV • Biosynthesis

3.12 Autotrophy is supported in most phototrophic and chemolithotrophic bacteria by the Calvin cycle, in which the enzyme RuBisCO plays a key role. The reduction of N_2 to NH_3 is called nitrogen fixation and is catalyzed by the enzyme nitrogenase.

Q How might the ability to fix CO_2 and N_2 help a bacterium be more competitive in its environment?

3.13 Polysaccharides are important structural components of cells and are biosynthesized from activated forms of their monomers. Gluconeogenesis is the production of glucose from nonsugar precursors.

Q What is the importance of the enzyme ribonucleotide reductase in the metabolism of sugars? What is the difference between "free" and "activated" glucose?

3.14 Amino acids are formed from carbon skeletons to which ammonia is added from glutamate, glutamine, or a few other amino acids. Nucleotides are biosynthesized using carbon skeletons from several different sources.

Q Name two common enzymes that function to incorporate NH_3 into the cell. How do their reaction mechanisms differ?

3.15 Fatty acids are synthesized from the three-carbon precursor malonyl-ACP, and fully formed fatty acids are attached to glycerol to form lipids. Only the lipids of *Bacteria* and *Eukarya* contain fatty acids.

Q Describe the process by which a fatty acid such as palmitate (a C_{16} straight-chain saturated fatty acid) is synthesized in a cell.

APPLICATION QUESTIONS

1. For the following reactions identify the electron donor and the electron acceptor, define the type of metabolism and whether the reaction is aerobic or anaerobic, and calculate the free-energy change under standard conditions (see Sections 3.2 and 3.3):

$$4\,H_2 + 2\,CO_2 \rightleftharpoons Acetate^- + H^+ + 2\,H_2O$$
$$Acetate^- + SO_4^{2-} + 3\,H^+ \rightleftharpoons H_2S + 2\,CO_2 + 2\,H_2O$$
$$H_2S + 2\,O_2 \rightleftharpoons SO_4^{2-} + 2\,H^+$$

2. Using the data in Figure 3.4, predict the sequence of electron carriers in the electron transport chain of an organism growing aerobically that has the following electron carriers: ubiquinone, cytochrome *a*, cytochrome *b*, NADH, cytochrome *c*, FAD.
3. Explain the following observation in light of the redox tower: Cells of *Escherichia coli* fermenting glucose grow faster when NO_3^- is supplied to the culture (NO_2^- is produced), and then grow even faster (and stop producing NO_2^-) when the culture is highly aerated. Calculate the standard free-energy change of the relevant metabolic reactions.
4. A new, experimental drug is developed that stops ATP from being hydrolyzed to ADP and inorganic phosphate in only *Bacteria* and *Archaea*. Discuss at least two cellular processes that would be affected by inhibiting ATP hydrolysis, and what the outcome would be for *Bacteria* and *Archaea* treated with this drug.

CHAPTER GLOSSARY

Activation energy the energy required to bring the substrate of an enzyme to the reactive state

Anabolic reactions (anabolism) the sum total of all biosynthetic reactions in the cell

Anaerobic respiration a form of respiration in which the terminal electron acceptor is not O_2

ATP (adenosine triphosphate) a nucleotide that is the primary form in which chemical energy is conserved and utilized in cells

ATPase (ATP synthase) a multiprotein enzyme complex embedded in the cytoplasmic membrane that catalyzes the synthesis of ATP coupled to dissipation of the proton motive force

Autotroph an organism capable of biosynthesizing all cell material from CO_2 as the sole carbon source

Calvin cycle the series of biosynthetic reactions by which most phototrophs and most aerobic chemolithotrophs convert CO_2 into organic compounds

Catabolic reactions (catabolism) biochemical reactions leading to energy conservation (usually as ATP) by the cell

Chemolithotroph an organism able to oxidize inorganic compounds (such as H_2, Fe^{2+}, S^0, or NH_{4+}) as energy sources (electron donors)

Chemoorganotroph an organism that obtains its carbon and reducing power from organic chemicals

Chemotroph an organism that uses chemical reactions as its source of energy

Citric acid cycle (CAC) a cyclical series of reactions resulting in the conversion of acetate to two molecules of CO_2

Coenzyme a small and loosely bound nonprotein molecule that participates in a reaction as part of an enzyme

Electron acceptor a substance that can accept electrons from an electron donor, becoming reduced in the process

Electron donor a substance that can donate electrons to an electron acceptor, becoming oxidized in the process

Endergonic a reaction that requires free energy

Enzyme a protein (or in some cases an RNA) catalyst that functions to speed up chemical reactions

Exergonic a reaction that releases free energy

Fermentation anaerobic catabolism in which an organic compound is both an electron donor and an electron acceptor and ATP is produced by substrate-level phosphorylation

Ferredoxin an electron carrier with an extremely electronegative reduction potential

Free energy (*G*) energy available to do work; $G^{0\prime}$ is free energy under standard conditions

Glycolysis a biochemical pathway in which glucose is oxidized to pyruvate, which is either used in respiration or fermented (also called the Embden–Meyerhof–Parnas pathway)

Glyoxylate cycle a modification of the citric acid cycle in which isocitrate is cleaved to form succinate and glyoxylate during growth on two-carbon electron donors such as acetate

Heterotroph an organism that uses organic compounds as a carbon source

Nicotinamide adenine dinucleotide (NAD^+/NADH) a cytoplasmic coenzyme that shuttles electrons between various enzymes that catalyze redox reactions

Nitrogenase the enzyme complex required to reduce N_2 to NH_3 in biological nitrogen fixation

Nitrogen fixation the reduction of N_2 to NH_3 by the enzyme nitrogenase

Oxidative phosphorylation the production of ATP from a proton motive force that is formed by electron transport reactions driven by the oxidation of an electron donor and reduction of an external electron acceptor (see also respiration)

Pentose phosphate pathway a series of reactions in which pentoses are catabolized to generate precursors for nucleotide biosynthesis or to synthesize glucose

Photophosphorylation the production of ATP from a proton motive force formed from light-driven electron transport

Phototroph an organism that uses light as its source of energy

Photosynthetic reaction center the site at which light energy is used to excite an electron to a more electronegative state so that it can be donated to the electron transport chain

Proton motive force (pmf) a source of energy resulting from the separation of protons and hydroxyl ions across the cytoplasmic membrane, generating a membrane electrochemical potential

Redox reaction A chemical reaction in which electrons are transferred between two molecules

Reducing power the ability to donate electrons in metabolic reactions

Reduction potential (E_0') the inherent tendency, measured in volts under standard conditions, of a compound to donate or to accept electrons

Respiration the process in which an electron donor is oxidized and electrons are passed through electron transport reactions to an external electron acceptor in order to generate a proton motive force and synthesize ATP by oxidative phosphorylation

RuBisCO ribulose bisphosphate carboxylase, a key enzyme for CO_2 fixation by the Calvin cycle

Substrate-level phosphorylation the production of ATP by the direct transfer of an energy-rich phosphate molecule from a phosphorylated organic compound to ADP

4 Microbial Growth and Its Control

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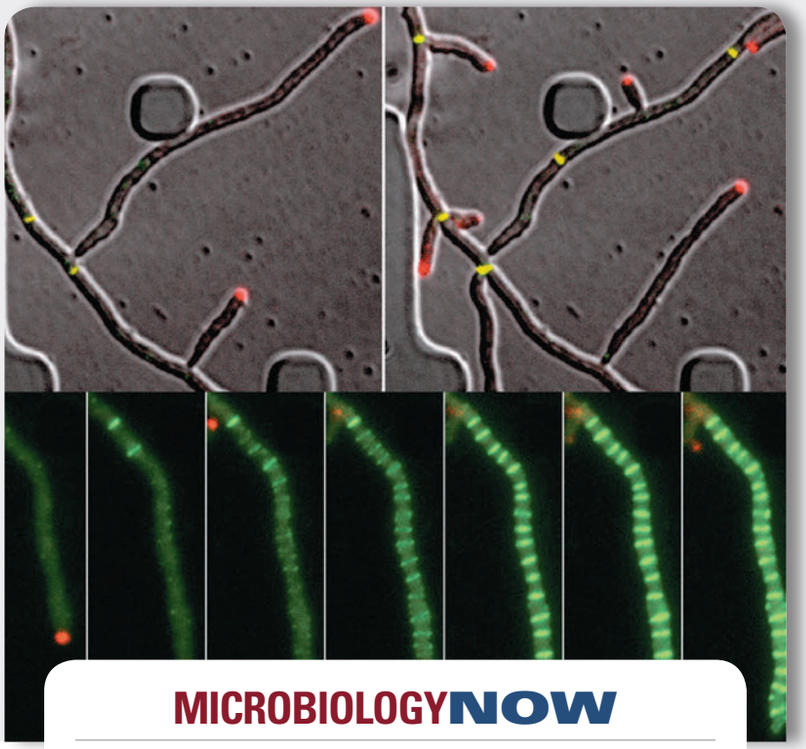
III Environmental Effects on Growth: Temperature 162

IV Environmental Effects on Growth: pH, Osmolarity, and Oxygen 167

V Controlling Microbial Growth 173



MICROBIOLOGYNOW

Growing Their Own Way

When thinking of microbial growth we typically think of binary fission, the splitting of one cell into two, and indeed this form of growth is common and widespread in the microbial world. But microbial growth takes many different forms. *Streptomyces*, for example, have a complex life cycle, as antibiotic producers often do. *Streptomyces* are common soil bacteria that produce secondary metabolites such as those called *geosmin*, which give soil its characteristic odor. *Streptomyces* also produce a wide range of other secondary metabolites, including many clinically useful antibiotics. Indeed, the antibiotic *streptomycin* provided the first cure for tuberculosis, and its discovery earned the soil microbiologist Selman Waksman a Nobel Prize in 1952. Progress in understanding the *Streptomyces* life cycle is now being made using techniques to grow and image *Streptomyces* in microfluidic chambers.

The life cycle of *Streptomyces* begins with vegetative growth, during which filamentous cells grow by elongating from the tip without cell division. This elongation without division can be visualized (upper image set) in stains where a red fluorescent protein has been fused to a protein that localizes to the growth tip. Cell division is visualized by a yellow fluorescent protein fused to FtsZ, a protein that mediates septa formation.

Nutrient limitation triggers the formation of aerial hyphae and sporulation. *Streptomyces* sporulation proceeds through multiple fission (lower image set). That is, one filamentous cell divides to form many spores. As seen in the image, many septa are formed simultaneously, resulting in up to 20 spores. Genetic analyses, facilitated by live imaging of growing cells, has revealed that dynamin-like proteins are required during this process. Dynamin is present in eukaryotic cells where it mediates membrane remodeling. This discovery in *Streptomyces* suggests that dynamin-like proteins may have similar functions in bacteria and shows that we still have a lot to learn about microbial growth.

Source: Schlimpert, S., et al. 2017. Two dynamin-like proteins stabilize FtsZ rings during *Streptomyces* sporulation. *Proc. Natl. Acad. Sci. USA* E6176.

In previous chapters we discussed fundamental aspects of microbiology including cell structure and function (Chapter 2) and the principles of microbial metabolism (Chapter 3). In this chapter we explore the areas of microbial growth and cultivation. We will also learn about environmental factors that influence microbial growth and consider some of the major techniques for the control and prevention of microbial growth.

In the first few sections we consider how microbes are grown in laboratory culture and how microbial growth is measured. Recall that when we use the term *microbial growth* we are referring to an increase in population size as the result of cell division (◀ Section 1.2). Culturing microbes and assessing their growth are major events in the daily routine of many microbiologists and microbiology laboratories.

I • Culturing Microbes and Measuring Their Growth

Microorganisms require a suite of nutrients as foodstuffs to produce new microbial cells. Several methods exist for quantifying cell numbers in either natural environments or laboratory cultures.

4.1 Feeding the Microbe: Cell Nutrition

Because the metabolic capacities of microbes differ, their nutrient requirements also differ. However, all microbes require a core set of nutrients. Some nutrients, called *macronutrients*, are required in large amounts, while others, called *micronutrients*, are required in minute amounts. We begin by dissecting the cell to reveal its chemical composition and then consider the nutrients that all cells require.

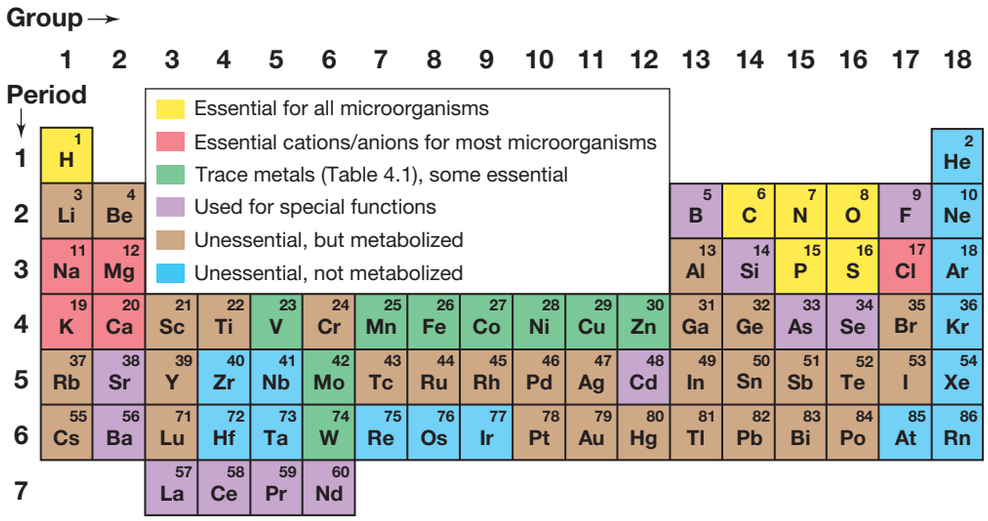
Chemical Makeup of a Cell

A single cell of *Escherichia coli* weighs, on average, just about 10^{-12} g, and over 75% of that mass is water. However, when considering the composition of cells, it is typical to refer to cell dry weight, which for *E. coli* is about 184×10^{-15} g (184 fg) per cell. Just a handful of the chemical elements predominate in living systems. The elements carbon (C), oxygen (O), nitrogen (N), hydrogen (H), phosphorus (P), and sulfur (S) account for about 96% of the dry weight of an average bacterial cell (**Figure 4.1**), and these six elements are universally required by all life forms. The next 3.7% of a cell's mass is composed of potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), chlorine (Cl), and iron (Fe) (Figure 4.1*c*), and these elements are required by most (but not all) microorganisms. In addition, at least another 20 elements can be found in cells of *E. coli* (Figure 4.1*a*) and in cells across the three domains of life. In total, microbes can metabolize 62 different elements in one way or another (Figure 4.1*a*).

Cells are comprised mostly of macromolecules, including proteins, lipids, polysaccharides, lipopolysaccharides, and nucleic acids, which together comprise more than 96% of the dry weight of an *E. coli* cell (Figure 4.1*b*). The vast majority of these are proteins and RNAs (Figure 4.1*b*). Interestingly, as important as DNA is to a cell (the cell genome), it contributes a very small percentage of a cell's dry weight (Figure 4.1*b*).

Carbon, Nitrogen, and Other Macronutrients

Carbon and nitrogen are present in large amounts in all cells. Heterotrophs (organisms that require organic carbon) obtain carbon from the breakdown of organic polymers or from the direct uptake of their monomeric constituents: the amino acids, fatty acids, organic acids, sugars, nitrogen bases, and other organic compounds. Some microbes are autotrophs and can synthesize organic compounds from carbon dioxide (CO_2). The bulk of nitrogen in nature



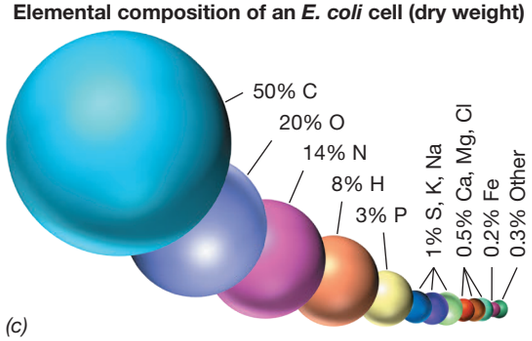
(a)

Macromolecular composition of a cell

Macromolecule	Percent of dry weight
Protein	55
Lipid	9.1
Polysaccharide	5.0
Lipopolysaccharide	3.4
DNA	3.1
RNA	20.5

(b)

Elemental composition of an *E. coli* cell (dry weight)



(c)

Figure 4.1 Elemental and macromolecular composition of a bacterial cell. *(a)* A microbial periodic table of the elements. With the exception of those shown in row 7, other elements in row 7 and elements in rows beyond row 7 are not known to be metabolized. *(b)* Relative abundance of macromolecules in a bacterial cell. Data from Escherichia coli *and* Salmonella typhimurium: *Cellular and Molecular Biology.* ASM, Washington, DC (1996). *(c)* Distribution of the elements in an average *E. coli* cell (dry weight). "Other" includes in order of abundance: Se, B, Cu, Mn, Zn, F, Si, As, Cs, Sr, Ba, V, Co, W, and Ni.

exists in proteins, ammonia (NH_3), nitrate (NO_3^-), or nitrogen gas (N_2). Virtually all microorganisms can use NH_3 as their nitrogen source and many can also use nitrate (NO_3^-). Some microbes can use organic nitrogen sources, such as amino acids, and a few can use N_2 (the nitrogen-fixing bacteria).

In addition to C and N (and O and H from H_2O), many other macronutrients are needed by cells but typically in smaller amounts (Figure 4.1*c*). Phosphorus is required for nucleic acids and phospholipids and is usually assimilated by microbes as inorganic phosphate (PO_4^{3-}). Sulfur is present in the amino acids cysteine and methionine and also in several vitamins, including thiamine, biotin, and lipoic acid. Most microbes can assimilate inorganic forms of S such as sulfate (SO_4^{2-}) or sulfide (H_2S) while others assimilate S from organic sulfur compounds. Potassium (K) is required for the activity of several enzymes, whereas magnesium (Mg) stabilizes ribosomes, membranes, and nucleic acids and is also required for the activity of many enzymes. Calcium (Ca) and sodium (Na) are essential nutrients for only a few organisms. For example, most marine organisms have a strict requirement for NaCl.

Micronutrients: Trace Metals and Growth Factors

Many enzymes require a metal ion or small organic molecule as a cofactor in order to catalyze their specific reaction (◀ Section 3.5). For this reason, the growth of microorganisms requires various metals, and chief among these is iron (Fe). Iron is present in cytochromes and several other enzymes that play major roles in cellular respiration or related oxidation–reduction reactions. Besides iron, many other metals may be required or otherwise metabolized by microorganisms (Figure 4.1*a*). Collectively these metals are called *trace metals* because they are required in such small amounts. **Table 4.1** lists the major trace metals and other trace elements of life and examples of enzymes or other molecules in which each is found. Enzymes with a trace metal requirement may be synthesized by the cell but will not function properly if the cell is starved for these metals.

Growth factors differ from trace metals in that they are *organic* micronutrients rather than metals (Table 4.1). Vitamins, which typically function as coenzymes (◀ Section 3.5), are the most frequently required growth factors, and a few common ones are shown in Table 4.1. While some microbes are able to biosynthesize all of their own growth factors, many organisms (including animals) need to assimilate diverse growth factors from the environments (or from their diet). Besides vitamins, other common growth factors can include amino acids, purines, pyrimidines, and several other organic molecules.

Growth factor requirements vary widely among microorganisms. For example, cyanobacteria are autotrophic microbes that inhabit aquatic environments such as lakes and oceans; they can synthesize all of their own growth factors and need little if any supplementation. By contrast, the lactic acid bacteria *Streptococcus*, *Lactobacillus*, and *Leuconostoc* inhabit environments rich in nutrients, such as certain foods and in the guts of animals (▶ Sections 16.6 and 24.2), and as a result have evolved to rely on their organic-rich environments for these required nutrients.

If a cell is to grow and divide, it must take up macronutrients and micronutrients from the environment and transform them into new cell material. Hence, to grow microbes in the laboratory, we need to satisfy their growth requirements. If the nutrients, trace metals, and growth factors that a microbe needs are not made available to it, the organism will not grow. In the next section we consider how these

TABLE 4.1 Micronutrients needed by microorganisms[a]

I. Trace elements		II. Growth factors	
Element	**Function**	**Growth factor**	**Function**
Boron (B)	Autoinducer for quorum sensing in bacteria; also found in some polyketide antibiotics	PABA (*p*-aminobenzoic acid)	Precursor of folic acid
		Folic acid	One-carbon metabolism; methyl transfers
Cobalt (Co)	Vitamin B_{12}; transcarboxylase (only in propionic acid bacteria)	Biotin	Fatty acid biosynthesis; some CO_2 fixation reactions
Copper (Cu)	In respiration, cytochrome *c* oxidase; in photosynthesis, plastocyanin, some superoxide dismutases	B_{12} (Cobalamin)	One-carbon metabolism; synthesis of deoxyribose
Iron (Fe)[b]	Cytochromes; catalases; peroxidases; iron–sulfur proteins; oxygenases; all nitrogenases	B_1 (Thiamine)	Decarboxylation reactions
		B_6 (Pyridoxine)	Amino acid/keto acid transformations
Manganese (Mn)	Activator of many enzymes; component of certain superoxide dismutases and of the water-splitting enzyme in oxygenic phototrophs (photosystem II)	Nicotinic acid (Niacin)	Precursor of NAD^+
		Riboflavin	Precursor of FMN, FAD
		Pantothenic acid	Precursor of coenzyme A
Molybdenum (Mo)	Certain flavin-containing enzymes; some nitrogenases, nitrate reductases, sulfite oxidases, DMSO–TMAO reductases; some formate dehydrogenases	Lipoic acid	Decarboxylation of pyruvate and α-ketoglutarate
		Vitamin K	Electron transport
Nickel (Ni)	Most hydrogenases; coenzyme F_{430} of methanogens; carbon monoxide dehydrogenase; urease	Coenzymes M and B	Methanogenesis[c]
		F_{420} and F_{430}	Methanogenesis[c]
Selenium (Se)	Formate dehydrogenase; some hydrogenases; the amino acid selenocysteine		
Tungsten (W)	Some formate dehydrogenases; oxotransferases of hyperthermophiles		
Vanadium (V)	Vanadium nitrogenase; bromoperoxidase		
Zinc (Zn)	Carbonic anhydrase; nucleic acid polymerases; many DNA-binding proteins		

[a]Not all trace elements or growth factors are needed by all organisms, and many growth factors are biosynthesized and not required from the environment.
[b]Iron is typically needed in larger amounts than the other trace metals shown.
[c]The production of methane (CH_4) by methanogens (*Archaea*).

important factors are taken into account to support the robust growth of microbes in laboratory culture media.

Check Your Understanding

- Which four chemical elements make up the bulk of a cell's dry weight?
- Which two classes of macromolecules contain most of a cell's nitrogen?
- Differentiate between trace metals and growth factors. How are these used by the cell?

4.2 Growth Media and Laboratory Culture

Laboratory cultures of microorganisms are grown in **culture media** (singular, medium), nutrient solutions tailored to the particular organism to be grown (◀ Section 1.13). Because laboratory culture of a microbe is required for its detailed study, careful attention must be paid to the selection and preparation of media for laboratory culture to be successful. Culture media must be sterilized before use, and sterilization is typically achieved by heating the medium under pressure in an *autoclave*. We discuss the operation and principles of the autoclave in Section 4.17, along with other methods for sterilizing culture media and laboratory utensils and other devices.

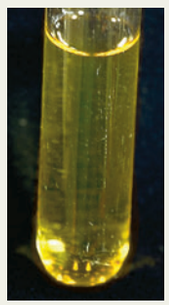
Classes of Culture Media

Although thousands of recipes for microbial culture media exist, two broad classes of culture media are widely used in microbiology: *defined media* and *complex media*. **Defined media** are prepared by adding precise amounts of pure inorganic or organic chemicals to distilled water. Therefore, the *exact composition* of a defined medium (in both a qualitative and quantitative sense) is known. Of major importance in any culture medium is the carbon source because all cells need large amounts of carbon to make new cell material (Figure 4.1). The particular carbon source and its concentration depend on the organism to be cultured. **Table 4.2** lists recipes for four different culture media. Some defined media, such as the one listed for *Escherichia coli*, are considered "simple" because they contain only a single carbon source. In such a medium, *E. coli* biosynthesizes all of its cellular materials from glucose.

For culturing many microorganisms, knowledge of the exact composition of a medium is not essential. In these instances, complex media may suffice and may even be advantageous. **Complex media** are made from digests of microbial, animal, or plant products, such as milk protein (casein), beef (beef extract), soybeans (tryptic soy broth), yeast cells (yeast extract), or any of a number of other highly nutritious substances (Table 4.2). For example, the media originally used by Pasteur in his classic early studies in microbiology were complex media containing yeast extract (◀ Section 1.11). Such digests are commercially available in dehydrated form and need only be hydrated with distilled water to form a culture medium. However, the disadvantage of a complex medium is that its exact nutritional composition is unknown.

Culture media are sometimes prepared that are selective or differential (or both), especially media used in medical microbiology. A *selective medium* contains compounds that inhibit the growth of

TABLE 4.2 Examples of culture media for microorganisms with simple and demanding nutritional requirements[a]

Defined culture medium for *Escherichia coli*	Defined culture medium for *Leuconostoc mesenteroides*	Complex culture medium for either *E. coli* or *L. mesenteroides*	Defined culture medium for *Thiobacillus thioparus*
K_2HPO_4 7 g	K_2HPO_4 0.6 g	Glucose 15 g	KH_2PO_4 0.5 g
KH_2PO_4 2 g	KH_2PO_4 0.6 g	Yeast extract 5 g	NH_4Cl 0.5 g
$(NH_4)_2SO_4$ 1 g	NH_4Cl 3 g	Peptone 5 g	$MgSO_4$ 0.1 g
$MgSO_4$ 0.1 g	$MgSO_4$ 0.1 g	KH_2PO_4 2 g	$CaCl_2$ 0.05 g
$CaCl_2$ 0.02 g	Glucose 25 g	Distilled water 1000 ml	KCl 0.5 g
Glucose 4–10 g	Sodium acetate 25 g	pH 7	$Na_2S_2O_3$ 2 g
Trace elements (Fe, Co, Mn, Zn, Cu, Ni, Mo) 2–10 μg each	Amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) 100–200 mg of each		Trace elements (as in first column) 2–10 μg each
Distilled water 1000 ml	Purines and pyrimidines (adenine, guanine, uracil, xanthine) 10 mg of each		Distilled water 1000 ml
pH 7	Vitamins (biotin, folate, nicotinic acid, pyridoxal, pyridoxamine, pyridoxine, riboflavin, thiamine, pantothenate, *p*-aminobenzoic acid) 0.01–1 mg of each		pH 7
	Trace elements (as in first column) 2–10 μg each		Carbon source: CO_2 from air
	Distilled water 1000 ml		
	pH 7		
(a)		*(b)*	

[a]The photos are tubes of *(a)* the defined medium for *Escherichia coli*, and *(b)* the complex medium described. Note how the complex medium is colored from the various organic extracts and digests that it contains. Photo credits: Cheryl L. Broadie and John Vercillo, Southern Illinois University at Carbondale.

some microorganisms but not others. Selective media are commercially available for the isolation of certain common gut pathogens, such as the enteric bacteria *Salmonella* or those strains of *E. coli* that cause foodborne illnesses. For example, bile salts are often added to culture media for the selective isolation of these bacteria because bile salts kill many bacteria that are unable to grow in the gut. A *differential medium* is one to which an indicator (typically a dye) is added, which reveals by a color change whether a particular metabolic reaction has occurred during growth. For example, the detection of lactic acid bacteria, which secrete lactic acid and cause the pH to drop, can be facilitated by incorporating a pH-sensitive dye into the culture medium that changes color when conditions become acidic. Differential media are useful for distinguishing bacteria and are widely used in clinical diagnostic microbiology and in microbial taxonomy. Differential and selective media are discussed in a clinical microbiology context in Chapter 29.

Nutritional Requirements and Biosynthetic Capacity

Of the four culture medium recipes in Table 4.2, three are defined and one is complex. The complex medium, which is rich in nutrients, is easiest to prepare and it supports growth of both *Escherichia coli* (a common enteric bacterium) and *Leuconostoc mesenteroides* (a lactic acid bacterium often associated with fermented foods), the examples used in Table 4.2. By contrast, only *E. coli* grows in the simple defined medium because *L. mesenteroides* has more nutritional requirements (it requires more growth factors) than *E. coli*. The nutritional needs of *L. mesenteroides* can be satisfied by preparing either a highly supplemented defined medium, a laborious undertaking because of all the individual nutrients that need to be added (Table 4.2), or by preparing a complex medium, a much easier and quicker operation.

The fourth medium listed in Table 4.2 supports growth of the bacterium *Thiobacillus thioparus*, an aerobic sulfur-oxidizing chemolithotroph (◀ Section 3.11). *T. thioparus* derives all of its carbon from CO_2 (that is, it is autotrophic) and conserves energy from the oxidation of sulfur compounds such as thiosulfate ($Na_2S_2O_3$). *T. thioparus* is common in low-nutrient sulfur-rich environments where it only has access to inorganic nutrients. Thus, *T. thioparus* has evolved to biosynthesize all of its needed growth factors and does not require organic nutrients of any type.

The take-home lesson from Table 4.2 is that different microorganisms may have vastly different nutritional requirements. For successful cultivation, it is necessary to understand an organism's physiology and nutritional requirements and then supply it with the nutrients it needs in both the proper form and amount.

Laboratory Culture

Laboratory media can be either liquid (Table 4.2) or solid (**Figure 4.2**). Culture media are solidified with *agar*, an algal polysaccharide first

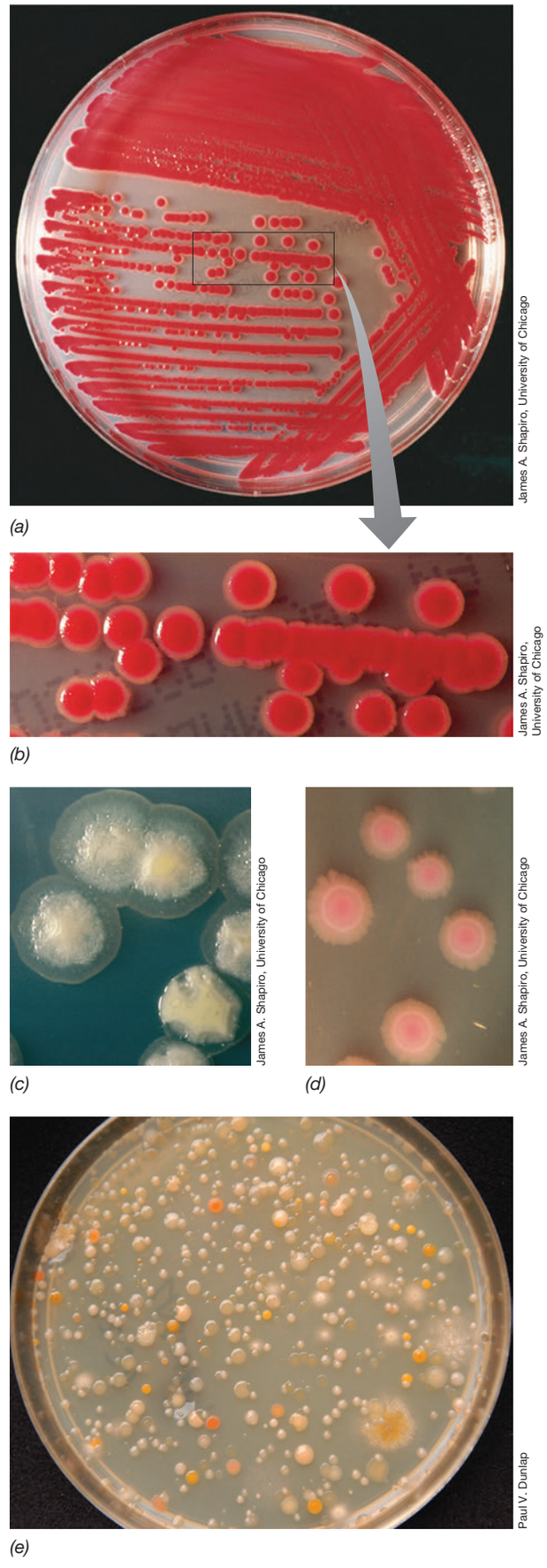
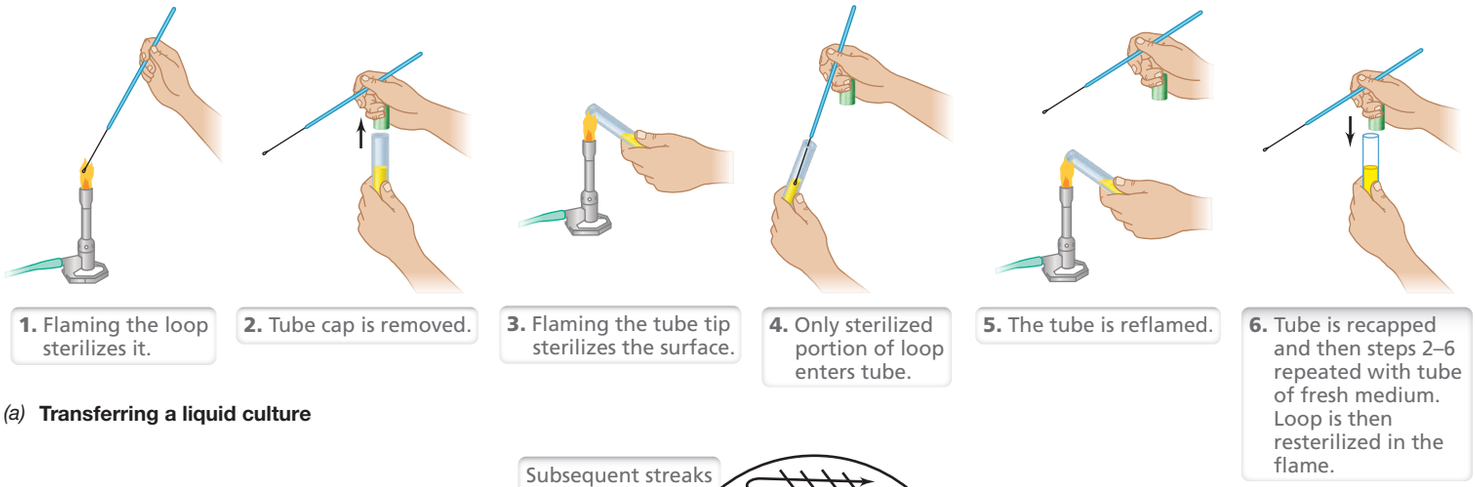


Figure 4.2 Bacterial colonies. Colonies are visible masses of cells formed from the division of one or a few cells and can contain over a billion (10^9) individual cells. *(a) Serratia marcescens*, grown on MacConkey agar. *(b)* Close-up of colonies outlined in part *a*. *(c) Pseudomonas aeruginosa*, grown on trypticase soy agar. *(d) Shigella flexneri*, grown on MacConkey agar. *(e)* An agar plate containing many different bacterial colonies that developed from plating a dilution of seawater.

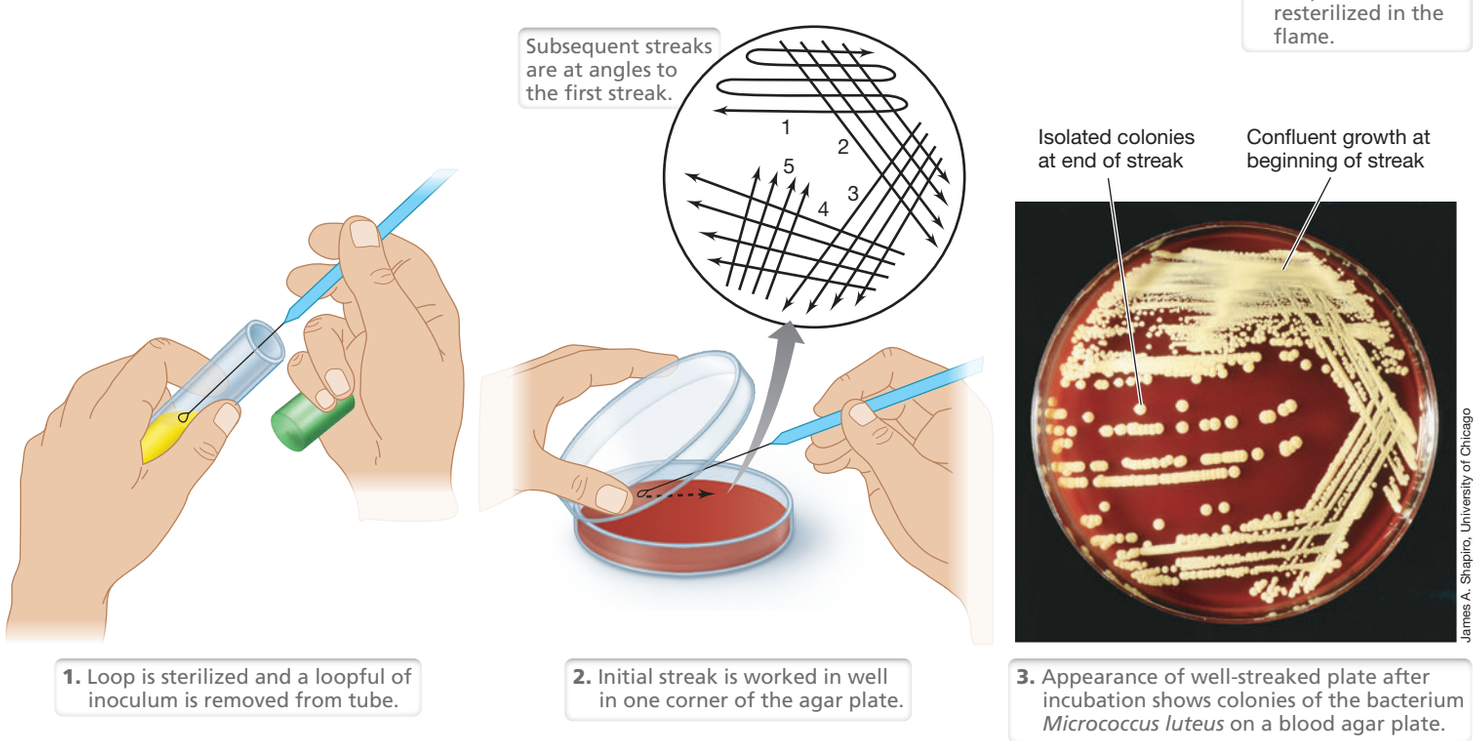
used in the classical studies of Robert Koch (◀ Section 1.12). Solid media immobilize cells so that as they grow, they accumulate in a pile to form visible, isolated cell masses called *colonies* (Figure 4.2). Microbial colonies vary in shape, color, texture, and size depending on the organism, the culture conditions, the nutrient supply, and other physiological parameters. Colony appearance is consistent for a given organism on a particular type of medium. Hence, **colony morphology**—the visible characteristics of a colony—can sometimes be used to identify microorganisms but is used routinely to determine if a culture is pure (only one microbe is present), contaminated (undesired organisms co-occur with a desired organism), or mixed (many microbes are present). Plates inoculated from a mixed culture (such as a natural sample, Figure 4.2*e*) or from a contaminated pure culture will typically contain more than one colony type.

Propagation of microbial cultures requires **aseptic technique** (**Figure 4.3**), a series of steps by which microbes are transferred between growth media without contamination. Contamination can be introduced from microbes in the air, in liquid droplets, or on surfaces. With liquid medium, the goal is to transfer a microbial culture while protecting the culture vessel from air currents or contact with nonsterile surfaces (Figure 4.3*a*). With agar plates, the plan is basically the same but with a greater emphasis on keeping the surface of the agar protected from aerosols or particulate matter in air (Figure 4.3*b*). A mastery of aseptic technique is required for maintaining pure cultures, as airborne contaminants are virtually

Mastering Microbiology
Art Activity: Figure 4.3*b* Streaking a Petri plate



(a) **Transferring a liquid culture**



(b) **Streaking a Petri plate**

Figure 4.3 **Aseptic technique.** *(a)* Liquid media: After the tube is recapped at the end, the loop is resterilized. *(b)* Solid media: making a streak plate to obtain pure cultures. The plate cover is opened just enough to permit streaking manipulations. In streaking a plate, the microbial cells are separated by the streaking process to yield widely separated single cells that then grow and divide to form colonies.

everywhere, even in what may appear to be a very clean microbiology laboratory.

The primary method for obtaining *pure cultures* that contain a single microbe, and of verifying culture purity, is to perform the *streak plate* technique (Figure 4.3*b*). In this method, an *inoculating loop* is used to spread a sample across a solidified medium on an agar plate. Any microbes that are able to grow will form colonies, and colony morphology can be examined to determine the number and type of microbes present.

Many techniques for obtaining pure cultures have been developed, and some of the more common ones will be discussed in Chapter 19. But for now, we turn our attention away from general methods of culturing microbes to explore methods for quantifying their growth.

Check Your Understanding

- In which medium shown in Table 4.2, defined or complex, do you think *Escherichia coli* would grow the fastest?
- What is meant by the word sterile? Why is aseptic technique necessary for successful maintenance of pure cultures in the laboratory?
- How many *E. coli* cells would be present in a single bacterial colony whose dry weight was 0.2 mg?

4.3 Microscopic Counts of Microbial Cell Numbers

Assessing cell numbers gives quantitative information on the state of a laboratory microbial culture or a microbial community in nature. Several methods for enumerating a microbial population have been developed, each having their own strengths and caveats. We begin with the classic "total count" carried out by microscopic examination of a culture or natural sample.

Total Cell Count

Total counts of microbial numbers in a culture or natural sample can be done by simply observing and enumerating the cells present by a *microscopic cell count*. Microscopic counts can be performed either on samples dried on slides or on liquid samples. Dried samples can be stained to increase contrast between cells and their background (◀ Section 1.8) when observed by bright-field microscopy, but a phase-contrast microscope is essential for observing unstained preparations. With liquid samples, counting chambers consisting of a grid with squares of known area etched on the surface of a glass slide are used (**Figure 4.4**). When the coverslip is placed on the chamber, each square on the grid has a precise volume. The number of cells per unit area of grid can be counted under the microscope, giving a measure of the number of cells per small chamber volume. The number of cells per milliliter of suspension is calculated by employing a conversion factor based on the volume of the chamber sample (Figure 4.4).

Microscopic counting is a quick and easy way of estimating microbial cell numbers. However, it has several limitations that restrict its usefulness. For example, without special staining techniques, dead cells cannot be distinguished from live cells, and precision is difficult to achieve, even when replicate counts are made. Moreover, small cells are often difficult to see under the microscope, which can lead to erroneous counts, and cell suspensions of low density (less than about 10^6 cells/milliliter) will have few if any cells in a microscope field unless the sample is first concentrated and resuspended in a small volume. Finally, motile cells must be killed (usually with formaldehyde) or otherwise immobilized before counting, and debris in the sample may easily be mistaken for microbial cells.

Microscopic Cell Counts in Microbial Ecology

Despite the many potential caveats, microbial ecologists often use microscopic cell counts on natural samples. But they do so by using stains to visualize the cells, often stains that yield phylogenetic or other key information about the cells, such as their metabolic properties (▶ Section 19.5).

Many fluorescing stains can be used in a general way. For example, the stain DAPI (◀ Section 1.8 and Figure 1.25*e*) stains all cells because it binds tightly to DNA. Other general fluorescent stains can differentiate live from dead cells by detecting whether a cell's cytoplasmic membrane is intact or not. By contrast, fluorescent stains that are highly specific for certain organisms or groups of related organisms can be prepared by attaching fluorescent dyes to specific nucleic acid probes. For example, *phylogenetic stains* that stain only species of *Bacteria* or only species of *Archaea* can be used in combination with

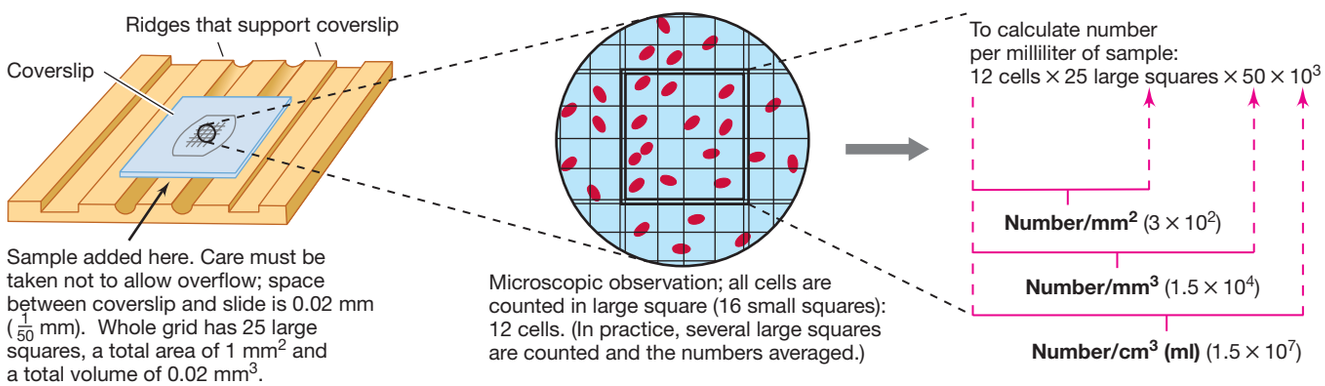


Figure 4.4 Direct microscopic counting procedure using the Petroff–Hausser counting chamber. A phase-contrast microscope is typically used to count the cells to avoid the necessity for staining.

nonspecific stains to determine the proportion of each domain present in a sample (▶ Section 19.5). Other fluorescent probes have been developed that target genes encoding enzymes that catalyze specific metabolic processes; if a cell is stained by one of these probes, a metabolism can be inferred that may reveal the cell's ecological role in the microbial community. In all of these cases, if cells in the sample are present in only low numbers, for example in a sample of ocean water, this limitation can be overcome by first concentrating the cells on a filter and then counting them after staining.

Because they are easy to do and often yield useful baseline information, microscopic cell counts are common in ecological studies of natural microbial environments. We pursue this theme in more detail in Chapter 19. In the next section we consider quantifying microbes using growth-based methods.

Check Your Understanding

- What are some of the problems that can arise when unstained preparations are enumerated in microscopic counts?
- Using microscopic techniques, how might you determine the number of *Archaea* present in an alpine lake?

4.4 Viable Counting of Microbial Cell Numbers

A **viable** cell is one that is alive and able to grow. Some viable microbes are able to grow on laboratory culture media; thus, while microscopic counts often fail to distinguish viable microbes from dead microbes, we can use culture media to perform viable counts. A **viable count** is performed by spreading microbes on solid media and counting colonies, and hence is often called a **plate count** because agar plates are required. The assumption made in a viable count is that each viable cell will grow and divide to yield a single colony, and hence, colony numbers are taken as an measure of cell numbers.

Methods for Viable Counts

There are at least two ways of performing a plate count: the *spread-plate* method and the *pour-plate* method (**Figure 4.5**). In the spread-plate method, a volume (usually 0.1 ml or less) of an appropriately diluted culture is spread over the surface of an agar plate using a sterile glass spreader. In the pour-plate method, a known volume (usually 0.1–1.0 ml) of culture is pipetted into an empty sterile Petri plate. Molten agar medium, tempered to just above gelling temperature (50°C), is then added and gently mixed before allowing the agar to solidify.

With both the spread-plate and pour-plate methods, it is important that the number of colonies developing on or in the medium not be too many or too few. On crowded plates some cells may not form colonies, and some colonies may fuse, leading to erroneous measurements. If the number of colonies is too small, the statistical significance of the calculated count will be low. The usual practice, which is most valid statistically, is to count colonies only on plates that contain between 30 and 300 colonies.

A major assumption of the viable plate count technique is that one cell forms one colony. However, we have already learned that some bacteria are filamentous and many form clusters or sticky biofilms (Chapter 1). For example, a bacterial filament might contain 10 or more cells, but since these cells are firmly attached together, such a filament would form only one colony. Hence, data from viable counts are typically expressed as the number of *colony-forming units* (CFU) obtained rather than the actual number of viable cells, to account for clumps containing more than one viable cell.

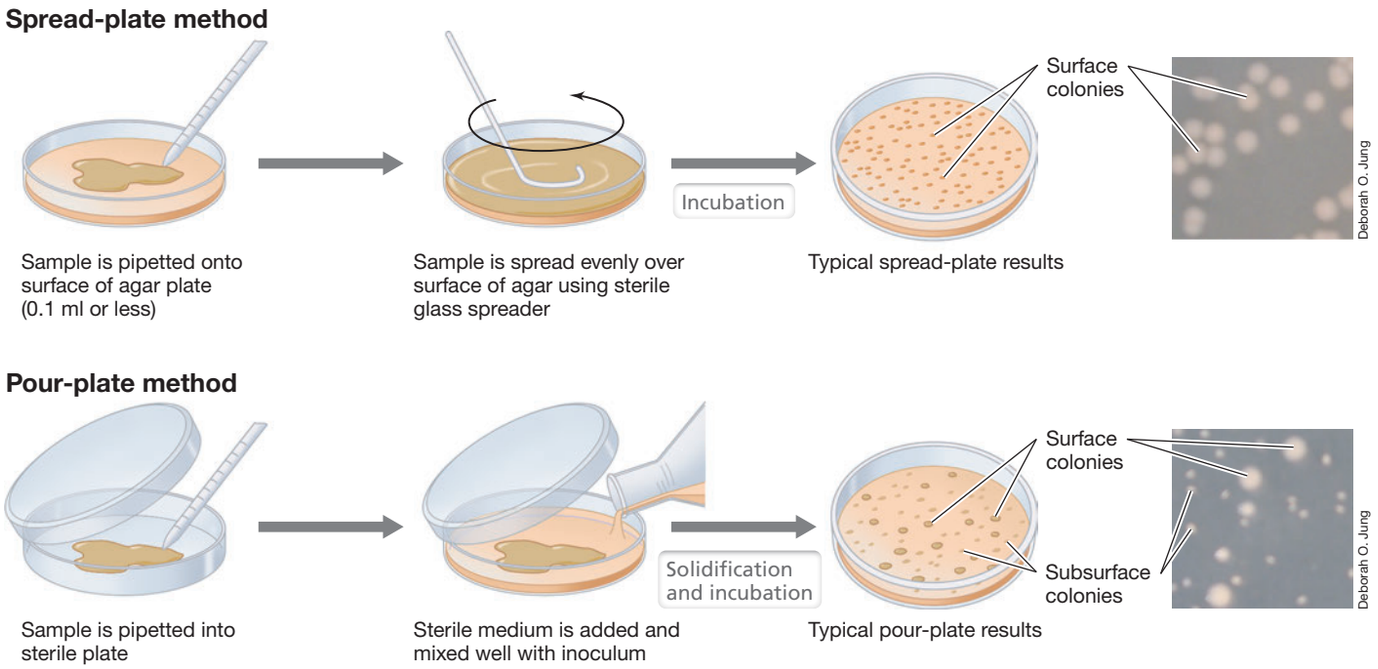


Figure 4.5 Two methods for the viable count. Only surface colonies form in the spread plate technique. By contrast, in the pour-plate method, colonies form within the agar as well as on the agar surface. On the far right are photos of colonies of *Escherichia coli* formed from cells plated by the spread-plate method (top) or the pour-plate method (bottom).

Diluting a Sample

Samples often contain thousands, millions, or even billions of viable bacteria, and so samples must typically be diluted to obtain a suspension that will yield a countable number of colonies. A series of 10-fold dilutions are commonly used prior to performing viable plate counts (**Figure 4.6**). To make a 10-fold dilution, 1 ml of sample can be added to 9 ml of diluent (usually water, a dilute salts solution, or growth medium). In this way, one milliliter of this 10-fold dilution contains 0.1 ml (10^{-1} ml) of the original sample and 0.9 ml of diluent.

With dense cultures, several *serial* dilutions are needed to yield a countable number of colonies (Figure 4.6). For example, if a sample is suspected to contain more than 10^5 cells then the sample can be diluted 1000-fold by performing three successive 10-fold dilutions ($1/10 \times 1/10 \times 1/10 = 1/1000$). In this way, one milliliter of a 1000-fold dilution contains 0.001 ml (10^{-3} ml) of sample and 0.999 ml of diluent. To determine the number of cells in the sample, the number of colonies observed is divided by the amount of sample plated. Hence, if 1 ml of a 1000-fold dilution is plated, this is equivalent to plating 0.001 ml of the original sample. If 159 colonies form from this sample, the original sample must have contained 159×10^3 (which is equivalent to 1.59×10^5) CFU (Figure 4.6). Because the number of bacteria in a sample is often not known ahead of time, it is usually necessary to make multiple dilutions to ensure a countable number of colonies (Figure 4.6).

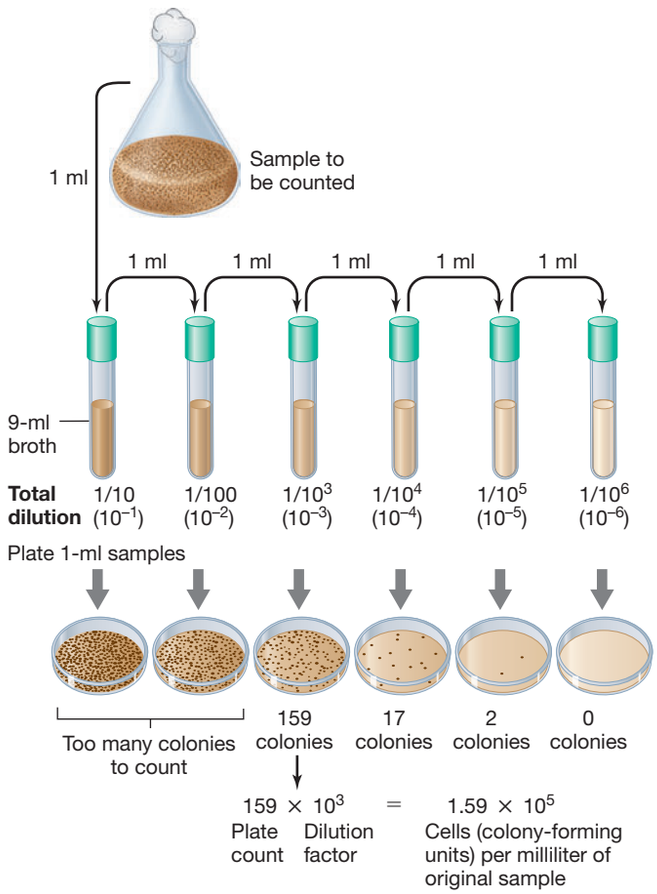


Figure 4.6 Procedure for viable counting using serial dilutions of the sample and the pour-plate method. The sterile liquid used for making dilutions can simply be water, but a solution of mineral salts or actual growth medium may yield a higher recovery. The dilution factor is the reciprocal of the dilution.

Applications of the Plate Count

Despite the caveats associated with viable counting, the procedure is quick and easy to do and so is widely used in microbiology. For example, in food, dairy, medical, and aquatic microbiology, viable counts are employed routinely. The method has the virtue of high sensitivity, because as few as one viable cell per sample plated can be detected. This feature allows for the sensitive detection of microbial contamination of foods or other materials.

The use of highly selective culture media and growth conditions allows the plate count to be used to target particular species in a sample containing many organisms. For example, a complex medium containing 10% NaCl is very useful in isolating species of potentially pathogenic *Staphylococcus* from skin, because the salt inhibits growth of most other skin bacteria. In practical applications such as in the food industry, viable counting performed using both a complex medium and a selective medium on the same sample allows for simultaneous quantitative and qualitative assessments of food quality and safety. That is, with a single food sample, the complex medium yields a total cell count—a relative indicator of freshness and shelf life—while the selective medium indicates the presence or absence of a particular pathogen that may be transmitted in the food.

Targeted counting is also common in wastewater and other water analyses. For instance, enteric bacteria such as *Escherichia coli* originate from feces and are easy to recover from natural samples using selective media; if enteric bacteria are detected in a water sample from a swimming site, for example, their presence is a signal that the water contains fecal matter and is therefore unsafe for human contact.

Caveats to Plate-Counting Methods

Direct microscopic counts of natural samples typically reveal far more microbes than are recoverable on any single culture medium. Thus, although a very sensitive technique, plate counts can be highly unreliable when used to assess total cell numbers of natural samples, such as soil and water. In microbiology, this has been referred to as *the great plate count anomaly*.

Why do plate counts reveal lower numbers of cells than direct microscopic counts? The reason is that different organisms, even those present in a very small natural sample, have vastly different requirements for nutrients and growth conditions in laboratory culture (Sections 4.1 and 4.2 and Table 4.2). Thus, one medium and set of growth conditions can only be expected to support the growth of one subset of the total microbial community. For example, if a sample has 10^9 viable cells but only 10^6 them are capable of growing on a given culture medium, then the viable plate count will miss 99.9% of the viable cell population, a vast underestimation of the actual number of organisms present in the sample.

Plate count results thus carry a large caveat. Plate counts targeted to specific organisms using highly selective media (Section 4.2), as in, for example, the microbial analysis of sewage or food, can often yield quite reliable data, since the physiology of the targeted organisms is known and so the recovery of viable cells is near 100%. By contrast, "total" cell counts of the same samples using a single medium and set of growth conditions may be, and usually are,

underestimates of actual cell numbers by one to several orders of magnitude. Because of this problem, a wide variety of molecular methods have been developed to detect and quantify specific organisms in natural samples without the need for cultivating them in the laboratory. We explore these methods and the important information they can reveal in Chapter 19.

Check Your Understanding

- Why is a viable count more sensitive than a microscopic count? What major assumption is made in relating plate count results to cell number?
- Describe how you would dilute a bacterial culture by 10^{-7}.
- Explain the "great plate count anomaly."

4.5 Turbidimetric Measures of Microbial Cell Numbers

Though small, microbial cells are large enough to scatter light, and the amount of light scattered in a cell suspension is proportional to the number of cells present. A cell suspension looks cloudy (turbid) to the eye because cells scatter light that passes through the suspension. The more cells that are present, the more light is scattered, and hence the more turbid the suspension. Hence, *turbidity* measurements offer a rapid and widely used approach to estimating cell numbers in a solution.

Optical Density and Its Relationship to Cell Numbers

Turbidity is measured with a *spectrophotometer*, an instrument that measures the unscattered light that passes through a sample (**Figure 4.7**). A spectrophotometer employs a prism or diffraction grating to generate incident light of a specific wavelength (Figure 4.7*a*). Commonly used wavelengths for microbial turbidity measurements include 480 nm (blue), 540 nm (green), and 660 nm (red). Sensitivity is best at shorter wavelengths, but measurements of dense cell suspensions are more accurate at longer wavelengths.

The unit of turbidity is *optical density* (*OD*) at the wavelength specified, for example, OD_{540} for measurements at 540 nm (Figure 4.7). For unicellular organisms, optical density is proportional, within certain limits, to cell number. Turbidity readings can therefore be used as a substitute for total or viable counting methods. However, before this can be done, a standard curve must be prepared that

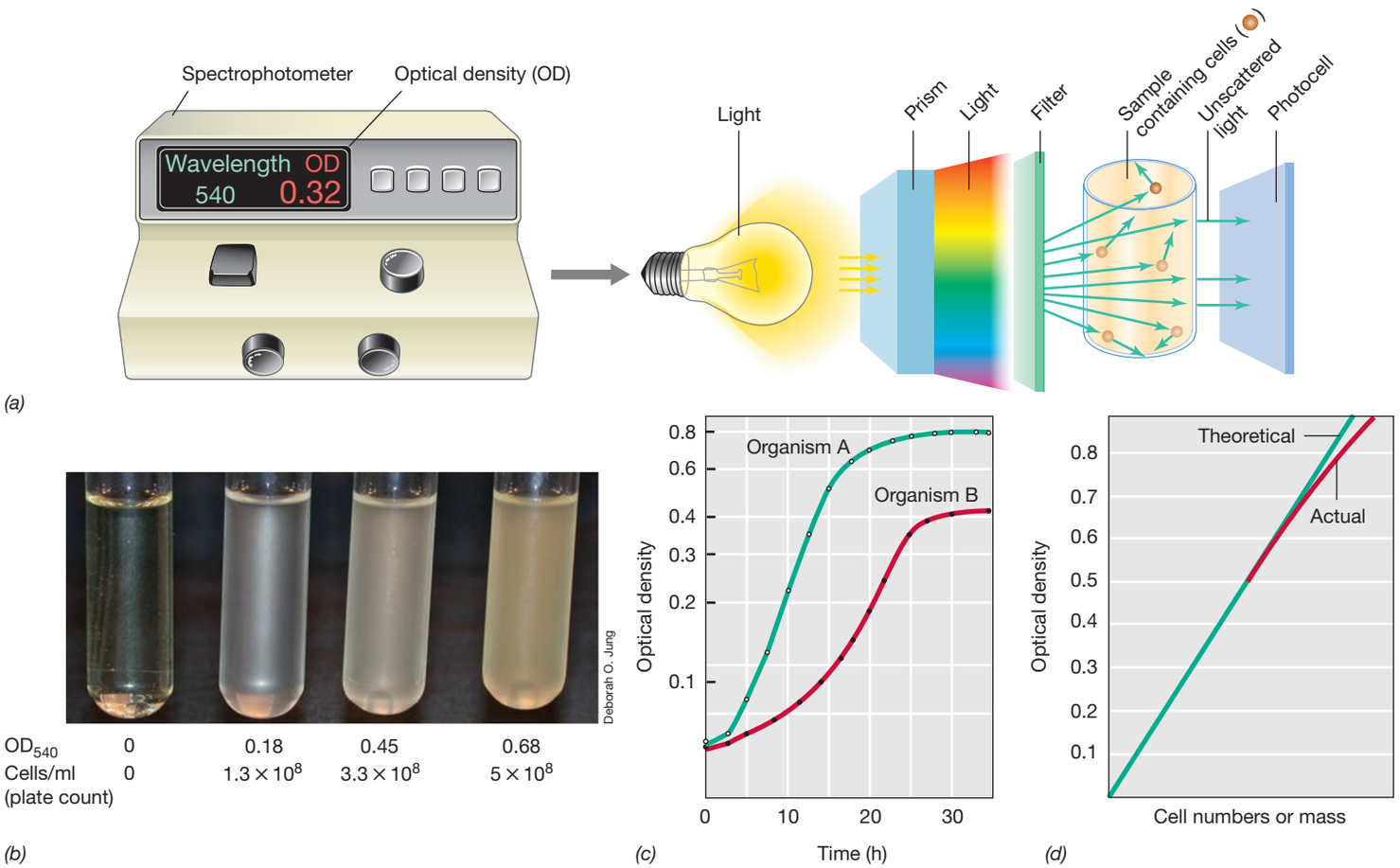


Figure 4.7 Turbidity measurements of microbial growth. *(a)* Measurements of turbidity are made in a spectrophotometer. The photocell measures incident light unscattered by cells in suspension and gives readings in optical density units. *(b)* Liquid cultures of *Escherichia coli*; the tube on the far left is sterile medium. The increasing (left to right) optical density (OD_{540}) of each culture is shown below the tube, as is the actual cell number measured in a viable count. *(c)* Typical growth curve data for two organisms growing at different growth rates. For practice, calculate the generation time of the two cultures using the formula $n = 3.3 (\log N_t - \log N_0)$ where N_t and N_0 are two different OD readings with a time interval t between the two (see Section 4.7). Which organism is growing faster, A or B? *(d)* Relationship between cell number or dry weight and turbidity readings. Note that the direct relationship between these values breaks down at high turbidities.

relates cell number (microscopic or viable count) to turbidity. As can be seen in such a plot, proportionality only holds within limits (Figure 4.7*d*). At high cell densities, light scattered away from the spectrophotometer's photocell by one cell can be scattered back toward the photocell by another, and as a result, the one-to-one correspondence between cell number and turbidity deviates from linearity. Nevertheless, when a standard has been prepared, turbidimetric estimates of bacterial numbers are very useful.

Other Issues with Turbidimetric Growth Estimates

On the one hand, turbidity measurements are quick and easy to perform and can be made without destroying or significantly disturbing the sample. For these reasons, turbidity measurements are widely employed to monitor growth of pure cultures of *Bacteria* and *Archaea* and can also be used with many microbial eukaryotes. With turbidimetric assays, the same sample can be checked repeatedly over time and the measurements plotted on a semilogarithmic plot versus time (Figure 4.7*c*) to measure growth of a microorganism over time.

On the other hand, turbidity measurements can occasionally be problematic. Although many microorganisms grow evenly distributed in suspensions in liquid medium, many do not. Some bacteria routinely form small to large clumps, and in such instances, OD measurements may be quite inaccurate as a measure of total microbial mass. In addition, many bacteria form biofilms on the sides of tubes or other growth vessels. Hence for OD measurements to be an accurate reflection of cell mass (and thus cell numbers) in a liquid culture, clumping and biofilms have to be minimized. This can be accomplished by stirring, shaking, or in some way keeping the cells well mixed during the growth process to prevent the formation of cell aggregates and the sticking of swimming cells to surfaces. Some bacteria are just naturally planktonic and stay well suspended in liquid medium for long periods. But if a solid surface is available, most bacteria will eventually develop a static biofilm, and accurately quantifying cell numbers by turbidity in such a case can be difficult or even impossible.

Check Your Understanding

- List two advantages of using turbidity as a measure of cell growth.
- Describe how you could use a turbidity measurement to tell how many colonies you would expect from plating a culture of a given OD.

II • Dynamics of Microbial Growth

Microorganisms that divide by binary fission grow at exponential rates into cell populations whose numbers over time are predictable from simple mathematical models. A few microbes grow by budding, and some can form sticky cell masses called biofilms.

In Part II of this chapter we consider the actual process of microbial growth. Here we consider the different ways in which microbes grow, explore the basic quantitative relationships displayed by growing microbial cultures, and introduce an important laboratory tool—the chemostat—for manipulating exponentially growing microbial cultures to better answer specific questions.

4.6 Binary Fission and the Microbial Growth Cycle

Growth is the result of cell division and is the ultimate process in the life of a microbial cell. In microbiology, growth is defined as *an increase in the number of cells*. As macromolecules accumulate in the cytoplasm of a cell, they assemble into major cell structures, such as the cell envelope, cytoplasmic membrane, ribosomes, and so on, eventually leading to cell division. In a growing culture of a rod-shaped bacterium such as *Escherichia coli*, cells elongate to approximately twice their original length and then form a partition that constricts the cell into two identical daughter cells (**Figure 4.8**). This process is called **binary fission** ("binary" to indicate that two cells have arisen from one).

The partition that forms between dividing cells is called a *septum* and results from the inward growth of the cell envelope from opposing directions; septum formation continues until the two daughter cells are pinched off. There are some variations in this general pattern of binary fission. In some bacteria, such as *Bacillus subtilis*, a septum forms without cell wall constriction (**Figure 4.9**), while in the budding bacterium *Caulobacter* (◀ Figure 2.14*a*, and see Figure 4.18) constriction occurs but no septum is formed.

When one cell eventually separates to form two cells (Figure 4.8), we say that *one generation* has occurred, and the time required for this process is called the **generation time** (or *doubling time*). Each daughter cell is essentially identical, having received a copy of the chromosome(s) and sufficient copies of all cellular materials required to begin life as an independent entity. Microbes differ in their doubling times, and the doubling time of any given microbe varies depending on growth conditions. Under optimal growth conditions, the generation time of *E. coli* is about 20 min. The fastest-growing

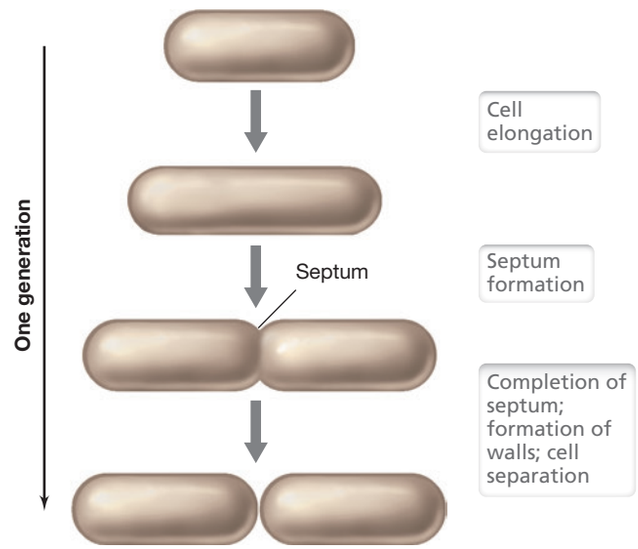


Figure 4.8 Binary fission in a rod-shaped bacterium. Cell numbers (and all components of the cells) double every generation.

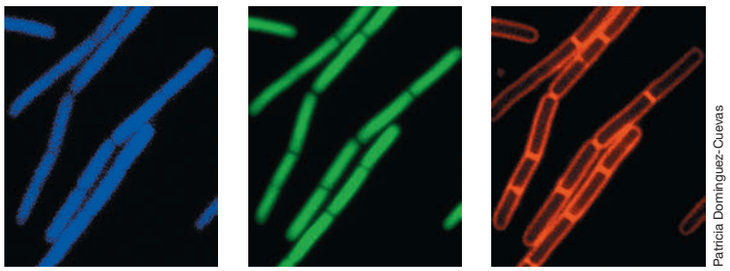


Figure 4.9 **Septa.** The septum that separates dividing cells of the bacterium *Bacillus subtilis* is clearly visible in this series of fluorescent micrographs. *(a)* DAPI stains the entire cell. *(b)* The green fluorescent protein lights up entire cells. *(c)* A dye that stains only the cytoplasmic membrane shows that septa contain membrane (and cell wall) material.

microbes known can double in less than 10 minutes while the slowest can have generation times of several months or even longer.

The Microbial Growth Cycle

Under optimal conditions, growth by binary fission can cause a rapid increase in the number of cells present, but conditions are rarely optimal, even in laboratory culture. Typically, microbes are grown in **batch culture**, which describes the growth of microbes in a fixed volume of liquid enclosed within a container such as a test tube or a flask. The nutrients in such a culture flask are finite and cannot support growth indefinitely. Hence, in batch culture microbes typically exhibit a growth cycle called the microbial **growth curve** (**Figure 4.10**). The growth curve is composed of four phases: lag phase, exponential phase, stationary phase, and decline phase.

Lag and Exponential Phases

When a microbial culture is inoculated into fresh growth media (Section 4.2), there is typically an initial pause during which the cells do not grow. This period between inoculation and the onset of growth is called the **lag phase** (Figure 4.10). Microbes exhibit a lag phase because transfer to fresh medium represents new environmental conditions for the cells, which need to alter their metabolic state to respond to these new conditions.

The lag phase may be brief or extended depending on the previous history of the cells (old culture, young culture, type of growth medium, and so on), the choice of growth medium, and the growth conditions. For example, if an actively growing culture is transferred into a new flask containing the same type of medium under the same growth conditions, there will be very little lag. This is because the cell needs to make very few metabolic adjustments to this new condition. However, if the inoculum is from an old culture, there is usually a longer lag because cell viability may be low, or the cells may need to synthesize many enzymes and macromolecules before they can begin growing. A long lag is also observed when a microbial culture is transferred from a nutrient-rich to a nutrient-poor culture medium. In order to grow, cells must have a complete set of enzymes to biosynthesize the essential metabolites absent from the nutrient-poor medium. The more enzymes and molecules that a cell must manufacture in order to grow in a new environment, the longer the lag.

The **exponential phase** of growth is the period when the growing cell population doubles at regular intervals (Figure 4.10). This phase is also called *balanced growth* because cells are as close to being metabolically identical throughout this period as they can be. Hence, in order to minimize experimental variation, most experiments are performed with microbes taken from cultures in the exponential phase of growth. Rates of exponential growth vary greatly and are influenced by choice of media, growth conditions, and the organism itself. Exponential growth continues until conditions in the batch culture can no longer sustain growth.

Stationary and Death Phases

In a batch culture, exponential growth cannot continue forever. Consider the fact that a single cell weighing one-trillionth (10^{-12}) of a gram that doubled unabated every 20 min for 48 hours would produce a population of cells that weighed 4000 times more than planet Earth. Obviously, this is impossible because the carrying capacity of an environment will always limit growth of the organisms within it. In the case of batch cultures, growth is limited because of either nutrient depletion or the accumulation of microbial waste products. When exponential growth ceases for one (or both) of these reasons, the population enters *stationary phase* (Figure 4.10).

In **stationary phase**, there is no net increase or decrease in cell number and thus the growth rate of the population is zero. During stationary phase, cellular metabolism shifts away from growth as the cell prepares for maintenance and survival. Despite

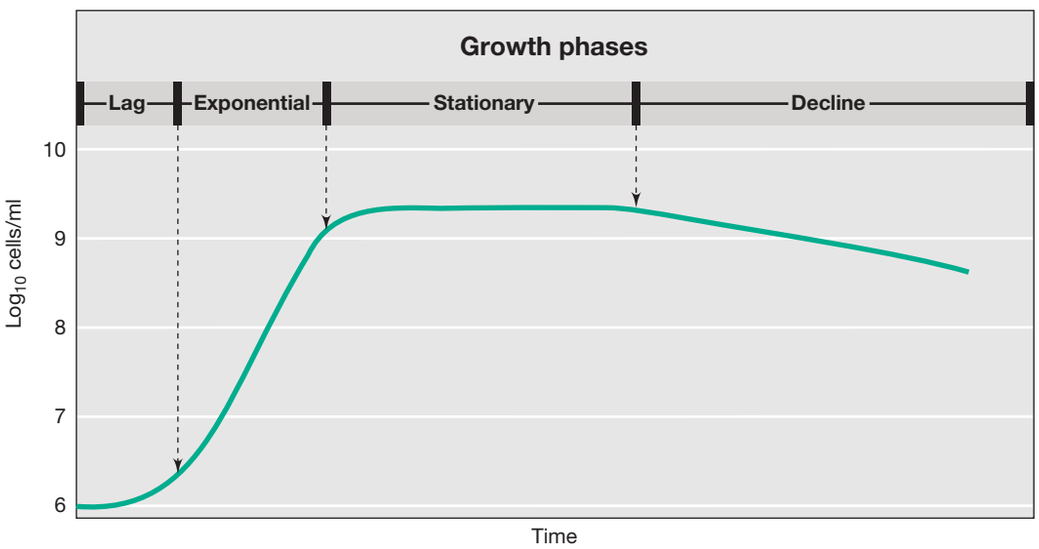


Figure 4.10 **Typical growth curve for a bacterial population.** A viable count measures the cells in the culture that are capable of reproducing. Optical density (turbidity), a quantitative measure of light scattering by a liquid culture (see Figure 4.7), increases with the increase in cell number.

growth arrest, energy metabolism and biosynthetic processes in stationary phase cells may continue, but typically at a greatly reduced rate. Eventually, the population will enter the **decline phase** of the growth cycle as the total number of cells decreases due to cell death. Cell division may still occur for some cells in the population during the stationary and decline phases, but this meager increase in number is balanced by the death of other cells. Some microbial cultures exhibit *cryptic growth* during stationary and decline phases as subpopulations of cells adapt to cannibalize and reuse resources released from dying cells. Also, if a culture is prevented from drying, some number of cells may remain for months or even years.

We now consider how exponential growth can be put on a quantitative footing using some basic mathematical relationships and how these relationships can be exploited in laboratory studies of growing microbial cultures.

Check Your Understanding

- Under what conditions would a lag phase not occur?
- Why do cells enter stationary phase?
- Define the term generation. What is meant by the term generation time?

4.7 Quantitative Aspects of Microbial Growth

During cell division, one cell becomes two. During that doubling time (the generation time), both the total cell *number* and cell *mass* double (Figure 4.8). As we will see, cell numbers in a bacterial culture can quickly become very large, and so it is necessary to deal with the topic of microbial growth using quantitative methods.

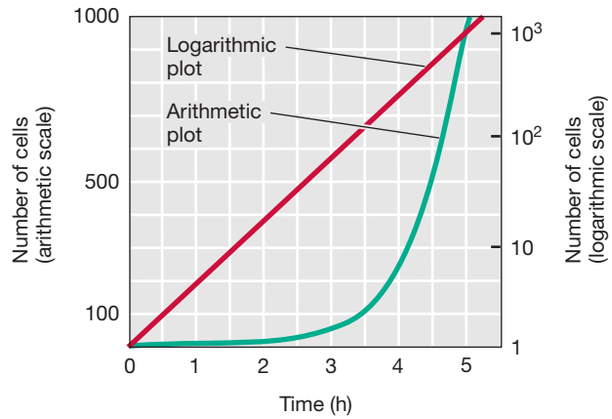
Plotting Growth Data

Exponential growth of a population occurs during a period when cell number doubles at regular intervals (defined as the doubling or generation time, Section 4.6). For example, consider a single cell that initiates exponential growth with a generation time of 30 minutes (**Figure 4.11**). When we plot the change in cell number over time on arithmetic (linear) coordinates, we obtain an upwardly sweeping curve with a continuously increasing slope (Figure 4.11*b*). By contrast, when the cell number is plotted on a logarithmic ($\log_{10}$) scale as a function of time (a *semilogarithmic* graph) the points fall on a straight line (Figure 4.11*b*). This straight-line function reflects the fact that the cells are growing exponentially and that the population is doubling in a constant time interval.

Semilogarithmic graphs are also convenient for estimating the generation time of a culture from actual growth data, since generation times can be inferred directly from the graph as shown in **Figure 4.12**. For example, the generation time can be determined by finding the time it takes for the number of cells to double (Figure 4.12). In a more formal sense, we define generation time (g) as the time (t) per generation (n), which is $g = t/n$. Hence, a population that takes six hours ($t = 6$ h) to double once ($n = 1$) has a generation time of 6 h (Figure 4.12*a*). Several of the terms and expressions useful in quantifying microbial growth are defined in Figure 4.12*c*.

Time (h)	Generation number (*n*)	Total number of cells
0	0	1
0.5	1	2
1	2	4
1.5	3	8
2	4	16
2.5	5	32
3	6	64
3.5	7	128
4	8	256
4.5	9	512
5	10	1024

(a)



(b)

Figure 4.11 The rate of growth of a microbial culture. *(a)* Data for a cell population that doubles every 30 min. *(b)* Data plotted on arithmetic (left ordinate) and logarithmic (right ordinate) scales.

The Mathematics of Bacterial Growth

Starting with a single cell and considering the fact that the cell number of an exponentially growing culture doubles every generation, the number of cells present at generation n can be expressed as 2^n. For example, after 3 generations there will be $2^3 = 8$ cells, after 10 generations there will be $2^{10} = 1024$ cells, and so on (Figure 4.11*a*). If exponential growth began with two cells instead of one, the number of cells at any generation would be doubled (for example, $2 \times 2^{10} = 2048$ cells), and if we started with three cells, the number of cells at any generation would be tripled ($3 \times 2^{10} = 3072$ cells). Hence, it is possible to calculate the number of cells (N) in an exponentially growing culture at any point in time from the following expression:

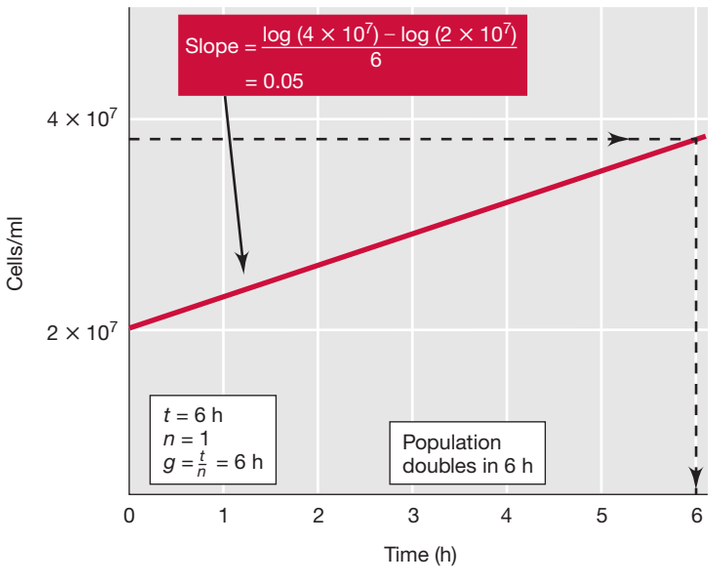
$$N_t = N_0 2^n \quad (1)$$

where N_t is cell number at time t, N_0 is the initial cell number (the number at time 0), and n is the number of generations during the period of exponential growth.

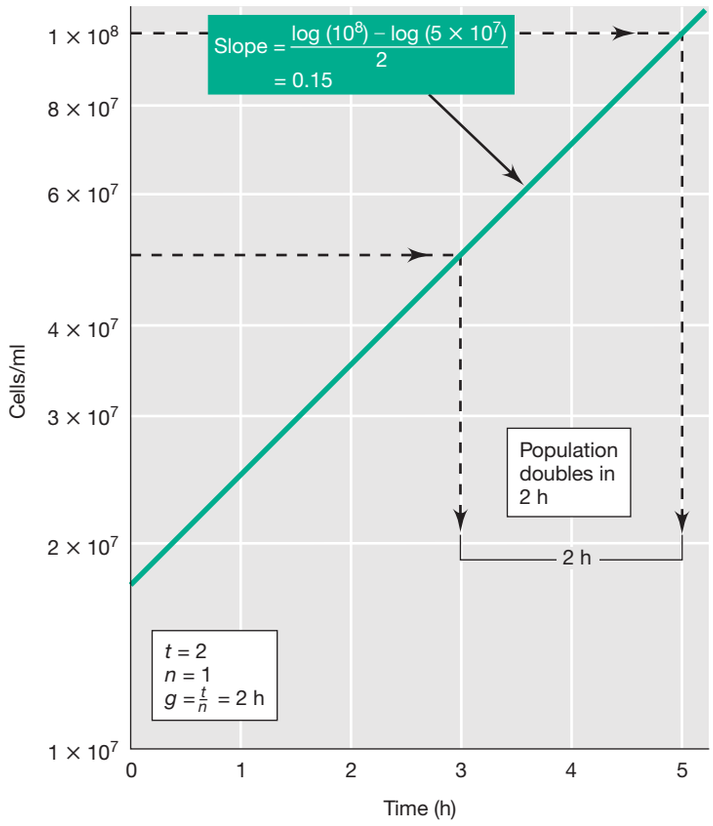
The equation $N_t = N_0 2^n$ can be solved for n by taking the logarithms of both sides and using algebra to yield

$$n = [(\log N_t - \log N_0)/\log 2] \quad (2)$$

This equation makes it possible to calculate generation times in terms of measurable quantities, N_t and N_0. Values for N_t and N_0 can



(a)



(b)

Exponential Growth Equations

$g = t/n$
$N_t = N_0 2^n$
$n = (\log N_t - \log N_0)/0.301$
$N_t = N_0 e^{kt}$
$k = 0.693/g$

g: *generation time*
t: *time*
$\boldsymbol{N_t}$: cell number at time *t*
$\boldsymbol{N_0}$: starting cell number
n: number of generations
k: specific growth rate

(c)

Figure 4.12 **Calculating microbial growth parameters.** Method of estimating the generation times (*g*) of exponentially growing populations with *g* of *(a)* 6 h and *(b)* 2 h from data plotted on semilogarithmic graphs. The slope of each line is equal to 0.301/*g*, and *n* is the number of generations in the time *t*. All numbers are expressed in scientific notation; that is, 10,000,000 is 1×10^7, 60,000,000 is 6×10^7, and so on. *(c)* Useful expressions for analyzing microbial growth.

be determined from the viable or microscopic counting methods described earlier; alternatively, OD readings from a spectrophotometer can be used for these calculations (Sections 4.3–4.5 describe methods for quantifying cell numbers). As an example, consider actual growth data from the plot in Figure 4.12*b*, in which $N_t = 10^8$, $N_0 = 5 \times 10^7$, and $t = 2$ h:

$$n = [(\log N_t - \log N_0)/\log 2]$$
$$n = [\log(10^8) - \log(5 \times 10^7)]/\log 2$$
$$n = (8 - 7.69)/\log 2 = (0.301)/0.301 = 1$$

Thus, in this example $n = 1$ and so we can use the equation $g = t/n$ to determine that $g = 2$ h. If exponential growth continued for another 2 h, the cell number would be 2×10^8. Two hours later, the cell number would be 4×10^8, and so on.

The value of *g* can also be calculated by evaluating the slope of the line on a semilogarithmic plot of exponential growth. On such a plot (Figure 4.12*b*) the *slope* is defined as $[(\log N_t - \log N_0)/t]$. Combining this with expression (2) and the understanding that $g = t/n$, the slope of the curve reduces to $[\log 2/g]$, or simply, $0.301/g$. In the example shown in Figure 4.12*b*, the slope is $0.15\ h^{-1}$, and since $g = 0.301/\text{slope}$, the generation time is $[0.301/0.15\ h^{-1}] = 2$ h.

Specific Growth Rate

Other expressions are often useful in describing exponential growth, and chief among these is the *specific growth rate* (*k*). The specific growth rate expresses the rate at which the population is growing *at any instant* (by contrast, *g* is the mean time required for the cell population to double); *k* is expressed in units of reciprocal hours (h^{-1}).

The specific growth rate is a function of the change in cell number (dN/dt) over time and is expressed as $dN/dt = kN$, where the specific growth rate (*k*) indicates the rate at which the cell number (*N*) is increasing at any point in time. Integration of this equation using natural logarithms ($\log_e$), gives the expression $N_t = N_0 e^{kt}$. To estimate *k*, the $\log_{10}$ of both sides of this equation is taken to obtain the expression $\log N_t = kt/2.303 + \log N_0$ ($\log_{10}$ is used such that *N* can be plotted against *t* in a semilog plot, Figure 4.12). From this equation, $[(\log N_t - \log N_0)/t]$ reduces to $(k/2.303)$; that is, the slope of the line on a semilogarithmic plot of growth can be calculated as $k/2.303$. Also, since $0.301/g = k/2.303$, *k* can be expressed directly as $0.693/g$.

Armed with knowledge of *n* and *t*, one can calculate *g* and *k* for different microorganisms growing under various conditions (Figure 4.12). This is often useful for optimizing culture conditions for

a newly isolated organism and for testing the positive or negative effect of some treatment on a bacterial culture. For example, comparison with an unamended control allows factors that stimulate or inhibit growth to be identified by measuring their effect on the various growth parameters presented here.

Consequences of Exponential Growth

During exponential growth, the increase in cell number is initially rather slow but increases at an ever-faster rate. In the later stages of exponential growth, this results in an explosive increase in cell numbers. For example, in the experiment shown in Figure 4.11, the rate of cell production in the first 30 min of growth is 1 cell per 30 min. However, between 4 and 4.5 h of growth, the rate of cell production is 256 cells per 30 min, and between 5.5 and 6 h of growth it is 2048 cells per 30 min. Because of this, cell numbers in laboratory cultures of bacteria can quickly become very large, and population sizes of $>10^9$ cells/ml are common.

Besides being a theoretical consideration, exponential growth can have implications in everyday life. Consider something we have all experienced, the spoilage of milk. The lactic acid bacteria responsible for the soured flavor of spoiled milk contaminate the milk during its collection and exist in fresh, pasteurized milk in low numbers; these organisms grow slowly at refrigerator temperature (4°C) but much faster at room temperature. If a bottle of fresh milk stands at room temperature overnight, some lactic acid is made, but not enough to affect milk quality. However, if week-old milk, which now contains a week's worth of slow bacterial growth (and thus much higher cell numbers), is left standing under the same conditions, a huge amount of lactic acid is made, and spoilage results.

Check Your Understanding

- What is a *semilogarithmic* plot and what information can we derive from it?
- For an exponentially growing culture that increases from 5×10^6 cells/ml to 5×10^8 cells/ml in 8 h, calculate g, n, and k.
- For testing a bacterium's response to an inhibitory substance, why would g be useful information?

4.8 Continuous Culture

Up to this point our consideration of microbial population growth has been confined to *batch cultures*. The environment in a batch culture is constantly changing because of nutrient consumption and waste production. These limitations can be circumvented in a *continuous culture device*. The most common type of continuous culture device is the **chemostat** (Figure 4.13). Unlike a batch culture, which is a *closed* system, a continuous culture is an *open* system. In a chemostat, a known volume of sterile medium is added at a constant rate while an equal volume of spent culture medium (which also contains cells) is removed at the same rate (Figure 4.13). Once in equilibrium, the culture volume, cell number, and nutrient/waste product status remain constant, and the culture attains *steady state*.

The Chemostat and the Concept of Steady State

A chemostat enables control over both the specific growth rate (k, Figure 4.14) and growth yield (biomass per ml, Figure 4.15) of a microbial culture. In a chemostat, fresh sterile medium is added to a culture vessel and spent media washed out at equal rates, resulting in a culture that maintains a fixed volume (Figures 4.13 and 4.14). The supply of medium is defined by the dilution rate (D) which is expressed as F/V, where F is the flow rate (volume per unit time), and V is the culture volume. In the chemostat, the specific growth rate is controlled by the *dilution rate* (D), and the growth yield is controlled by the *concentration of a limiting nutrient* in the fresh medium added to the vessel.

In a chemostat at steady state, cells grow at the same rate that they are removed by outflow from the system; that is, $k = D$ (Figure 4.14). At steady state, a large number of cells are all competing for a limiting nutrient. The nutrient added in the fresh medium is consumed rapidly by the cells within the chemostat, thereby limiting their growth rate. As the flow of nutrients increases, the cells can grow faster and they continue to keep pace with the dilution rate until it is so fast that it exceeds the maximal growth rate of the organism. At this point, the cells can no longer keep up and they are washed out of the culture vessel (Figure 4.14).

While the specific growth rate is controlled by D, the growth yield is controlled

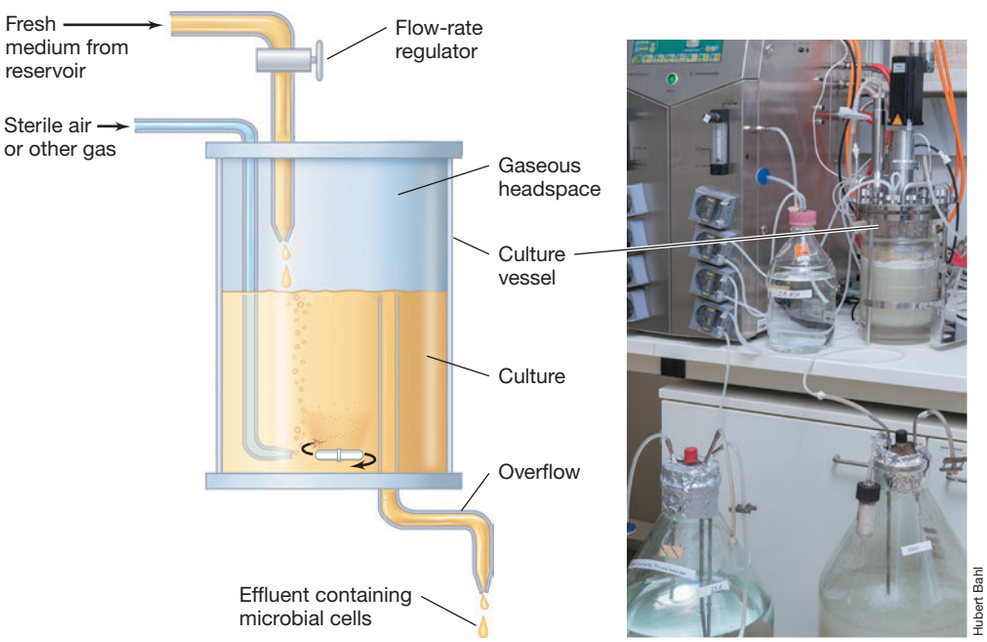


Figure 4.13 Continuous culture device (chemostat). The population density is controlled by the concentration of a limiting nutrient in the reservoir, and the growth rate is controlled by the dilution rate; both parameters are set by the experimenter. *(a)* Chemostat components. *(b)* Photo of a chemostat setup.

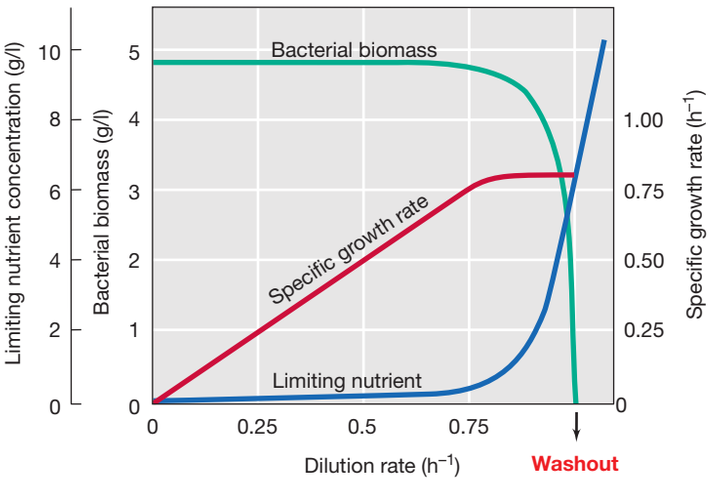


Figure 4.14 Steady-state relationships in the chemostat. The dilution rate is determined from the flow rate and the volume of the culture vessel. Note that at a high dilution rate, growth cannot balance dilution, and the population washes out. Note also that although the population density (bacterial biomass) remains constant and the concentration of the growth-limiting nutrient remains near zero during steady state, the specific growth rate can vary over a wide range.

by the concentration of the limiting nutrient in the medium (Figure 4.15). As shown in Figure 4.14, a change in *D* does not change growth yield in terms of biomass present until washout occurs. However, more nutrients in the medium mean that more cells can be made (Figure 4.15) but has no impact on the growth rate (except at very low nutrient concentrations when cells are starving). Thus, by varying *D* or the concentration of the growth-limiting nutrient, one can establish cultures that are growing exponentially at a defined growth rate and a defined cell density.

Experimental Uses of the Chemostat

A practical advantage of the chemostat is that a cell population can be maintained in the exponential growth phase for long periods—weeks or even months. Exponential phase cells are usually most desirable for physiological experiments. Such cells are available at any time in the chemostat, and the vessel can be sampled repeatedly. Chemostat cultures have been used extensively to study bacterial physiology and have also been used in studies of microbial ecology and evolution. For example, because the chemostat can mimic the low substrate concentrations often found in nature, it is possible to ask which organisms in mixed cultures of known composition compete best at various specific growth rates or when particular nutrients are limiting. Chemostats have also been used for the direct enrichment and isolation of bacteria from nature. From a natural sample, one can select a stable population under the chosen conditions of nutrient concentration and *D* and then slowly increase *D* until a single organism remains. In this way, microbiologists studying the growth rates of various soil bacteria isolated a bacterium with a 6-min doubling time—the fastest-growing bacterium known!

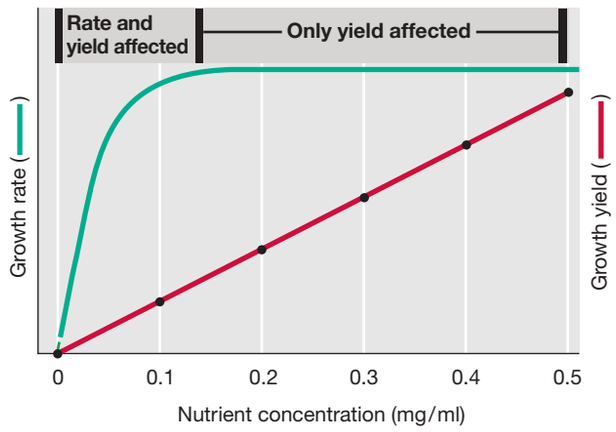


Figure 4.15 The effect of nutrients on growth in a chemostat. Relationship between nutrient concentration, growth rate, and growth yield (bacterial biomass) in a chemostat culture. Only at low nutrient concentrations are both growth rate and growth yield affected.

Check Your Understanding

- How do microbial populations in a chemostat differ from microorganisms in a batch culture?
- What happens in a chemostat if the dilution rate exceeds the maximal specific growth rate of the organism?
- Do pure cultures have to be used in a chemostat?

4.9 Biofilm Growth

Thus far we have considered microbial growth in liquid suspensions only. Such growth of free-floating or free-swimming cells is called *planktonic growth*. Planktonic growth is a natural state for many microbes, and most laboratory cultures are maintained in this state. However, *sessile growth*, meaning growth while attached to a surface, is quite common in the microbial world. Microbes that are attached to surfaces often develop into *biofilms*. Biofilm growth has tremendous importance in medical and industrial applications, and it allows microbes to exhibit different properties from those of planktonic cells.

Biofilm Formation

A **biofilm** is a population of cells enmeshed in a polysaccharide matrix that is attached to a surface (**Figure 4.16**). Biofilms form in stages, beginning with the *attachment* of planktonic cells to a surface. Attachment is often mediated by flagella, fimbriae, or pili (Chapter 2). *Colonization* of the surface starts when microbes begin to grow and produce sticky extracellular polysaccharides (EPS). Microbial colonization and growth on the surface causes changes in the biofilm that lead to development. During *development*, cells in the biofilm begin to change their metabolism. These changes can cause biofilms to develop complex systems of mushroomlike columns and channels that trigger metabolic differentiation of microbes at the surface of the biofilm from those at its base. Finally, *dispersal* of cells from a mature biofilm allows microbes to colonize new sites (Figure 4.16). Dispersal is usually prompted by changes in the environment, such as nutrient limitation or other forms of stress.

Biofilms can be studied in a *flow chamber*, in which liquid media flows continuously between two layers of glass. Flow chambers are designed so that biofilm growth can be monitored microscopically

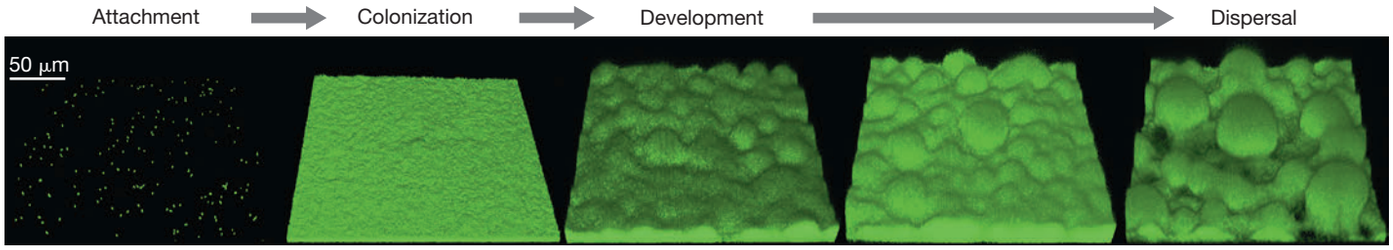


Figure 4.16 ***Pseudomonas aeruginosa* biofilm development.** Confocal scanning laser micrographs of a developing *Pseudomonas aeruginosa* biofilm in a flow cell continuously irrigated with nutrient-rich medium. *P. aeruginosa* cells first attach to the glass surface (day 0), then rapidly grow and move on the surface to cover the entire surface (day 1); by day 4 mushroom-shaped microcolonies over 0.1 mm high have developed.

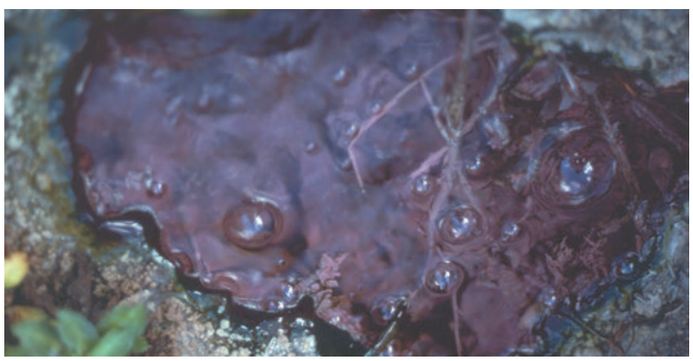
over time (Figure 4.16). *Pseudomonas aeruginosa* is a model organism that has been used to study biofilms. *P. aeruginosa* is a relevant model because it forms biofilms in the lungs of humans with the genetic disease *cystic fibrosis*. Biofilm formation allows cells of *P. aeruginosa* to persist and resist removal. Biofilms also tend to make bacteria more resistant to drugs because they provide a penetration barrier and promote metabolic differentiation. Hence, biofilms allow complex microbial interactions not observed in planktonic microbes.

Biofilms and Humans

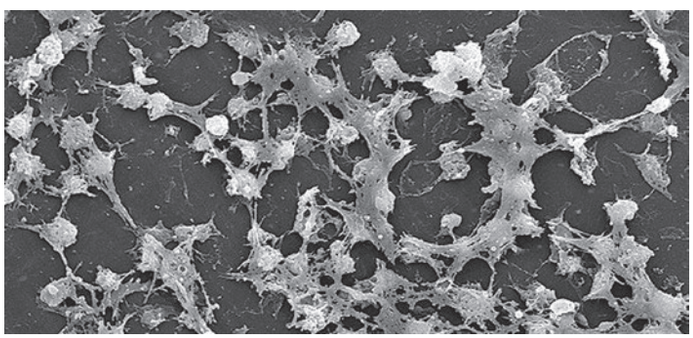
Biofilms are a common growth form for bacteria in nature because the intensely interwoven nature of the structure prevents harmful chemicals from reaching cells deep within the biofilm structure. Biofilms also provide a physical barrier that protects cells from grazing by protists and allows them to remain in a safe and favorable habitat. Some biofilms form multilayered sheets with different organisms present in the individual layers, and these biofilms are called *microbial mats*. Mats composed of various phototrophic and chemotrophic bacteria are common in the outflows of hot springs (**Figure 4.17*a***) and marine intertidal regions and can form crusty matlike structures quickly in puddles of water that stay moist for as little as a few days.

Biofilms affect many aspects of our lives, including human health. For example, biofilms have been implicated in difficult-to-treat infections of human joints, implanted medical devices such as artificial heart valves and joints, and indwelling devices, such as catheters (Figure 4.17*b*). Biofilms are also responsible for the formation of dental caries (cavities) and are a cause of gum disease. In industrial and municipal settings, biofilms can cause fouling, plugging, and corrosion in pipes used to transport liquids. Biofilms can even form in fuel storage tanks, where they contaminate fuel by producing corrosive chemicals such as H_2S. Finally, the formation of biofilms on the hulls of ships is a major problem because they decrease speed and energy efficiency and thereby increase shipping times and shipping costs.

Biofilms are of tremendous significance and so we will revisit different aspects of the biology of biofilms in Chapters 7 and 20.



(a)



(b)

Figure 4.17 Examples of biofilms. *(a)* A microbial mat of the purple phototrophic bacterium *Thermochromatium tepidum* that developed in a small sulfidic hot spring in Yellowstone National Park (USA). *(b)* Scanning electron micrograph of a biofilm of cells of *Staphylococcus aureus* that formed on an indwelling catheter.

Check Your Understanding

- What are the stages and events that occur during biofilm formation?
- Why is biofilm formation such a major problem in human medicine?

4.10 Alternatives to Binary Fission

Planktonic cells that grow by binary fission undergo *balanced growth* during exponential phase. All cells in such a culture are nearly identical, genetically and metabolically, and the uniformity of such cultures makes them desirable as experimental systems. We have already learned that cells within biofilms do not exhibit balanced growth because their metabolic characteristics and growth rates vary widely depending on their position within the biofilm. Here we consider microbes that do not display binary fission and whose growth dynamics are not well explained by standard growth equations.

Budding Cell Division

Although binary fission, which produces identical daughter cells, is common among *Bacteria* and *Archaea*, many bacteria grow by other means. Budding bacteria, for example, exhibit unequal cell growth and produce daughter cells that have different characteristics (**Figure 4.18**). In **budding division** a new cell emerges and buds off from a mother cell, the latter of which retains its original identity (Figure 4.18).

Some budding bacteria form cytoplasmic extensions such as stalks or hyphae, and classic examples are the genera *Caulobacter* (◀ Figure 2.14) and *Hyphomicrobium* (Figure 4.18). *Hyphomicrobium* forms a long stalk from which a new cell emerges and buds. In the case of *Caulobacter*, the mother cell is attached to a surface by its stalk. The daughter cell that buds off is motile; it lacks a stalk and has a flagellum that allows it to disperse by swimming motility. Other budding bacteria such as the aquatic bacterium *Ancalomicrobium* produce multiple appendages that resemble arms extending away from the cell (▶ Figure 15.53*b*). The appendages increase the surface-to-volume ratio of the cell (◀ Section 1.3), which increases its ability to extract nutrients from oligotrophic (very dilute) habitats. Many budding bacteria also have distinctive life cycles, and we consider these and the group as a whole in Section 15.18.

Hyphal Growth and Multiple Fission

Actinomycetes are gram-positive filamentous bacteria that are common in soils and grow as long thin filaments called *hyphae*; species of the genus *Streptomyces* are typical actinomycetes (**Figure 4.19**). **Hyphal growth** occurs only at the tip of an elongating filament and is unlike binary fission because growth of the cell is not linked directly to cell division. That is, cells elongate and replicate DNA as they grow but do not produce septa. Instead, hyphae form cross-walls away from the point of cell growth. These cross-walls do not define independent cells but instead allow transport to occur between adjacent compartments in the hyphal filament (see also page 144). Hyphae often weave together to form *mycelia*, which form from complex hyphal filaments, and mature mycelia often form *arthrospores*. Arthrospores are survival structures but differ from endospores in their mechanism of development and their lack of resistance to heat and harsh chemicals.

Arthrospores develop by *multiple fission*, in which a single hyphal filament forms many septa simultaneously along its length. This causes many cells to form all at once along the filament, each of which ultimately differentiates into a mature arthrospore. Multiple fission is also seen in certain cyanobacteria (a defining feature of the *Pleurocapsales*; see ▶ Figure 15.2*b*). Cells of the cyanobacterium *Stanieria*, for example, begin about 1 μm in diameter and grow to as large as 30 μm in diameter before undergoing multiple fission, a process in which the large cell subdivides into tens (or even hundreds) of smaller cells, each of which begins the replication cycle anew. Finally, some bacteria even form intracellular offspring. Bacteria such as the giant-celled *Epulopiscium* (◀ Figure 1.6*a*) grow by forming multiple daughter cells within the cytoplasm of the mother cell. Once the daughter cells are mature, they burst out of the mother cell and begin a new round of daughter cell production.

Now we turn our attention from microbial growth itself to environmental characteristics that control microbial growth. We will see that the microbial world is ripe with exceptional organisms able to grow under punishing conditions that can only be labeled extreme.

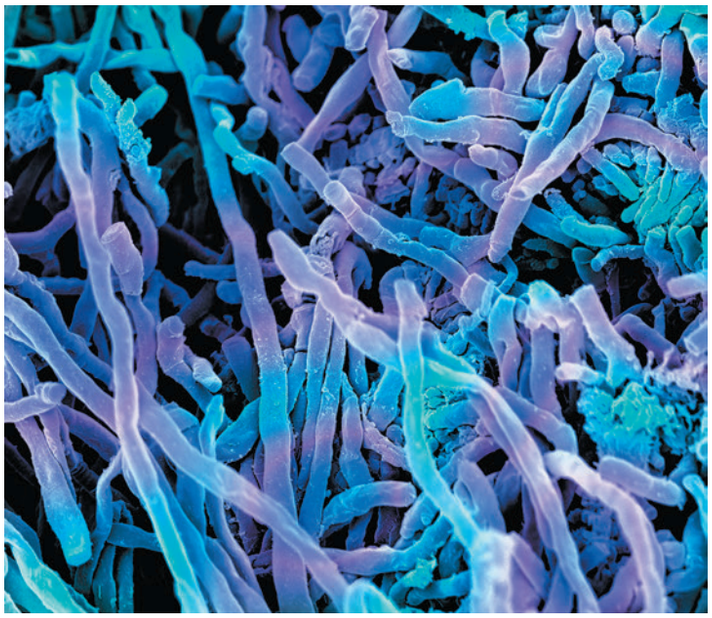


Figure 4.19 Colorized scanning electron micrograph of cells of *Streptomyces*, a filamentous bacterium that produces arthrospores. Various species of *Streptomyces* are major producers of antibiotics.

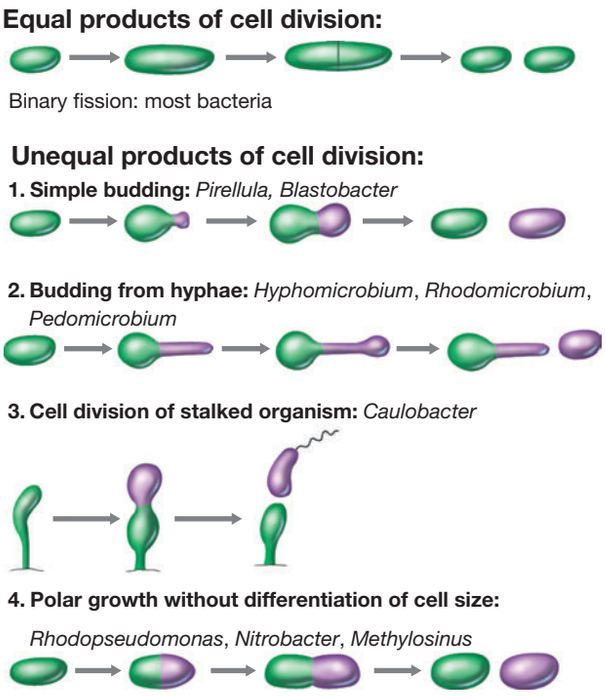


Figure 4.18 Cell division in different morphological forms of bacteria. The contrast is shown between cell division in conventional bacteria (cells that divide by binary fission) and in various budding and stalked bacteria.

Check Your Understanding

- Why would it be incorrect to apply the growth equations in Figure 4.12 to an organism like *Stanieria*?
- In a solid environment such as soil, what advantage might hyphal growth provide to a bacterium relative to those that grow by binary fission?
- Compare and contrast the life cycle of *Caulobacter* to the development of a biofilm.

III • Environmental Effects on Growth: Temperature

Having the right set of chemical nutrients in a culture medium is only half the secret of growing microbial cells; the other half is to ensure that the environment is suitable for growth. Temperature is a key factor, but what's warm for one organism may be cold for another.

Even when a microbe is provided with an optimal array of its required nutrients, growth is not a sure thing unless the chemical and physical state of its environment is also suitable. Four environmental factors control microbial growth in a major way: *temperature*, *pH*, *water availability*, and *oxygen*. If any one of these factors is beyond the limits that an organism can tolerate, growth will not occur, even in an otherwise ideal culture medium. In Parts III and IV of this chapter we examine these important environmental factors, beginning with temperature, the key factor affecting the growth and survival of microorganisms.

4.11 Temperature Classes of Microorganisms

At either too cold or too hot a temperature, microorganisms will not be able to grow and may even die. The minimum and maximum temperatures supporting growth vary greatly among different organisms and usually reflect the temperature range and average temperature of the environments the organisms inhabit.

Cardinal Temperatures

Temperature affects microorganisms in two opposing ways. As temperatures rise, the rate of enzymatic reactions increases and growth becomes faster. However, above a certain temperature, proteins or other critical cell components may be denatured or otherwise irreversibly damaged. For every microorganism there is a *minimum* temperature below which growth is not possible, an *optimum* temperature at which growth is most rapid, and a *maximum* temperature above which growth is not possible. These three temperatures, called the **cardinal temperatures** (Figure 4.20), are characteristic of any given microorganism and can differ dramatically between different species. For example, some organisms have growth temperature optima near 0°C, whereas the optima for others can be higher than 100°C. The temperature range throughout which microbial growth is possible is even wider than this, from as low as −15°C to at least 122°C. However, no single organism can grow over this whole temperature range, as the range for any given organism is typically less than 40°C.

Mastering Microbiology
Art Activity: Figure 4.20 The cardinal temperatures: minimum, optimum, and maximum

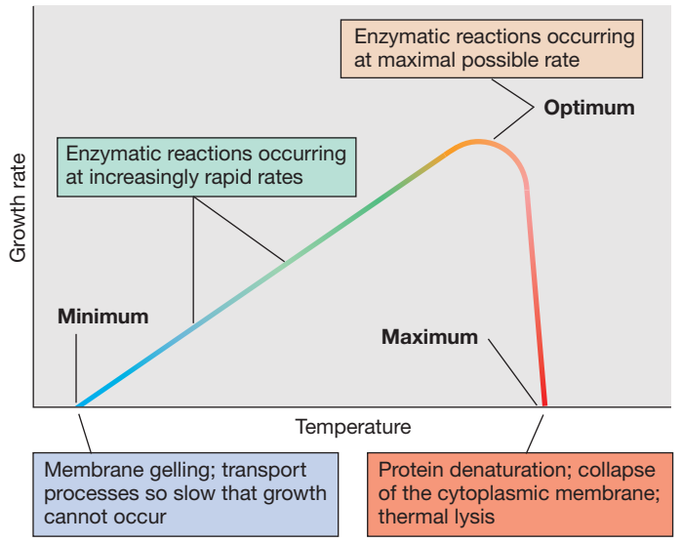


Figure 4.20 The cardinal temperatures: minimum, optimum, and maximum. The actual values may vary greatly for different organisms (see Figure 4.21).

The maximum growth temperature of an organism reflects the temperature above which denaturation of one or more essential cell components, such as a key enzyme, occurs. The factors controlling an organism's minimum growth temperature are not as clear. However, the cytoplasmic membrane must remain in a semifluid state for nutrient transport and bioenergetic functions to take place. That is, if an organism's cytoplasmic membrane stiffens to the point that it no longer functions properly in transport or can no longer develop or consume a proton motive force, the organism cannot grow. In contrast to the minimum and maximum, the growth temperature *optimum* reflects a state in which all or most cellular components are functioning at their maximum rate and typically lies closer to the maximum than to the minimum (Figures 4.20 and 4.21).

Temperature Classes of Organisms

Although there is a continuum of organisms, from those with very low temperature optima to those with high temperature optima, it is possible to distinguish four broad classes of microorganisms in relation to their growth temperature optima: *psychrophiles*, with low temperature optima; *mesophiles*, with midrange temperature optima; *thermophiles*, with high temperature optima; and *hyperthermophiles*, with very high temperature optima (Figure 4.21).

Mesophiles are widespread in nature and are the most commonly studied microorganisms. Mesophiles are found in the intestines of endothermic (warm-blooded) animals and in terrestrial and aquatic environments in temperate and tropical latitudes. *Escherichia coli* is a typical mesophile, and its cardinal temperatures have been precisely defined. The optimum temperature for most strains of *E. coli* is 39°C, the maximum is 48°C, and the minimum is 8°C. Thus, the temperature *range* for *E. coli* is about 40 degrees (Figure 4.21).

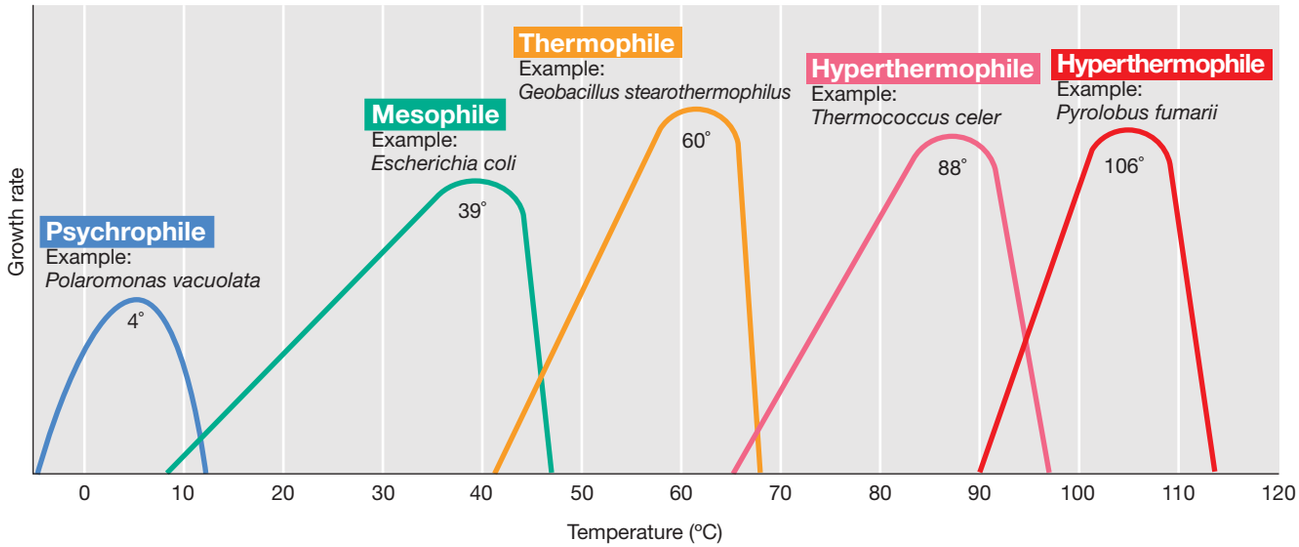


Figure 4.21 Temperature and growth response in different temperature classes of microorganisms. The temperature optimum of each example organism is shown on the graph.

Psychrophiles and thermophiles are found in unusually cold and unusually hot environments, respectively. Hyperthermophiles are found in extremely hot habitats such as hot springs, where temperatures can be as hot as 100°C, and deep-sea hydrothermal vents, where temperatures can exceed 100°C. We now consider these fascinating microbes and examine some of the physiological problems they face and some of the biochemical solutions they have evolved to thrive under these extreme conditions.

Check Your Understanding

- How does a hyperthermophile differ from a psychrophile?
- What are the cardinal temperatures for *Escherichia coli*? To what temperature class does it belong?
- *E. coli* can grow at a higher temperature in a complex medium than in a defined medium. Why?

4.12 Microbial Life in the Cold

Because humans live and work in places where temperatures are moderate, it is natural to consider very hot and very cold environments as "extreme." However, many microbial habitats are indeed very hot or very cold, and organisms that inhabit these environments—called *extremophiles* (◀ Section 1.5 and Table 1.2)—actually thrive in these punishing environments. We consider the biology of these fascinating organisms here and in the next section.

Cold Environments

Much of Earth's surface is cold. The oceans, which make up over half of Earth's surface, have an average temperature of 5°C, and the depths of the open oceans have constant temperatures of 1–3°C. Vast areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer (**Figure 4.22**). These cold environments support diverse microbial life, as do glaciers (Figure 4.22*e*), where the networks of liquid water channels that run through and under the glacier are teeming with microorganisms. Even in solidly frozen materials there remain small pockets of liquid water where solutes have concentrated and microorganisms can metabolize and grow, albeit very slowly.

In considering cold environments as microbial habitats, it is important to distinguish between environments that are *constantly* cold and those that are only *seasonally* cold. The latter, characteristic of temperate climates, may have summer temperatures as high as 40°C. A temperate lake, for example, may have ice cover in the winter, but the water may remain at 0°C for only a relatively brief time. By contrast, Antarctic lakes contain a permanent ice cover several meters thick (Figure 4.22*d*), and the water column below the ice in these lakes remains at 0°C or colder year-round. Marine sediments and glaciers are also constantly cold, as are subglacial lakes—lakes deep beneath the glacier surface—and all of these are teeming with microbial life. It is thus not surprising that the best examples of microbes well adapted to cold temperatures have emerged from these environments.

Mastering Microbiology
Art Activity: Figure 4.21 Temperature and growth responses in different temperature classes of microorganisms

Psychrophilic and Psychrotolerant Microorganisms

A **psychrophile** is a microbe with an optimal growth temperature of 15°C or lower, a maximum growth temperature below 20°C, and a minimum growth temperature of 0°C or lower. By contrast, microbes that grow at 0°C but have optima of 20–40°C are called **psychrotolerant**. Psychrophiles are found in environments that are constantly cold and may even be killed by warming to moderate temperatures. For this reason, the laboratory study of psychrophiles requires that great care be taken to ensure that they never warm up during sampling, transport to the laboratory, isolation, or other manipulations.

Psychrophilic algae and bacteria often grow in dense masses within and under sea ice (frozen seawater that forms seasonally) in polar regions (Figure 4.22*a–c*). They can also be found on the surfaces of permanent snowfields and glaciers where they impart a

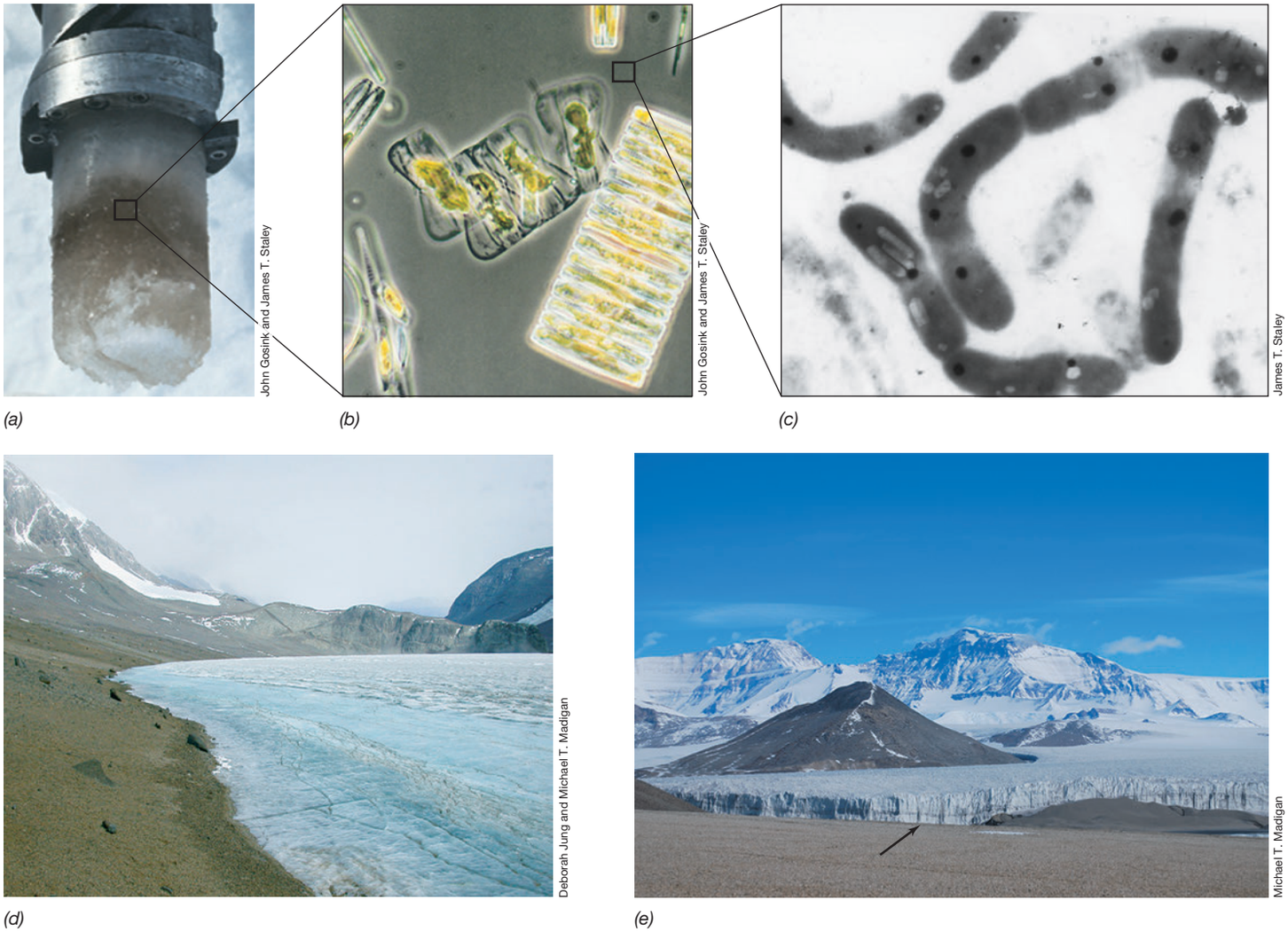


Figure 4.22 **Antarctic microbial habitats and microorganisms.** *(a)* A core of frozen seawater from McMurdo Sound, Antarctica. The core is about 8 cm wide. Note the dense coloration due to pigmented microorganisms. *(b)* Phase-contrast micrograph of phototrophic microorganisms from the core shown in part *a*. Most organisms are either diatoms or green algae (both eukaryotic phototrophs). *(c)* Transmission electron micrograph of *Polaromonas*, a gas vesiculate bacterium that lives in sea ice and grows optimally at 4°C. Cells are about 0.8 μm in diameter. *(d)* Lake Bonney, McMurdo Dry Valleys, Antarctica. Although the lake is permanently ice-covered, the water column under the ice contains a diverse array of *Bacteria*, *Archaea*, and microbial eukaryotes. *(e)* Garwood Glacier, McMurdo Dry Valleys, Antarctica. The edge of the glacier (arrow) is about 20 m high. Glaciers and subglacial lakes are teeming with microbial life.

distinctive coloration to the surface (**Figure 4.23**). The snow alga *Chlamydomonas nivalis* is an example of this, the carotenoid pigment astaxanthin in its spores (Figure 4.23 inset) being responsible for the brilliant red color of the snow surface. This alga grows within the snow as a green-pigmented vegetative cell and then sporulates. As the snow dissipates by melting, erosion, and ablation (evaporation and sublimation), the spores become concentrated on the surface. Related species of snow algae contain different carotenoid pigments, and thus fields of snow algae can also be green, orange, brown, or purple.

Several psychrophilic *Bacteria* and a few psychrophilic *Archaea* have been isolated, and some of these show very low growth temperature optima. The permafrost bacterium *Planococcus halocryophilus* grows slowly at −15°C, the lowest growth temperature documented for any bacterium. However, theoretical considerations of bacterial metabolism suggest that the lower temperature limit for bacterial metabolism is likely to be considerably colder than this. For example, microbial respiration (as measured by CO_2 production) has been measured in tundra soils at nearly −40°C. At a temperature of −20°C, pockets of liquid water can exist in "frozen" materials, and studies have shown that enzymes from cold-active bacteria still function under such conditions. Growth at such temperatures, if possible, would be extremely slow. However, if an organism can grow, even if only at a very low rate, it can remain competitive and maintain a population in its habitat.

Psychrotolerant microorganisms are more widely distributed in nature than are psychrophiles and can be isolated from soils and water in temperate climates as well as from meat, dairy products,

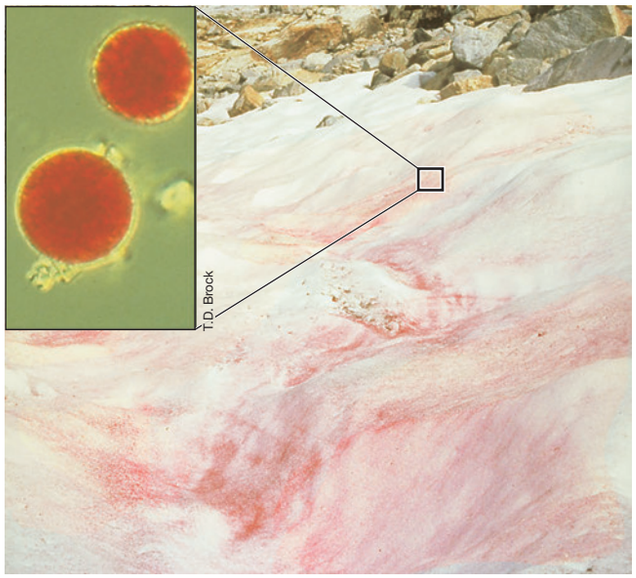


Figure 4.23 Snow algae. Snow bank in the Sierra Nevada, California, with red coloration caused by the presence of snow algae. Inset: photomicrograph of red-pigmented spores of the snow alga *Chlamydomonas nivalis*. Spores are about 18 μm in diameter. The spores germinate to yield motile green algal cells.

cider, vegetables, and fruit stored at standard refrigeration temperatures (4°C). Although psychrotolerant microorganisms grow at 0°C, most do not grow well, and one must often wait weeks before visible growth is seen in laboratory cultures. By contrast, the same organism cultured at 30°C may grow at rates similar to that of many mesophiles. Various *Bacteria*, *Archaea*, and microbial eukaryotes are psychrotolerant.

Molecular Adaptations to Life in the Cold

Psychrophiles produce enzymes that function—often optimally—in the cold and that may be denatured or otherwise inactivated at even very moderate temperatures. The molecular basis for this is not entirely understood, but clearly it is linked to protein structure. Several cold-active enzymes whose structure is known show a greater content of α-helix and lesser content of β-sheet secondary structure (▶ Section 6.7) than do enzymes that show little or no activity in the cold. Because β-sheet secondary structures tend to be more rigid than α-helices, the greater α-helix content of cold-active enzymes allows these proteins greater flexibility for catalyzing their reactions at cold temperatures. Cold-active enzymes also tend to have greater polar and lesser hydrophobic amino acid content (▶ Figure 6.27 for structures of amino acids) and lower numbers of weak bonds, such as hydrogen and ionic bonds, compared with the corresponding enzyme from mesophiles. Collectively, these molecular features are likely to keep cold-active enzymes flexible and functional under cold conditions.

Another characteristic feature of psychrophiles is that their cytoplasmic membranes remain functional at low temperatures. Cytoplasmic membranes from psychrophiles tend to have a higher content of unsaturated and shorter-chain fatty acids, and this helps the membrane remain in a semifluid state at low temperatures to carry out important transport and bioenergetic functions. Some psychrophilic bacteria even contain *polyunsaturated* fatty acids; unlike monounsaturated or fully saturated fatty acids that tend to stiffen at low temperatures, polyunsaturated fatty acids remain flexible even at very cold temperatures.

Other molecular adaptations to cold temperatures include "cold shock" proteins and cryoprotectants, and these are not limited to psychrophiles. Cold shock proteins are a type of molecular chaperone (▶ Section 6.11) and have several functions that include maintaining cold-sensitive proteins in an active form or binding specific mRNAs and facilitating their translation under cold conditions. Cryoprotectants include dedicated antifreeze proteins or specific solutes—such as glycerol or certain sugars—that are produced in large amounts at cold temperatures; these agents help prevent the formation of ice crystals that can puncture the cytoplasmic membrane. Highly psychrophilic bacteria often produce abundant levels of exopolysaccharide cell surface slime, and these slime layers confer cryoprotection as well.

Although "freezing" temperatures may prevent microbial growth, they do not necessarily cause death. Indeed, just the opposite may occur, and this has been exploited for the preservation of bacterial cells in microbial culture collections. Cells suspended in growth medium containing 10% dimethyl sulfoxide (DMSO) or glycerol as a cryoprotectant and frozen at −80°C (ultracold freezer) or −196°C (liquid nitrogen) remain viable in the frozen state for years.

Check Your Understanding

- How do psychrotolerant organisms differ from psychrophilic organisms?
- What molecular adaptations to cold temperatures are seen in the cytoplasmic membrane of psychrophiles? Why are they necessary?

4.13 Microbial Life at High Temperatures

Microbial life flourishes in high-temperature environments, from sun-heated soils and pools of water to boiling hot springs, and the organisms that live in these environments are typically highly adapted to their environmental temperature. We examine these organisms now and pick up on them again in several later chapters.

Thermal Environments

Organisms whose growth temperature optimum exceeds 45°C are called **thermophiles** and those whose optimum exceeds 80°C are called **hyperthermophiles** (Figure 4.21). The surface of soils subject to full sunlight can be heated to above 50°C at midday, and some surface soils may warm to as high as 70°C. Fermenting materials such as compost piles and silage can also reach temperatures of 70°C. Thermophiles abound in such environments. The most extreme high-temperature environments in nature, however, are hot springs, and these are home to a huge diversity of thermophiles and hyperthermophiles.

Many terrestrial hot springs have temperatures at or near boiling, while those at the bottom of the ocean, called *hydrothermal vents*, can have temperatures of 350°C or greater. Hot springs are found throughout the world, but they are especially abundant in the western United States, New Zealand, Iceland, Japan, Italy, Indonesia,

Central America, and central Africa. The largest concentration of hot springs in the world is in Yellowstone National Park, Wyoming (USA). Although some hot springs vary widely in temperature, many have nearly constant high temperatures, varying less than a degree or two over many years. In addition, different springs have different chemical compositions and pH values. In habitats hotter than 65°C, only prokaryotic cells can thrive (**Table 4.3**), but the diversity of *Bacteria* and *Archaea* in such environments is often extensive.

Hyperthermophiles and Thermophiles

A variety of hyperthermophiles inhabit boiling hot springs (**Figure 4.24**), including both chemoorganotrophic and chemolithotrophic species. Growth rates of hyperthermophiles can be studied in the field by immersing a microscope slide into a spring and then examining it microscopically over time. The slide is an excellent surface for microbial attachment and subsequent growth, and so small microbial colonies form (Figure 4.24*b*) and growth rates can be calculated from cell number data. Ecological studies such as this have shown that growth rates in boiling springs are often quite high, with generation times (*g*) as short as 1 h not uncommon.

Cultures of diverse hyperthermophiles have been obtained, and a variety of morphological and physiological types of both *Bacteria* and *Archaea* are known. Some hyperthermophilic *Archaea* have growth-temperature optima above 100°C, while no species of *Bacteria* have yet been discovered that grow above 95°C. Growing laboratory cultures of organisms with optima above the boiling point requires pressurized vessels that permit temperatures in the growth medium to rise above 100°C without boiling. The most heat-tolerant organisms known inhabit hydrothermal vents, with the most thermophilic example thus far being *Methanopyrus*, a methane-producing genus of *Archaea* capable of growth at up to 122°C (▶ Section 17.12).

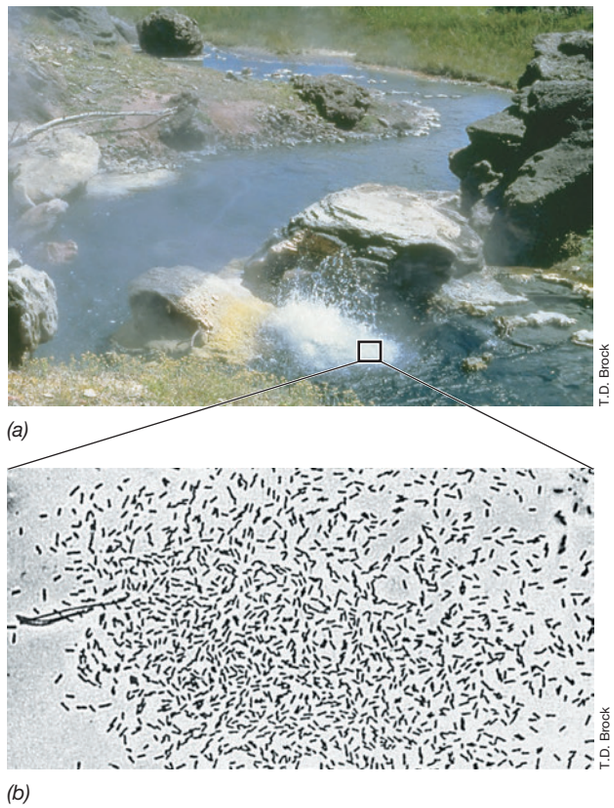


Figure 4.24 Growth of hyperthermophiles in boiling water. *(a)* Boulder Spring, a small boiling spring in Yellowstone National Park. This spring is superheated, having a temperature 1–2°C above the boiling point. The mineral deposits around the spring consist mainly of silica and sulfur. *(b)* Photomicrograph of a microcolony of *Archaea* that developed on a microscope slide immersed in such a boiling spring.

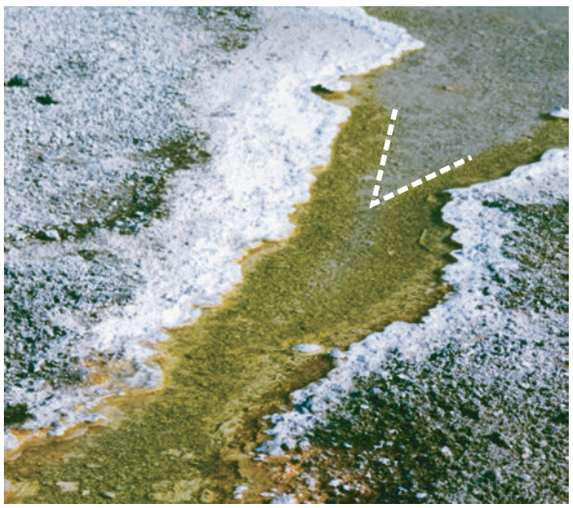
TABLE 4.3 Presently known upper temperature limits for growth of living organisms

Group	Upper temperature limits (°C)
Macroorganisms	
Animals	
Fish and other aquatic vertebrates	38
Insects	45–50
Ostracods (crustaceans)	49–50
Plants	
Vascular plants	45 (60 for one species)
Mosses	50
Microorganisms	
Eukaryotic microorganisms	
Protozoa	56
Algae	55–60
Fungi	60–62
Bacteria and Archaea	
Bacteria	
Cyanobacteria	73
Anoxygenic phototrophs	70–73
Chemoorganotrophs/chemolithotrophs	95
Archaea	
Chemoorganotrophs/chemolithotrophs	122

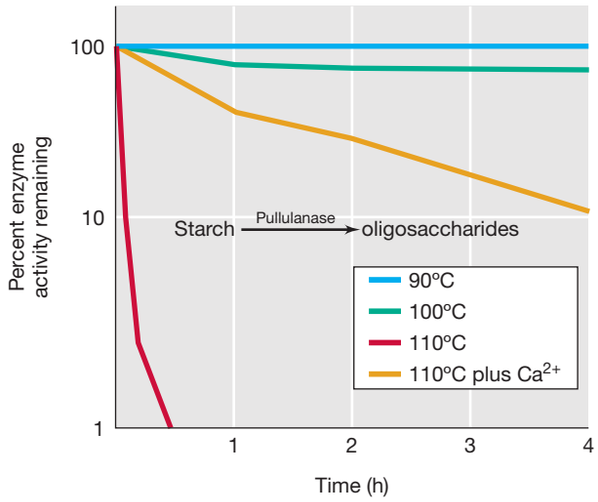
In contrast to hyperthermophiles, thermophiles (optima 45–80°C) inhabit moderately hot or intermittently hot environments. As boiling water leaves a hot spring, it gradually cools, setting up a thermal gradient. Along this gradient, microorganisms become established, with different species growing in the different temperature ranges (**Figure 4.25*a***). By studying the species distribution along such natural thermal gradients, it has been possible to determine the upper temperature limits for various classes of microbes (Table 4.3). Thermophilic *Bacteria* and *Archaea* have also been found in artificial thermal environments, such as hot water heaters. Hot water discharges from power plants and other artificial thermal sources also provide sites where thermophiles can flourish.

Protein and Membrane Stability at High Temperatures

How do thermophiles and hyperthermophiles survive high temperatures? First, their enzymes and other proteins are much more heat-stable than are those of mesophiles and actually function *optimally* at high temperatures. The heat stability of an enzyme from a hyperthermophile is often due to subtle changes in amino acid sequence from the corresponding enzyme of a mesophile, and these changes affect protein structure and function to resist heat denaturation. Heat-stable proteins also typically show increased ionic bonding



(a)



(b)

Figure 4.25 Hot spring microbes and their heat-stable enzymes. *(a)* Characteristic V-shaped pattern (shown by the dashed white lines) formed by cyanobacteria at the upper temperature for phototrophic life, 70–73°C, in the thermal gradient formed from a boiling hot spring in Yellowstone National Park. The pattern develops because the water cools more rapidly at the edges than in the center of the channel. *(b)* In the spring source, hyperthermophiles thrive, and some have been used as sources of heat-stable enzymes, such as pullulanase from *Pyrococcus* (*Archaea*). Ca^{2+} stabilizes the enzyme above the boiling point.

between basic and acidic amino acids and have highly hydrophobic interiors, factors that also prevent unfolding. Finally, solutes such as di-inositol phosphate, diglycerol phosphate, and mannosylglycerate are produced at high levels in certain hyperthermophiles, and these are thought to help stabilize their proteins against thermal denaturation.

Enzymes from thermophiles and hyperthermophiles have significant commercial uses. Heat-stable enzymes catalyze biochemical reactions at high temperatures and are in general more stable than enzymes from mesophiles, thus prolonging the shelf life of commercial enzyme preparations (Figure 4.25*b*). A classic example of this is the DNA polymerase isolated from *Thermus aquaticus*—*Taq polymerase*—used to automate the repetitive steps in the polymerase chain reaction (PCR), a technique for amplifying DNA and a major tool of modern biology (▶ Section 12.1). Several other heat-stable enzymes are commercially available for specific industrial applications (Figure 4.25*b*).

Besides enzymes and other macromolecules in the cell, the cytoplasmic membranes of thermophiles and hyperthermophiles must be heat-stable. Heat naturally works to peel apart the lipid bilayer that makes up the cytoplasmic membrane (◀ Section 2.1). In thermophiles and most hyperthermophilic *Bacteria*, the cytoplasmic membrane has a higher content of long-chain and saturated fatty acids and a lower content of unsaturated fatty acids than are found in the cytoplasmic membranes of mesophiles. Saturated fatty acids form a stronger hydrophobic environment than do unsaturated fatty acids, and longer-chain fatty acids have a higher melting point than shorter-chain fatty acids; collectively, these properties increase membrane stability.

Hyperthermophiles, most of which are *Archaea*, do not contain fatty acids in their membranes but instead have C_{40} hydrocarbons composed of repeating units of isoprene bonded by ether linkage to glycerol phosphate (◀ Figure 2.3). In addition, however, the architecture of the cytoplasmic membranes of many hyperthermophiles takes a unique twist: The membrane forms a lipid *monolayer* rather than a lipid *bilayer* (◀ Figure 2.3*c*). The monolayer structure covalently links both halves of the membrane and prevents it from melting at the high growth temperatures of hyperthermophiles.We consider other aspects of heat stability in hyperthermophiles, including that of DNA stability, in Chapter 17.

Check Your Understanding

- Which phylogenetic domain includes species with optima of >100°C? What special techniques are required to culture them?
- How does the membrane structure of hyperthermophilic *Archaea* differ from that of *Escherichia coli* and why is this structure helpful for growth at high temperature?
- What is *Taq* polymerase and why is it important?

IV • Environmental Effects on Growth: pH, Osmolarity, and Oxygen

Although temperature has an overarching effect on microbial growth, pH, salinity, and atmospheric conditions also control growth of laboratory cultures and natural populations. The best conditions for one organism may differ considerably from those for another.

As we have seen, temperature has a major effect on the growth of microorganisms. But many other environmental factors can affect microbial growth as well, including pH, osmolarity, and oxygen.

4.14 Effects of pH on Microbial Growth

Acidity or alkalinity of a solution is expressed by its **pH** on a logarithmic scale in which neutrality is pH 7 (Figure 4.26). pH values less than 7 are *acidic* and those greater than 7 are *alkaline*. In analogy to a temperature range (Figure 4.21), every microorganism has a pH range, typically about 2–3 pH units, within which growth is possible. Also, each organism shows a well-defined pH optimum, where growth occurs best. Most natural environments have a pH between 3 and 9, and organisms with pH growth optima in this range are most common. Terms used to describe organisms that grow best in particular pH ranges are shown in Table 4.4.

Acidophiles

Organisms that grow optimally at a pH value in the range termed *circumneutral* (pH 5.5 to 7.9) are called **neutrophiles**. For example, the bacterium *Escherichia coli* is a neutrophile (Table 4.4). By contrast, organisms that grow best below pH 5.5 are called **acidophiles**. There are different classes of acidophiles, some growing best at moderately acidic pH and others at very low pH. Many fungi and bacteria grow best at pH 5 or even below, while a more restricted number grow best below pH 3. An even more restricted group grow best below pH 2 and those with pH optima below 1 are extremely rare. Most acidophiles cannot grow at pH 7 and many cannot grow at pH values more than two units above their optimum.

A critical factor governing acidophily is the stability of the cytoplasmic membrane. When the pH is raised to neutrality, the cytoplasmic membranes of strongly acidophilic bacteria are destroyed and the cells lyse. This indicates that these organisms are not just acid-*tolerant* but that high concentrations of protons are actually *required* for cytoplasmic membrane stability. For example, the most acidophilic microbe known is *Picrophilus oshimae*, a species of *Archaea* that grows optimally at pH 0.7 and 60°C. Above pH 4, cells of *P. oshimae* spontaneously lyse. As one would predict, *P. oshimae* inhabits extremely acidic thermal soils associated with volcanic activity.

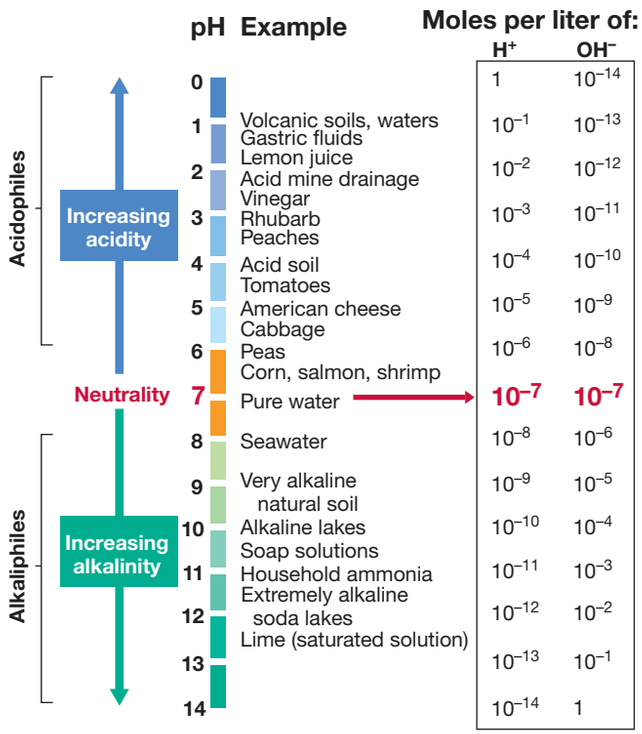


Figure 4.26 The pH scale. Although some microorganisms can live at very low or very high pH, the cell's internal pH remains near neutrality.

TABLE 4.4 Relationships of microorganisms to pH

Physiological class (optima range)	Approximate pH optimum for growth	Example organism[a]
Neutrophile (pH > 5.5 and < 8)	7	*Escherichia coli*
Acidophile (pH < 5.5)	5	*Rhodopila globiformis*
	3	*Acidithiobacillus ferrooxidans*
	1	*Picrophilus oshimae*
Alkaliphile (pH ≥ 8)	8	*Chloroflexus aurantiacus*
	9	*Bacillus firmus*
	10	*Natronobacterium gregoryi*

[a] *Picrophilus* and *Natronobacterium* are *Archaea*; all others are *Bacteria*.

Alkaliphiles

A few extremophiles have very high pH optima for growth, sometimes as high as pH 10, and some of these can still grow, albeit poorly, at even higher pH. Microorganisms showing pH optima of 8 or higher are called **alkaliphiles**. Alkaliphilic microorganisms are typically found in highly alkaline habitats, such as soda lakes and high-carbonate soils. The best-studied alkaliphilic bacteria are certain *Bacillus* species, such as *Bacillus firmus*. This organism is alkaliphilic but has an unusually broad range for growth, from pH 7.5 to 11. Some extremely alkaliphilic microbes are also halophilic (salt-loving), and most of these are *Archaea* (▶ Section 17.1). Some phototrophic purple bacteria (▶ Sections 15.4 and 15.5) are also strongly alkaliphilic. Certain alkaliphiles have commercial uses because they excrete hydrolytic enzymes such as proteases and lipases that maintain their activities at alkaline pH. These enzymes are added to laundry detergents to remove protein and fat stains, respectively, from clothing.

Managing membrane bioenergetics is an obvious problem for alkaliphiles. *B. firmus* uses sodium (Na^+) rather than H^+ to drive transport reactions and rotate its flagellum; that is, it forms a *sodium* motive force instead of a *proton* motive force. Remarkably, however, *B. firmus* uses a proton motive force to drive ATP synthesis even though the external membrane surface is highly alkaline. Exactly how this happens is unclear, although it is thought that hydrogen ions are in some way kept very near the outer surface of the cytoplasmic membrane such that they cannot spontaneously combine with the abundant hydroxyl ions to form water.

Cytoplasmic pH and Buffers

The optimal pH for growth of an organism refers to the *extracellular* environment only; the *intracellular* pH must be maintained at a value consistent with the stability of macromolecules, a range of about 4 pH units from pH 5 to 9. Thus, despite conditions in their

habitats, extreme acidophiles and alkaliphiles maintain cytoplasmic pH values nearer to neutrality.

To prevent major shifts in pH during microbial growth in batch cultures, *buffers* are commonly added to culture media along with the nutrients required for growth. However, any given buffer works over only a relatively narrow pH range. For neutrophilic species, potassium phosphate (KH_2PO_4) or sodium bicarbonate ($NaHCO_3$) is often employed. Various organic buffers are available for the growth of acidophiles and alkaliphiles and are widely used for assaying enzymes extracted from cells. The buffer keeps the enzyme solution at optimal pH during the assay, thus ensuring that the enzyme remains catalytically active and unaffected by any protons or hydroxyl ions generated in the enzymatic reaction.

Check Your Understanding

- How does the concentration of H^+ change when a culture medium at pH 5 is adjusted to pH 9?
- What terms are used to describe organisms whose growth pH optimum is very high? Very low?
- In terms of pH, what class of organism is the bacterium *Escherichia coli*?

4.15 Osmolarity and Microbial Growth

Water is the solvent of life, and water availability is an important factor affecting the growth of microorganisms. Water availability depends not only on how moist or dry an environment is but also on the concentration of solutes (salts, sugars, or other substances) dissolved in the water that is present. Solutes bind water, making it less available to organisms. Hence, for organisms to thrive in high-solute environments, physiological adjustments are necessary. Water availability is expressed in terms of **water activity (a_w)**, the ratio of the vapor pressure of air in equilibrium with a substance or solution to the vapor pressure of pure water. Values of a_w vary between 0 (no free water) and 1 (pure water); some a_w values are listed in Table 4.5.

Water diffuses from regions of higher water concentration (lower solute concentration) to regions of lower water concentration (higher solute concentration) in the process of *osmosis*. The cytoplasm of a cell typically has a higher solute concentration than the environment, so the tendency for water is to diffuse into the cell. Under such conditions, the cell is said to be in *positive water balance*, which is the normal state of the cell. However, when a cell is placed in an environment where the solute concentration exceeds that of the cytoplasm, water will flow out of the cell. If a cell has no strategy to counteract this, it will become dehydrated and unable to grow.

Halophiles and Related Organisms

In nature, osmotic effects are of interest mainly in habitats with high concentrations of salts. Seawater contains about 3% sodium chloride (NaCl) plus small amounts of many other minerals and elements. Microorganisms that inhabit marine environments almost always show an NaCl requirement and grow optimally at the a_w of seawater, 0.98 (Table 4.6). Such organisms are called **halophiles**. The requirement for NaCl by halophiles is absolute and cannot be replaced by other salts, such as potassium chloride (KCl), calcium chloride ($CaCl_2$), or magnesium chloride ($MgCl_2$).

TABLE 4.5 Water activity of several substances

Water activity (a_w)	Material	Example organisms[a]
1.000	Pure water	*Caulobacter, Spirillum*
0.995	Human blood	*Streptococcus, Escherichia*
0.980	Seawater	*Pseudomonas, Vibrio*
0.950	Bread	Most gram-positive rods
0.900	Maple syrup, ham	Gram-positive cocci such as *Staphylococcus*
0.850	Salami	*Saccharomyces rouxii* (yeast)
0.800	Fruit cake, jams	*Zygosaccharomyces bailii* (yeast), *Penicillium* (fungus)
0.750	Salt lakes, salted fish	*Halobacterium, Halococcus*
0.700	Cereals, candy, dried fruit	*Xeromyces bisporus* and other xerophilic fungi

[a]Selected examples of *Bacteria* and *Archaea* or fungi capable of growth in culture media adjusted to the stated water activity.

Although halophiles require at least some NaCl for growth, the NaCl optimum varies with the organism and is habitat dependent (Figure 4.27). For example, marine microorganisms typically grow best with 1–4% NaCl, organisms from hypersaline environments (environments that are more salty than seawater) grow best at 3–12% NaCl, and organisms from extremely hypersaline environments require even higher levels of NaCl. Organisms isolated from brackish waters (a mixture of freshwater and seawater) may or may not be halophilic.

Mastering Microbiology
Art Activity: Figure 4.27 Effect of NaCl on growth of microorganisms of different salt tolerances or requirements

In contrast to halophiles, **halotolerant** organisms can tolerate some level of dissolved solutes but grow best in the absence of the

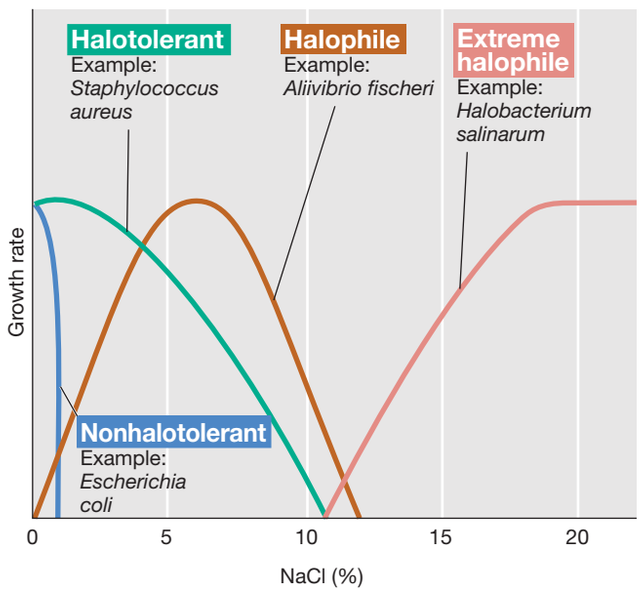
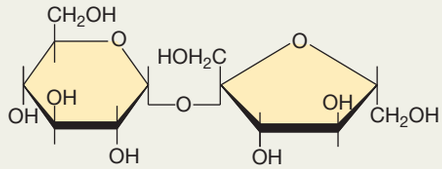


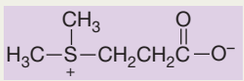
Figure 4.27 Effect of NaCl concentration on growth of microorganisms of different salt tolerances or requirements. The optimum NaCl concentration for marine microorganisms such as *Aliivibrio fischeri* is about 5%; for extreme halophiles, it is between 15 and 30%, depending on the organism.

TABLE 4.6 Compatible solutes of microorganisms

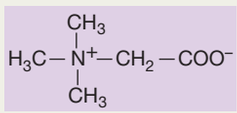
Organism group and example	Major cytoplasmic compatible solute(s)	Minimum a_w for growth[c]
Most nonphototrophic *Bacteria* (*Escherichia*) and freshwater cyanobacteria (*Anabaena*)	Amino acids (mainly glutamate or proline[a])/ sucrose, trehalose[b]	0.98
Marine cyanobacteria (*Synechococcus*)	αGlucosylglycerol[b]	0.92
Marine algae (*Phaeocystis*)	Mannitol,[b] various glycosides, dimethylsulfoniopropionate	0.92
Halotolerant *Bacteria* (*Staphylococcus*)	Amino acids	0.90
Salt lake cyanobacteria (*Aphanothece*)	Glycine betaine	0.75
Halophilic phototrophic purple *Bacteria* (*Halorhodospira*)	Glycine betaine, ectoine, trehalose[b]	0.75
Extremely halophilic *Archaea* (*Halobacterium*) and some *Bacteria* (*Salinibacter*)	KCl	0.75
Halophilic green algae (*Dunaliella*)	Glycerol	0.75
Haloalkaliphilic *Archaea* (*Natrinema*)	KCl	0.68
Xerophilic and osmophilic yeasts (*Zygosaccharomyces*)	Glycerol	0.62[d]
Xerophilic filamentous fungi (*Xeromyces*)	Glycerol	0.605[d]



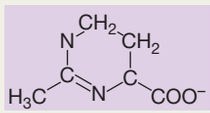
Sucrose



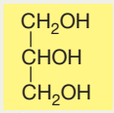
Dimethylsulfoniopropionate



Glycine betaine



Ectoine



Glycerol

[a]See Figure 6.27 for the structures of amino acids.
[b]Structures not shown. Like sucrose, trehalose is a C_{12} disaccharide; glucosylglycerol is a C_9 alcohol; mannitol is a C_6 alcohol.
[c]To achieve an osmotic a_w lower than about 0.77, solutes other than just NaCl are necessary; for example, other salts ($MgCl_2$, $MgSO_4$, or $CaCl_2$) or nonsalts, such as glycerol or sucrose. For most organisms listed (other than for the xerophiles), the lower a_w for growth can be extended downward somewhat by additional solutes.
[d]Growth of *Zygosaccharomyces* tested in high-sucrose medium. Germination of *Xeromyces* spores tested using matric water potential.

added solute (Figure 4.27). Halophiles capable of growth in very salty environments are called **extreme halophiles** (Figure 4.27). These organisms require very high levels of NaCl, typically 15–30%, for optimum growth and are often unable to grow at all at NaCl concentrations below this. Organisms able to live in environments high in sugar are called **osmophiles**, and those able to grow in very dry environments (made dry by lack of water rather than by dissolved solutes) are called **xerophiles**. Examples of these various classes of organisms are given in Table 4.6.

From growth data obtained from extremely halophilic representatives of all three domains of life, there appears to be a common lower water activity limit for living organisms, and this limit is 0.61. This lower limit is likely set by the physiochemical constraints on obtaining water in osmotic environments of a_w less than 0.6 that cannot be overcome through biochemical adaptations by the cell. *Matric water activity*, a measure of water bound to a surface, is measured in the same way as osmotic water activity but can drop to significantly lower than 0.6 and still contain viable microbial communities. For example, hyper-arid hot desert soils can have matric a_w values as low as 0.1 during daylight hours. But these environments absorb moisture at night and during rain events, and these increase the water activity to above 0.6, making conditions suitable for microbial metabolism and growth.

Compatible Solutes

When an organism is transferred from a medium of high a_w to one of low a_w, it maintains positive water balance by increasing its internal

solute concentration. This is possible either by pumping solutes into the cell from the environment or by synthesizing a cytoplasmic solute (Table 4.6). In either case, the solute must not inhibit biochemical processes in the cell and is thus called a **compatible solute**.

Compatible solutes are highly water-soluble organic molecules and include sugars, alcohols, and amino acid derivatives (Table 4.6). Glycine betaine, an analog of the amino acid glycine, is widely distributed among halophilic bacteria. Other common compatible solutes include sugars such as sucrose and trehalose, dimethylsulfoniopropionate (produced by marine algae), and glycerol, a common solute in xerophilic fungi, organisms that grow at the lowest water activities known (Table 4.6). In contrast to these organic solutes, KCl is the compatible solute of extremely halophilic *Archaea*, such as *Halobacterium* (▶ Section 17.1), and of a few extremely halophilic *Bacteria*.

The concentration of compatible solute in a cell is a function of the levels of solute in its environment, and adjustments are made in response to the challenge from external solutes. However, in any given organism, the maximal level of compatible solute tolerated is a genetically encoded characteristic. As a result, different organisms have evolved to thrive in habitats of different salinities (Tables 4.5 and 4.6). In fact, organisms designated as *nonhalotolerant*, *halotolerant*, *halophilic*, or *extremely halophilic* (Figure 4.27) are to some extent a reflection of their genetic capacity to produce or accumulate compatible solutes.

Check Your Understanding

- What is the a_w of pure water? What is the lower limit of a_w for life?
- What are compatible solutes, and when and why are they needed by the cell? What is the compatible solute of *Halobacterium*?

4.16 Oxygen and Microbial Growth

Oxygen (O_2) is an essential nutrient for many microbes; they are unable to metabolize or grow without it. Other microbes, by contrast, cannot grow in the presence of O_2 and may even be killed by it. We therefore see, just as we did for other environmental factors considered in this chapter, *classes* of microorganisms based on their needs or tolerance of O_2.

Oxygen Classes of Microorganisms

Microorganisms can be grouped according to their relationship with O_2 as outlined in Table 4.7. **Aerobes** can grow at full oxygen tensions (air is 21% O_2) and respire O_2 in their metabolism. **Microaerophiles**, by contrast, are aerobes that can use O_2 only when it is present at levels reduced from that in air (microoxic conditions). This is because of the limited capacity of these organisms to respire or because they contain some O_2-sensitive molecule such as an O_2-labile enzyme. Many aerobes are **facultative**, meaning that under the appropriate nutrient and culture conditions they can grow in the absence of O_2.

Some organisms cannot respire oxygen and are called **anaerobes**. There are two kinds of anaerobes: **aerotolerant anaerobes**, which can tolerate O_2 and grow in its presence even though they cannot respire, and **obligate anaerobes**, which are inhibited or even killed by O_2 (Table 4.7). *Anoxic* (O_2-free) microbial habitats are common in nature and include muds and other sediments, bogs, marshes, water-logged soils, intestinal tracts of animals, sewage sludge, the deep subsurface of Earth, and many other environments. Because there are many habitats for anaerobes, they are very common in nature and highly diverse. As far as is known, obligate anaerobiosis is characteristic of only three groups of microorganisms: a wide variety of *Bacteria* and *Archaea*, a few fungi, and a few protozoa.

Some of the best-known prokaryotic anaerobes are *Clostridium*, a genus of gram-positive endospore-forming *Bacteria*, and the methanogens, a group of methane-producing *Archaea*. Among obligate anaerobes, the sensitivity to O_2 varies greatly. Many clostridia, for example, although requiring anoxic conditions for growth, can tolerate traces of O_2 or even full exposure to air. Others, such as the methanogens, are killed rapidly by O_2 exposure.

Culture Techniques for Aerobes and Anaerobes

For the growth of aerobes, it is necessary to provide extensive aeration. This is because the O_2 that is consumed by the organisms

TABLE 4.7 Oxygen relationships of microorganisms

Group	Relationship to O_2	Type of metabolism	Example[a]	Habitat[b]
Aerobes				
Obligate	Required	Aerobic respiration	*Micrococcus luteus* (B)	Skin, dust
Facultative	Not required, but growth better with O_2	Aerobic respiration, anaerobic respiration, fermentation	*Escherichia coli* (B)	Mammalian large intestine
Microaerophilic	Required but at levels lower than atmospheric	Aerobic respiration	*Spirillum volutans* (B)	Lake water
Anaerobes				
Aerotolerant	Not required, and growth no better when O_2 present	Fermentation	*Streptococcus mutans* (B)	Oral cavity
Obligate	Harmful or lethal	Fermentation or anaerobic respiration	*Methanobacterium formicicum* (A)	Sewage sludge, anoxic lake sediments

[a]Letters in parentheses indicate phylogenetic status (B, *Bacteria*; A, *Archaea*). Representatives of either domain of prokaryotic cells are known in each category. Most eukaryotes are obligate aerobes, but facultative aerobes (for example, yeast) and obligate anaerobes (for example, certain protozoa and fungi) are known.
[b]Listed are typical habitats of the example organism; many others could be listed.

during growth is not replaced fast enough by diffusion from the air. Therefore, forced aeration of liquid cultures is needed and can be achieved by either vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium through a fine glass tube or porous glass disk.

For the culture of anaerobes, the problem is not to *provide* O_2 but to *exclude* it. Bottles or tubes filled completely to the top with culture medium and fitted with leakproof closures provide suitably anoxic conditions for organisms that are not overly sensitive to small amounts of O_2. A chemical called a *reducing agent* may be added to such vessels to remove traces of O_2 by reducing it to water (H_2O). An example is thioglycolate, which is present in thioglycolate broth, a medium commonly used to test an organism's requirements for O_2 (**Figure 4.28**).

Thioglycolate broth is a complex medium containing a small amount of agar, making the medium viscous but still fluid. After thioglycolate reacts with O_2 throughout the tube, O_2 can penetrate only near the top of the tube where the medium contacts air. Obligate aerobes grow only at the top of such tubes. Facultative organisms grow throughout the tube but grow best near the top. Microaerophiles grow near the top but not right at the top. Anaerobes grow only near the bottom of the tube, where O_2 cannot penetrate, whereas aerotolerant anaerobes grow throughout the tube. The redox indicator dye *resazurin* is present in thioglycolate broth to signal oxic regions; the dye is pink when oxidized and colorless when reduced and so gives a visual assessment of the degree of penetration of O_2 into the medium (Figure 4.28).

To remove all traces of O_2 for the culture of strict anaerobes, one can incubate tubes or plates in a glass jar flushed with an O_2-free gas or fitted with an O_2 consumption system (**Figure 4.29*a***). For manipulating cultures in an anoxic atmosphere, special enclosures called *anoxic glove bags* permit work with open cultures in completely anoxic atmospheres (Figure 4.29*b*).

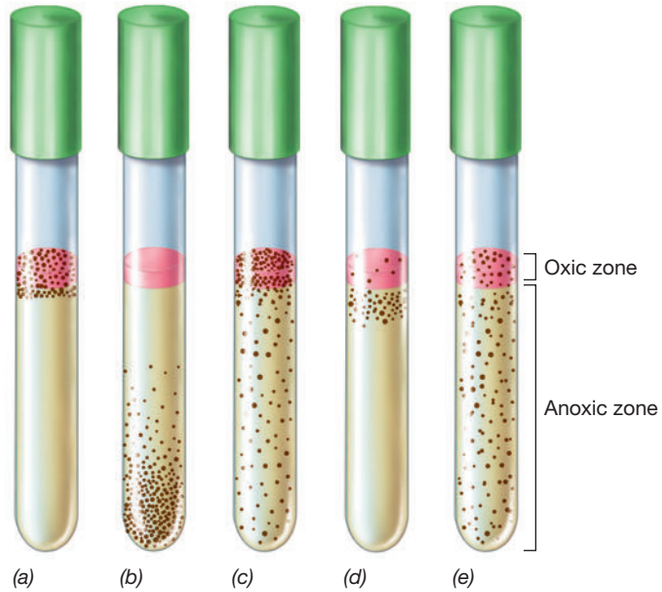


Figure 4.28 Growth versus O_2 concentration. From left to right, aerobic, anaerobic, facultative, microaerophilic, and aerotolerant anaerobe growth, as revealed by the position of microbial colonies (depicted in this drawing as black dots) within tubes of thioglycolate broth culture medium. A small amount of agar has been added to keep the liquid from becoming disturbed. The redox dye resazurin, which is pink when oxidized and colorless when reduced, has been added as a redox indicator. *(a)* O_2 penetrates only a short distance into the tube, so obligate aerobes grow only close to the surface. *(b)* Anaerobes, being sensitive to O_2, grow only away from the surface. *(c)* Facultative aerobes are able to grow in either the presence or the absence of O_2 and thus grow throughout the tube. However, growth is better near the surface because these organisms can respire O_2. *(d)* Microaerophiles grow away from the most oxic zone. *(e)* Aerotolerant anaerobes grow throughout the tube. Growth is not better near the surface because these organisms can only ferment. In nature, many different habitats exist for each of these oxygen classes. In addition, a single habitat, such as a soil particle, may support growth of both aerobes and anaerobes.

Figure 4.29 Incubation under anoxic conditions. *(a)* Anoxic jar. A chemical reaction in the envelope in the jar generates $H_2 + CO_2$. The H_2 reacts with O_2 in the jar on the surface of a palladium catalyst to yield H_2O; the final atmosphere contains N_2, H_2, and CO_2. *(b)* Anoxic glove bag for manipulating and incubating cultures under anoxic conditions. The airlock on the right, which can be evacuated and filled with O_2-free gas, serves as a port for adding and removing materials to and from the glove bag.

Reactants		Products	
$O_2 + e^-$	→	O_2^-	(superoxide)
$O_2^- + e^- + 2\,H^+$	→	H_2O_2	(hydrogen peroxide)
$H_2O_2 + e^- + H^+$	→	$H_2O + OH\bullet$	(hydroxyl radical)
$OH\bullet + e^- + H^+$	→	H_2O	(water)

Outcome:
$O_2 + 4\,e^- + 4\,H^+ \rightarrow 2\,H_2O$

Figure 4.30 Four-electron reduction of O_2 to H_2O by stepwise addition of electrons. All the intermediates formed are reactive and toxic to cells; water is not.

Why Is Oxygen Toxic?

Why are anaerobic microorganisms inhibited in their growth or even killed by oxygen? Molecular oxygen (O_2) per se is not toxic, but O_2 can be converted to toxic oxygen by-products, and it is these that can harm or kill cells not able to deal with them. These include *superoxide anion* (O_2^-), *hydrogen peroxide* (H_2O_2), and *hydroxyl radical* ($OH\bullet$). All of these are by-products of the reduction of O_2 to H_2O in respiration (**Figure 4.30**). Flavoproteins, quinones, and iron–sulfur proteins, electron carriers found in virtually all cells (◀ Section 3.8), also catalyze some of these reductions. Thus, regardless of whether it can respire O_2, an organism exposed to O_2 will experience toxic forms of oxygen, and if not destroyed, these molecules can wreak havoc in cells. For example, superoxide anion and $OH\bullet$ are strong oxidizing agents that can oxidize macromolecules and any other organic compounds in the cell. Peroxides such as H_2O_2 can also damage cell components but are not as toxic as O_2^- or $OH\bullet$.

Superoxide Dismutase and Other Enzymes That Destroy Toxic Oxygen

A major requirement for inhabiting an oxic world is to keep toxic oxygen molecules under control. Microbes accomplish this in much the same way as plants and animals do. Superoxide anion and H_2O_2 are the most abundant toxic oxygen species, and all aerobic and aerotolerant cells have enzymes that destroy these compounds (**Figure 4.31**). The enzymes catalase and peroxidase attack H_2O_2,

$H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$

(a) **Catalase**

$H_2O_2 + \mathbf{NADH} + H^+ \rightarrow 2\,H_2O + NAD^+$

(b) **Peroxidase**

$O_2^- + O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$

(c) **Superoxide dismutase**

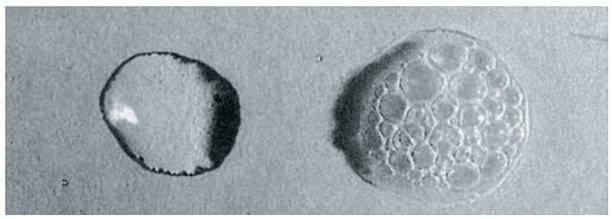
$4\,O_2^- + 4\,H^+ \rightarrow 2\,H_2O + 3\,O_2$

(d) **Superoxide dismutase/catalase in combination**

$O_2^- + 2\,H^+ + \text{rubredoxin}_{\text{reduced}} \rightarrow H_2O_2 + \text{rubredoxin}_{\text{oxidized}}$

(e) **Superoxide reductase**

Figure 4.31 Enzymes that destroy toxic oxygen species. *(a)* Catalases and *(b)* peroxidases are porphyrin-containing proteins, although some flavoproteins may consume toxic oxygen species as well. *(c)* Superoxide dismutases are metal-containing proteins, the metals being copper and zinc, manganese, or iron. *(d)* Combined reaction of superoxide dismutase and catalase. *(e)* Superoxide reductase catalyzes the one-electron reduction of O_2^- to H_2O_2.



T.D. Brock

Figure 4.32 Method for testing a microbial culture for the presence of catalase. A heavy loopful of cells from an agar culture was mixed on a slide (right) with a drop of 30% hydrogen peroxide. The immediate appearance of bubbles is indicative of the presence of catalase. The bubbles are O_2 produced by the reaction $H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$.

forming O_2 and H_2O, respectively (Figure 4.31 and **Figure 4.32**). Superoxide anion is destroyed by the enzyme *superoxide dismutase*, an enzyme that generates H_2O_2 and O_2 from two molecules of O_2^- (Figure 4.31*c*). Superoxide dismutase and catalase (or peroxidase) thus work in series to convert O_2^- to harmless products (Figure 4.31*d*).

Aerobes and facultative aerobes typically contain both superoxide dismutase and catalase. Superoxide dismutase is an essential enzyme for aerobes. Some aerotolerant anaerobes lack superoxide dismutase and use protein-free manganese complexes instead to carry out the dismutation of O_2^- to H_2O_2 and O_2. Such a system is not as efficient as superoxide dismutase, but it is sufficient to protect the cells from O_2^- damage. In some strictly anaerobic *Archaea* and *Bacteria*, superoxide dismutase is absent and instead the enzyme *superoxide reductase* functions to remove O_2^-. Unlike superoxide dismutase, superoxide reductase reduces O_2^- to H_2O_2 without the production of O_2 (Figure 4.31*e*), thus avoiding exposure of the organism to O_2.

Check Your Understanding

- How does an obligate aerobe differ from a facultative aerobe?
- How does a reducing agent work? Give an example of a reducing agent.
- How does superoxide dismutase or superoxide reductase protect a cell?

V • Controlling Microbial Growth

Chemical and physical agents used to control the growth of microorganisms include heat, radiation, and toxic chemicals. Microbial growth control—whether simple disinfection or actual sterilization—is part of our everyday lives and helps curb the transmission of infectious microbes.

Thus far in this chapter we have discussed microbial growth from the perspective of conditions that *promote* their growth. We close by considering the opposite side of the coin, microbial growth *control*.

Many aspects of microbial growth control have significant practical applications. For example, we routinely wash fresh fruits and vegetables to remove attached microorganisms and we inhibit microbial growth on body surfaces by washing. However, neither of these processes kills or removes all microorganisms. Only **sterilization**—the

killing or removal of all microorganisms (including viruses)—ensures that this is the case. In many circumstances, sterility is not required. In others, however, sterilization is absolutely essential.

4.17 General Principles and Microbial Growth Control by Heat

The effects of microorganisms can often be controlled by simply limiting or inhibiting growth. Methods for inhibiting microbial growth include *decontamination*, the treatment of an object or surface to make it safe to handle, and **disinfection**, a process that directly targets pathogens although it may not eliminate all microorganisms. Decontamination can be as simple as wiping off food utensils to remove food fragments (and their attached organisms) before using them, while disinfection requires agents called *disinfectants* that actually kill microorganisms or severely inhibit their growth. Physical methods of microbial growth control are used extensively in industry, medicine, and the home, and we consider three classes of physical controls in this section and the next: heat, radiation, and filtration. Of these three, heat is the most widely used method of physically treating an object or substance to render it sterile.

Heat Sterilization

The effectiveness of heat as a sterilant is quantified by the time required at a given temperature for a 10-fold reduction in the viability of a microbial population. This is called the *decimal reduction time* (*D*). The relationship between *D* and temperature is exponential, as the logarithm of *D* plotted against temperature yields a straight line (**Figure 4.33**). Moreover, heat killing proceeds more rapidly as the temperature rises. The type of heat is also important: Moist heat has better penetrating power than dry heat and, at a given temperature, inhibits growth or kills cells more quickly than does dry heat.

Another way to characterize the heat sensitivity of an organism is to measure its *thermal death time*, the time it takes to kill all cells at a given temperature. To determine the thermal death time, samples of a cell suspension are heated for different times, mixed with culture medium, and incubated. If all the cells have been killed, no growth is observed in the incubated samples. However, unlike a decimal reduction time measurement that is independent of the original cell number, the thermal death time is greatly affected by population size; a longer time is required to kill all cells in a large population than in a small one.

The presence of endospore-forming bacteria in a heat-treated sample can influence both the decimal reduction and thermal death times. Recall that the mature endospore is very dehydrated and contains calcium dipicolinate and small acid-soluble spore proteins (SASPs) that help confer heat stability on the structure (◀ Section 2.8). The medium in which heating takes place also influences the rate of killing, and this is especially relevant in food canning procedures. Microbial death is more rapid at acidic pH, and acidic foods such as tomatoes, fruits, and pickles are easier to sterilize than neutral-pH foods such as corn and beans. Moreover, high concentrations of sugars, proteins, and fats decrease heat penetration and usually increase the resistance of organisms to heat, whereas high salt concentrations may either increase or decrease heat resistance, depending on the organism.

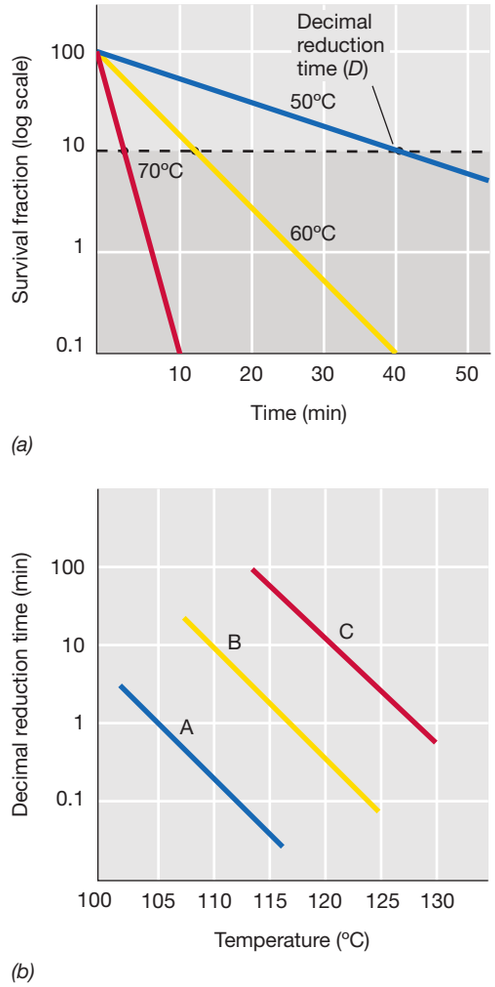
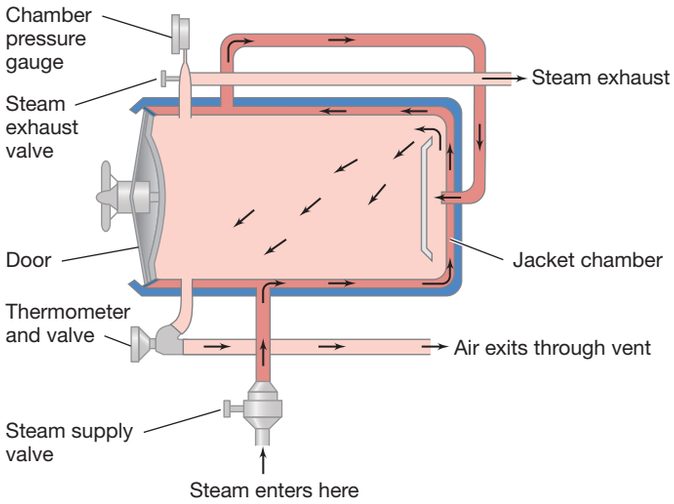


Figure 4.33 The effect of temperature on the heat killing of microorganisms. *(a)* The decimal reduction time (*D*) is the time at which only 10% of the original population of a given organism (in this case, a mesophile) remains viable at a given temperature. For 70°C, $D = 3$ min; for 60°C, $D = 12$ min; for 50°C, $D = 42$ min. *(b)* *D* values for model organisms of different temperature classes: A, mesophile; B, thermophile; C, hyperthermophile.

The Autoclave and Pasteurization

The **autoclave** is a sealed heating device that uses steam under pressure to kill microorganisms (**Figure 4.34**). Killing of heat-resistant endospores requires heating at temperatures above the boiling point of water at 1 atm. The autoclave places steam under a pressure of 1.1 kg/cm^2 (15 lb/in^2), which yields a temperature of 121°C. At 121°C, the time to achieve sterilization of small amounts of endospore-containing material is about 15 min (Figure 4.34*b*). If the object to be autoclaved is bulky or large volumes of liquids are to be sterilized, heat transfer to the interior is retarded, and thus the total heating time must be extended. Note that it is not the *pressure* inside the autoclave that kills the microorganisms but the *high temperatures* that are achieved when steam is placed under pressure.

Pasteurization, a process named for the famous early microbiologist Louis Pasteur (◀ Section 1.11), is not the same as



(a)

J. Martinko

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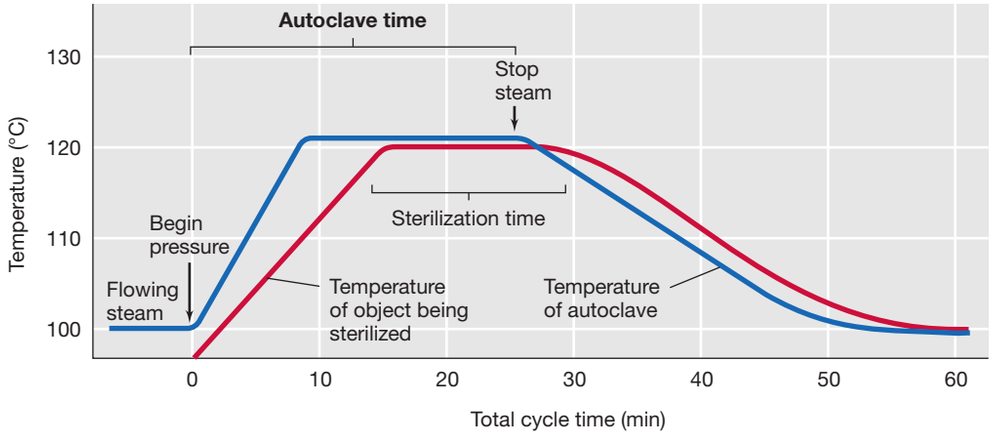


(b)

Figure 4.34 The autoclave and moist heat sterilization. *(a)* The flow of steam through an autoclave. *(b)* A typical autoclave cycle. The temporal heating profile of a fairly bulky object is shown. The temperature of the object rises and falls more slowly than the temperature of the autoclave. The temperature of the object must reach the target temperature and be held for 10–15 min to ensure sterility, regardless of the temperature and time recorded in the autoclave. *(c)* A modern research autoclave containing a pressure-lock door and automatic cycle controls.

sterilization. Pasteurization uses heat to significantly reduce rather than totally eliminate the microorganisms found in liquids, such as milk. At the temperatures and times standardized for the pasteurization of food products, all known pathogenic bacteria are killed. In addition, however, by decreasing the overall microbial load, pasteurization increases the shelf life of perishable liquids.

To pasteurize milk, the liquid is passed through a tubular heat exchanger. Careful control of flow rate and the size and temperature of the heat source raises the temperature of the milk to 71°C for 15 sec (or even higher temperatures for shorter time periods; see time–temperature relationships in Figure 4.33), after which it is rapidly cooled. This process is called *flash pasteurization*. *Ultrahigh-temperature* (*UHT*) *pasteurization* of milk requires heat treatment at 135°C for 1–2 sec and actually sterilizes the milk such that it can be stored at room temperature for long periods without spoilage.

Check Your Understanding

- Why is heat an effective sterilizing agent?
- What steps are necessary to ensure the sterility of material contaminated with bacterial endospores?
- Distinguish between the sterilization of microbiological media and the pasteurization of dairy products.

4.18 Other Physical Control Methods: Radiation and Filtration

In addition to heat, radiation, in particular ultraviolet (UV) light, X-rays, and gamma rays, are also effective microbial killing agents. However, each type of energy has a different mode of action and killing efficacy and thus their applications vary widely.

Ultraviolet and Ionizing Radiation

Ultraviolet radiation between 220 and 300 nm is absorbed by DNA and can cause mutations or have other serious effects on DNA that lead to death of the exposed organism (▶ Section 9.4). UV radiation is useful for disinfecting surfaces and air and is widely used to decontaminate and disinfect the work surface of laboratory laminar flow hoods equipped with a "germicidal" UV light (**Figure 4.35**) and air circulating in hospital and food preparation rooms. However, UV radiation has very poor penetrating power, limiting its use to the disinfection of exposed surfaces or air rather than bulk objects such as canned foods or surgical clothing.

Ionizing radiation is electromagnetic radiation of sufficient energy to produce ions and other reactive molecular species from molecules with which the radiation particles collide. The unit of ionizing radiation is the *roentgen*, and the standard for sterilization is the *absorbed radiation dose*, measured in *rads* (100 erg/g) or *grays* (1 Gy = 100 rad). Ionizing radiation is typically generated from X-ray sources or the radioactive nuclides ^{60}Co and ^{137}Cs. These nuclides produce X-rays or gamma rays, both of which have sufficient energy and penetrating power to kill microorganisms in bulk items such as food products and medical supplies.

Table 4.8 shows the dose necessary for a 10-fold reduction ($D10$) in number of selected microorganisms. The $D10$ value is analogous to the decimal reduction time (D) for heat sterilization, and the killing curve of ionizing radiation yields a similar plot (**Figure 4.36**; compare with Figure 4.33). As is also true of heat treatments, killing endospores with ionizing radiation is more difficult than killing vegetative cells, and viruses are more difficult to kill than bacteria (Table 4.8). In addition, microorganisms in general are much more resistant to ionizing radiation than are multicellular organisms. For example, the lethal radiation dose for humans can be as low as 10 Gy if delivered over a short time period.

In the United States, radiation is used for the sterilization of such diverse items as surgical supplies, plastic labware, drugs, and even tissue grafts. Certain foods and food products such as fresh produce, poultry, meat products, and spices are also routinely irradiated to ensure that they are sterile or at least free of pathogens and insects.

TABLE 4.8 Radiation sensitivity of some representative microorganisms

Type of microorganism	Characteristics	$D10$[a] (Gy)
Bacteria		
Clostridium botulinum	Gram-positive anaerobe; forms endospores	3,300
Deinococcus radiodurans	Gram-negative, radiation-resistant coccus	2,200
Lactobacillus brevis	Gram-positive, rod-shaped	1,200
Bacillus subtilis	Gram-positive aerobe; forms endospores	600
Escherichia coli	Gram-negative, rod-shaped	300
Salmonella enterica (typhimurium)	Gram-negative, rod-shaped	200
Fungi		
Aspergillus niger	Common mold	500
Saccharomyces cerevisiae	Baker's and brewer's yeast	500
Viruses		
Foot-and-mouth	Pathogen of cloven-hoofed animals	13,000
Coxsackie	Human pathogen	4,500

[a]$D10$ is the amount of radiation necessary to reduce the initial population or activity level 10-fold (1 logarithm, see Figure 4.36). Gy, grays. 1 Gy is equivalent to 100 rads. The lethal dose for humans is 10 Gy.

Filter Sterilization

Heat is an effective way to decontaminate most liquids, but not all substances are stable at high temperature and so heat-sensitive liquids that are not sterilized by radiation are typically sterilized by filtration. For sterilization, a filter with pores of average size 0.2 μm is a minimum requirement; however, even such tiny holes will not

Figure 4.35 A laminar flow hood. An ultraviolet light source prevents contamination of the hood when it is not in use. When the hood is in use, air is drawn into the cabinet through a HEPA filter. The filtered air inside the cabinet is exhausted out of the cabinet, preventing contamination of the inside of the hood. The cabinet provides a contaminant-free workspace for microbial and tissue culture manipulations.

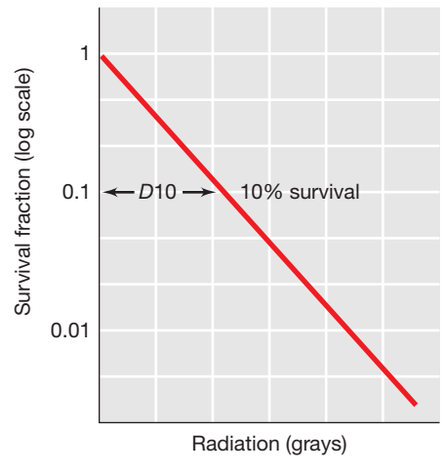
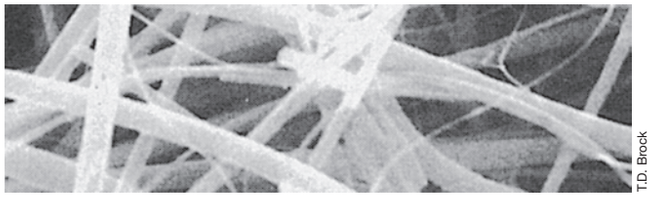


Figure 4.36 Relationship between the survival fraction and the radiation dose of a microorganism. The $D10$, which is the decimal reduction dose, can be interpolated from the data as shown.

trap most viruses. Commonly used filter pore sizes for the filter sterilization of small volumes, such as laboratory solutions, are 0.45 μm and 0.2 μm.

Several types of filters are in routine use in microbiology, including depth filters, membrane filters, and nucleopore filters. A depth filter is a fibrous sheet made from an array of overlapping paper or glass fibers that traps particles in the network of fibers (**Figure 4.37*a***). Depth filters are important in biosafety applications such as in a biological safety cabinet where air, both into and out of the cabinet, flows through a depth filter called a *HEPA filter*, or



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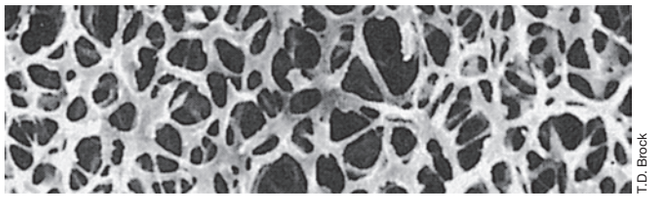

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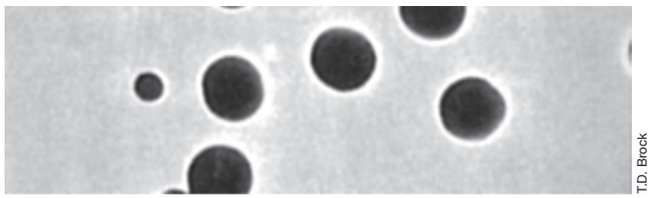

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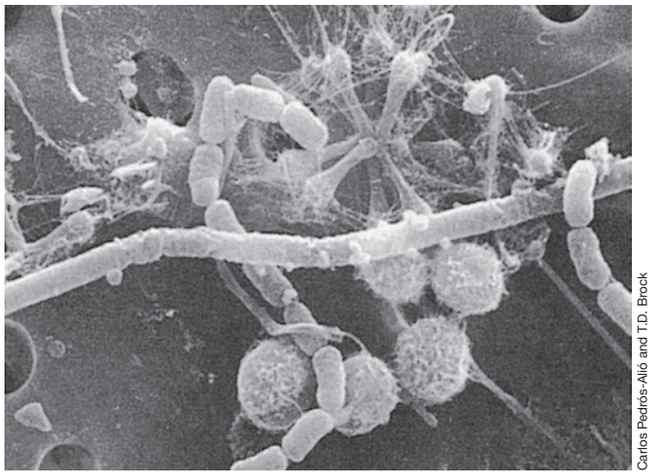

(d)

Figure 4.37 Microbiological filters. Scanning electron micrograph showing the structure of *(a)* a depth filter, *(b)* a conventional membrane filter, and *(c)* a nucleopore filter. *(d)* Scanning electron micrograph of various aquatic microbes trapped on a nucleopore 0.2-μm-pore-size membrane filter.

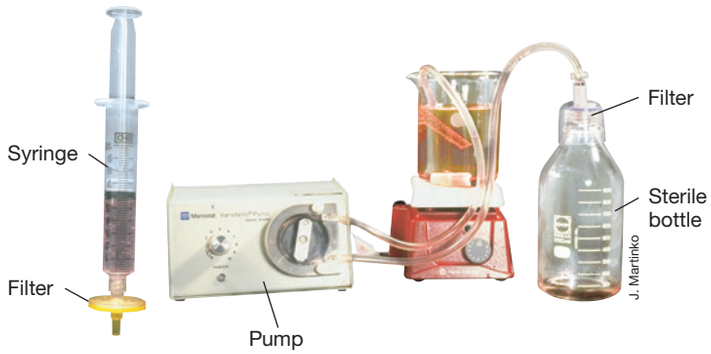

Figure 4.38 Membrane filters. Disposable, presterilized, and assembled membrane filter units. Left: a filter system designed for small volumes forced through the filter by a syringe. Right: a system employing a peristaltic pump for filtering larger volumes.

*h*igh-*e*fficiency *p*articulate *a*ir filter (Figure 4.37*a*). HEPA filters typically remove 0.3-μm or larger particles from an airstream with an efficiency of greater than 99.9%; however, this does not ensure sterilization.

Membrane filters are the most common filters used for liquid sterilization in the microbiology laboratory (Figure 4.37*b* and **Figure 4.38**). Membrane filters are composed of high-tensile-strength polymers manufactured in such a way as to contain a large number of tiny pores. Filtration is accomplished by using a syringe or a pump to force the liquid through the filtration apparatus into a sterile collection vessel (Figure 4.38). Another type of membrane filter is the nucleopore filter. Nucleopore filters are made from a thin polycarbonate film that is treated with radiation and then etched with a chemical, yielding very uniform-sized holes (Figure 4.37*c*). Nucleopore filters are commonly used to isolate specimens for scanning electron microscopy. Microorganisms removed by filtration from a liquid or a natural sample, such as lake water, can then be observed directly on the filter (Figure 4.37*d*).

Check Your Understanding

- Why is ionizing radiation more effective than UV radiation for sterilization of food products?
- Distinguish between the major types of sterilization filters used in the microbiology laboratory.

4.19 Chemical Control of Microbial Growth

Chemicals are routinely used to control microbial growth, and an **antimicrobial agent** is a natural or synthetic chemical that kills or inhibits the growth of microorganisms. Agents that actually kill are called *-cidal* agents, with a prefix indicating the type of microorganism killed. Thus, **bactericidal**, *fungicidal*, and *viricidal* agents kill bacteria, fungi, and viruses, respectively. Agents that do not kill but only inhibit growth are called *-static* agents, and include **bacteriostatic**, *fungistatic*, and *viristatic* compounds. We focus here on chemicals commonly used as disinfectants and reserve discussion of the activities of a very important class of chemicals—the antibiotics—for later (▶ Sections 8.11 and 28.5).

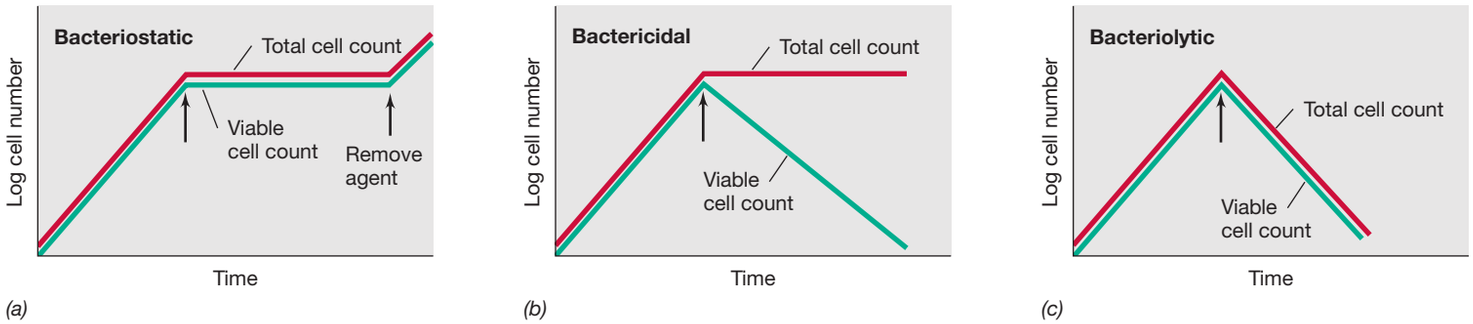

Figure 4.39 **Different types of antimicrobial agents.** *(a)* Bacteriostatic agents inhibit but do not kill. *(b)* Bactericidal agents kill. *(c)* Bacteriolytic agents lyse cells. In each graph, the arrow indicates the time at which a growth-inhibitory concentration of an antimicrobial agent was added to an exponentially growing culture. The turbidity and viable counts shown are characteristic of each type of agent.

Effect of Antimicrobial Agents on Growth

Mastering Microbiology
Art Activity: Figure 4.39 Different types of antimicrobial agents

Antibacterial agents are classified as -static, -cidal, or -lytic (cell lysing) by observing their effects on cultures using viable and turbidimetric growth assays (**Figure 4.39**). Bacteriostatic agents are typically inhibitors of some important biochemical process and bind relatively weakly; if the agent is removed, the cells can resume growing. Some antibiotics, such as the sulfonamides, work in this way. The clinical value of bacteriostatic antibiotics is their ability to keep a pathogenic bacterium from multiplying until the immune system can rid the body of the pathogen. By contrast, bactericidal agents, for example formaldehyde, bind tightly to their cellular targets and by definition kill the cell. However, the dead cells are not lysed, and total cell numbers, reflected in the turbidity of the culture, remain constant (Figure 4.39*b*). Bacteriolytic agents kill cells by lysing them, and this affects both viable and total cell numbers (Figure 4.39*c*). Examples of bacteriolytic agents would be a detergent that ruptures the cytoplasmic membrane or the antibiotic penicillin, which inhibits bacterial cell wall synthesis, resulting in cell lysis.

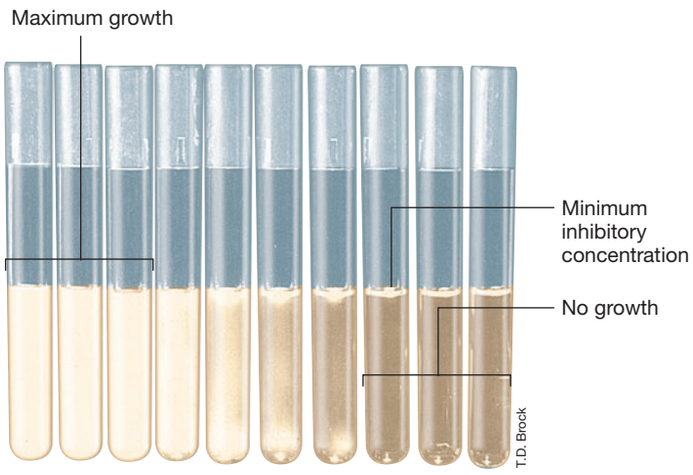

Figure 4.40 **Antimicrobial agent susceptibility assay using dilution methods.** The assay defines the minimum inhibitory concentration (MIC). A series of increasing concentrations (from left to right) of antimicrobial agent is prepared in the culture medium. Each tube is inoculated with a specific concentration of a test organism, followed by a defined incubation period. Growth, measured as turbidity, occurs in those tubes with antimicrobial agent concentrations below the MIC.

Assaying Antimicrobial Activity

Antimicrobial activity can be measured by determining the smallest amount of the agent needed to inhibit the growth of a test organism, a value called the **minimum inhibitory concentration (MIC)**. One way to determine the MIC of a given agent is to inoculate a series of tubes of liquid growth medium (**Figure 4.40**) containing a test organism and dilutions of the agent. Following incubation, the tubes are scored for growth (turbidimetrically), and the MIC is revealed as the lowest concentration of antimicrobial agent that completely inhibits growth.

Antimicrobial activity can also be assessed using solid media (**Figure 4.41**). Known amounts of an antimicrobial agent are added

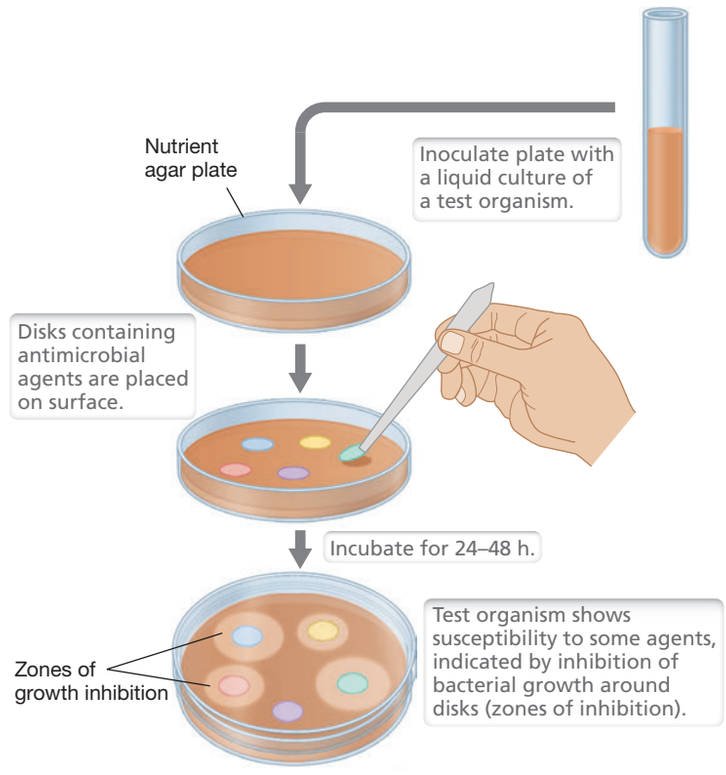

Figure 4.41 **Antimicrobial agent susceptibility assay using diffusion methods.** The antimicrobial agent diffuses from paper disks into the surrounding agar, inhibiting growth of susceptible microorganisms.

to filter-paper disks and the disks are arranged on the surface of a uniformly inoculated agar plate. During incubation, the agent diffuses from the disk into the agar, establishing a gradient; the farther a chemical diffuses away from a disk, the lower its concentration. Following an incubation period, a *zone of growth inhibition* forms around disks that released effective chemicals. The zone is a function of several factors, including the amount of antimicrobial agent added to the disk, its solubility and diffusion coefficient, and, most importantly, its overall effectiveness. The disk diffusion assay is routinely used to test clinically isolated pathogenic bacteria for their antibiotic susceptibility (▶ Section 28.5).

Chemical Antimicrobial Agents

Several antimicrobial agents are used to prevent the growth of human pathogens on inanimate surfaces and on external body surfaces. These include sterilants, disinfectants, sanitizers, and antiseptics (Table 4.9).

Sterilants destroy all microorganisms, including endospores. Chemical sterilants are used for decontamination or sterilization in situations where it is impractical or impossible to use heat or radiation. Hospitals, clinics, and laboratories, for example, must routinely decontaminate and sterilize heat-sensitive materials such as thermometers, lensed instruments, polyethylene tubing, catheters, and reusable medical and dental equipment. This process of *cold sterilization*, as it is called, employs gases such as ethylene oxide or aldehydes such as formaldehyde or glutaraldehyde to sterilize the devices.

Disinfectants are chemicals that kill microorganisms but not necessarily endospores and are primarily used on surfaces. For example, phenol and cationic detergents are used to disinfect floors,

TABLE 4.9 Antiseptics, sterilants, disinfectants, and sanitizers[a]

Agent	Mode of action	Use
Antiseptics (germicides)		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	Topical antiseptic
Phenol-containing compounds (hexachlorophene, triclosan, chloroxylenol, chlorhexidine)	Disrupts cytoplasmic membrane	Soaps, lotions, cosmetics, deodorants, topical disinfectants; paper, leather, and textile industries
Cationic detergents, especially quaternary ammonium compounds (benzalkonium chloride)	Disrupts cytoplasmic membrane	Soaps, lotions, topical disinfectants; metal and petroleum industries
Hydrogen peroxide (3% solution)	Oxidizing agent	Topical antiseptic
Iodophors (Betadine®)	Iodinates proteins, rendering them nonfunctional; oxidizing agent	Topical antiseptic
Octenidine	Cationic surfactant, disrupts cytoplasmic membrane	Topical antiseptic
Sterilants, disinfectants, and sanitizers		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	General-purpose disinfectant for virtually any surface
Cationic detergents (quaternary ammonium compounds, Lysol® and many related disinfectants)	Interacts with phospholipids	Disinfectant/sanitizer for medical instruments, food and dairy equipment
Chlorine gas	Oxidizing agent	Disinfectant for drinking water and electrical/nuclear cooling towers
Chlorine compounds (chloramines, sodium hypochlorite, sodium chlorite, chlorine dioxide)	Oxidizing agent	Disinfectant/sanitizer for medical instruments, food/dairy equipment, and in water purification
Copper sulfate	Protein precipitant	Algicide in swimming pools
Ethylene oxide (gas)	Alkylating agent	Sterilant for temperature-sensitive materials such as plastics
Formaldehyde	Alkylating agent	Diluted (3% solution) as surface disinfectant/sterilant; concentrated (37% solution) as sterilant
Glutaraldehyde	Alkylating agent	Disinfectant or sterilant as 2% solution
Hydrogen peroxide	Oxidizing agent	Vapor used as sterilant
Iodophors (Wescodyne®)	Iodinates proteins; oxidizing agent	General disinfectant
OPA (ortho-phthalaldehyde)	Alkylating agent	Powerful disinfectant used for sterilizing medical instruments
Ozone	Strong oxidizing agent	Disinfectant for drinking water
Peroxyacetic acid	Strong oxidizing agent	Disinfectant/sterilant
Phenolic compounds	Protein denaturant	General-purpose disinfectant
Pine oils (Pine-Sol®) (contains phenolics and detergents)	Protein denaturant	General-purpose disinfectant for household surfaces

[a]Alcohols, hydrogen peroxide, and iodophors can be antiseptics, disinfectants, sanitizers, or sterilants depending on concentration, length of exposure, and form of delivery.

tables, bench tops, walls, and so on (Table 4.9) and are important agents of infection control in hospitals and other medical settings. **Sanitizers**, by contrast, are less harsh than disinfectants and reduce microbial numbers but do not sterilize. Sanitizers are widely used in the food industry to treat surfaces such as mixing and cooking equipment, dishes, and utensils, and are also used for dry hand washing when water is unavailable. **Antiseptics**, often called **germicides**, are chemicals that kill or inhibit the growth of microorganisms but are sufficiently nontoxic to animals to be applied to living tissues. Most germicides are used for hand washing or for treating surface wounds (Table 4.9). Certain antiseptics are also effective disinfectants. Ethanol, for example, can be both an antiseptic and a disinfectant, depending on the concentration and exposure time employed.

Several factors affect the efficacy of any chemical antimicrobial agent. For example, many antimicrobial agents are bound and inactivated by organic matter; thus, disinfecting a kitchen countertop littered with spilled foods is more difficult than disinfecting a clean countertop. Furthermore, bacteria can form biofilms (Section 4.9), covering surfaces of tissue or soiled medical devices with microbial cells embedded in polysaccharides. Biofilms may slow or even completely prevent penetration of antimicrobial agents, reducing or negating their effectiveness. Thus, the ultimate efficacy of any antimicrobial agent must be determined empirically and under the actual conditions of use. Only by actually testing the chemical and assaying for microbial growth both before and after treatment can one be confident that the agent is working as it should.

Check Your Understanding

- Distinguish between the antimicrobial effects of -static, -cidal, and -lytic agents.
- Describe how the minimum inhibitory concentration of an antibacterial agent is determined.
- Distinguish between a sterilant, a disinfectant, and an antiseptic. What is cold sterilization?

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CHAPTER REVIEW

I • Culturing Microbes and Measuring Their Growth

4.1 Cells are primarily composed of the elements H, O, C, N, P, and S. Nutrients required by a cell in large amounts are called macronutrients while those required in very small amounts, such as trace elements or growth factors, are micronutrients. Proteins are the most abundant class of macromolecules in the cell.

Q What is the major difference between macronutrients and micronutrients? List a few macronutrients and micronutrients.

4.2 Culture media supply the nutritional needs of microorganisms and are either defined or complex. Other media, such as selective and differential media, are used for specific purposes. Many microorganisms can be grown in liquid or solid culture media, and pure cultures can be maintained if aseptic technique is practiced.

Q Why would the following medium not be considered a chemically defined medium: glucose, 5 grams (g); NH_4Cl, 1 g; KH_2PO_4, 1 g; $MgSO_4$, 0.3 g; yeast extract, 5 g; distilled water, 1 liter? What is aseptic technique and why is it necessary?

4.3 Total cell counts can be done under the microscope using counting chambers and are useful for assessing total cell numbers in a microbial habitat or laboratory culture. Certain stains can be used to target specific cell populations in a sample.

Q When assessing a culture's performance, in which growth phase would total cell counting be suitable, and in which growth phase would knowing the viability of the culture or sample become necessary?

4.4 Viable cell counts measure only the living population present in the sample with the assumption that each colony originates from the growth and division of a single cell. Depending on the growth medium and conditions employed, and the nature of the sample, viable counts can be fairly accurate assessments or highly unreliable.

Q How does a viable count differ from a total count?

4.5 Turbidity measurements are a rapid and useful method of measuring microbial growth and are based on the fact that cells in suspension scatter light. In order to relate a turbidity value to a cell number, a standard curve plotting these two parameters against one another must first be established.

Q How can turbidity be used as a measure of cell numbers?

II • Dynamics of Microbial Growth

4.6 Microbial growth is defined as an increase in cell numbers and is the final result of the doubling of all cell components prior to actual division that yields two daughter cells. Microorganisms show a characteristic growth pattern when inoculated into a fresh culture medium. There is usually a lag phase and then growth commences in an exponential fashion. As essential nutrients are depleted and/or toxic products accumulate, growth ceases and the population enters the stationary phase. Further incubation can lead to cell death.

Q How does the growth pattern of a population of bacteria inoculated into a fresh medium from an old culture differ from the growth pattern of a population inoculated into a fresh medium from a culture in the mid-exponential phase of growth?

4.7 Microbial cells undergo exponential growth, and a semilogarithmic plot of cell numbers with time can reveal the doubling time of the population. Various growth expressions can be calculated from cell number data obtained from an exponentially growing culture. Key expressions here are n, the number of generations; t, time; g, generation time; and k, the instantaneous growth rate constant.

Q How is the generation time (g) of an exponentially growing culture determined?

4.8 The chemostat is an open system used to maintain cell populations in exponential growth for extended periods. In a chemostat, the rate at which a culture is diluted with fresh growth medium controls the growth rate of the population, while the cell density (cells/ml) is controlled by the concentration of a growth-limiting nutrient present in the fresh medium.

Q How does a chemostat regulate growth rate and cell density independently?

4.9 Biofilms are an alternative growth mode to a suspended (planktonic) lifestyle and consist of a microbial community enmeshed in a sticky polysaccharide matrix attached to a surface. Biofilms promote intercellular communication and offer protection from predation, harmful substances, and being washed away.

Q What happens during each of the steps that occur in the formation of a microbial biofilm?

4.10 Most microorganisms grow by binary fission but some grow by budding. Budding bacteria show unequal cell growth and produce daughter cells that have different characteristics. Some bacteria such as the actinomycetes form long filaments called hyphae that grow from their tips.

Q What are the advantages of biofilms as a growth mode over a planktonic lifestyle?

III • Environmental Effects on Growth: Temperature

4.11 Temperature is a major environmental factor controlling microbial growth. An organism's cardinal temperatures are the minimum, optimum, and maximum temperatures at which it grows. Microorganisms can be grouped by their cardinal temperature from cold-loving to heat-loving as psychrophiles, mesophiles, thermophiles, and hyperthermophiles.

Q Examine the graph in Figure 4.21. Why is the optimum temperature for an organism usually closer to its maximum than its minimum?

4.12 Organisms with temperature optima below 15°C are called psychrophiles, and the most extreme representatives inhabit constantly cold environments. Psychrophiles synthesize macromolecules that remain flexible and functional at cold temperatures but that can be unusually sensitive to warm temperatures.

Q How do the proteins and lipids of psychrophiles differ from those of mesophiles?

4.13 Organisms with growth temperature optima between 45 and 80°C are called thermophiles while those with optima greater than 80°C are hyperthermophiles. These organisms inhabit hot environments that can have temperatures even above 100°C. Thermophiles and hyperthermophiles produce heat-stable macromolecules.

Q What are some important biotechnological uses from hyperthermophiles?

IV • Environmental Effects on Growth: pH, Osmolarity, and Oxygen

4.14 The acidity or alkalinity of an environment can greatly affect microbial growth. Some organisms grow best at low or high pH (acidophiles and alkaliphiles, respectively), but most organisms grow best between pH 5.5 and 8. The internal pH of a cell must stay relatively close to neutral to prevent the spontaneous destruction of macromolecules at pH extremes.

Q Concerning the pH of the environment and of the cell cytoplasm, in what ways are acidophiles and alkaliphiles different? In what ways are they similar?

4.15 The water activity of an aqueous environment is a function of its solute concentration. To survive in high-solute environments, organisms produce or accumulate compatible solutes to maintain positive water balance. Some microorganisms grow best at reduced water potential and some even require high levels of salts for growth.

Q How does a halophile maintain positive water balance while growing in a solution high in NaCl?

4.16 Aerobes require O_2 while anaerobes do not and may even be killed by O_2. Facultative organisms can live with or without O_2. Special techniques are needed to grow aerobic and anaerobic microorganisms. Several toxic forms of oxygen can form in the cell, but enzymes are present that neutralize most of them. Superoxide is a major toxic form of oxygen.

Q Contrast an aerotolerant and an obligate anaerobe in terms of sensitivity to O_2 and ability to grow in the presence of O_2. Compare and contrast the enzymes catalase, superoxide dismutase, and superoxide reductase in regard to their substrates and products.

V • Controlling Microbial Growth

4.17 Sterilization is the killing of all microbes including viruses, and heat is the most widely used method of sterilization. An autoclave employs moist heat under pressure to achieve temperatures above the boiling point of water. Pasteurization does not sterilize liquids, but it reduces the microbial load and kills pathogens.

Q Contrast the terms thermal death time and decimal reduction time. How would the presence of bacterial endospores affect either value? What time and temperature is necessary to ensure sterility in the autoclave?

4.18 Radiation can effectively inhibit or kill microorganisms. Ultraviolet radiation is used for decontaminating surfaces and air, whereas ionizing radiation is used for sterilization where penetration is required. Filters remove microorganisms from air or liquids. Membrane and nucleopore filters are used for sterilization of heat-sensitive liquids and to examine the contents of filtration by microscopy.

Q Describe the effects of a lethal dose of ionizing radiation at the molecular level. What type of filter would be used to filter sterilize a heat-sensitive liquid?

4.19 Chemicals that kill organisms are called -cidal agents while those that arrest growth but do not kill are called -static

agents. Antimicrobial agents are tested for efficacy by determining their ability to inhibit growth. Sterilants, disinfectants, and sanitizers are used to decontaminate nonliving material, while antiseptics (germicides) are used to reduce the microbial load on living tissues.

Q Describe the procedure for obtaining the minimum inhibitory concentration (MIC) for a chemical that is bactericidal for *Escherichia coli*. Contrast the action of disinfectants and antiseptics.

APPLICATION QUESTIONS

1. A medium was inoculated with 5×10^6 cells/ml of *Escherichia coli* cells. Following a 1-h lag, the population grew exponentially for 5 h, after which the population was 5.4×10^9 cells/ml. Calculate g and k for this growth experiment.
2. *Escherichia coli* but not *Pyrolobus fumarii* will grow at 40°C, while *P. fumarii* but not *E. coli* will grow at 110°C. What is happening (or not happening) to prevent growth of each organism at the nonpermissive temperature?
3. In which direction (into or out of the cell) will water flow in cells of *Escherichia coli* (an organism found in your large intestine) suddenly suspended in a solution of 20% NaCl? What if the cells were suspended in distilled water? If growth nutrients were added to each cell suspension, which (if either) would support growth, and why?

CHAPTER GLOSSARY

Acidophile an organism that grows best at low pH, typically below pH 5.5
Aerobe an organism that can use O_2 in respiration; some require O_2
Aerotolerant anaerobe a microorganism unable to respire O_2 but whose growth is unaffected by it
Alkaliphile an organism that has a growth pH optimum of 8 or higher
Anaerobe an organism that cannot use O_2 in respiration and whose growth is typically inhibited by O_2
Antimicrobial agent a chemical compound that kills or inhibits the growth of microorganisms
Antiseptic (germicide) a chemical agent that kills or inhibits growth of microorganisms and is sufficiently nontoxic to be applied to living tissues
Aseptic technique a series of steps taken to prevent contamination of laboratory cultures and media
Autoclave a sealed heating device that destroys microorganisms with temperature and steam under pressure
Bactericidal agent an agent that kills bacteria
Bacteriostatic agent an agent that inhibits bacterial growth
Batch culture a closed-system microbial culture of fixed volume
Binary fission cell division following enlargement of a cell to twice its minimum size
Biofilm colonies of microbial cells encased in a porous organic matrix and attached to a surface
Budding division a cell division process whereby new cell material is produced from a single point instead of along the entire cell
Cardinal temperatures the minimum, maximum, and optimum growth temperatures for a given organism
Chemostat a device that allows for the continuous culture of microorganisms with independent control of both growth rate and cell number
Colony morphology the visible appearance of a colony grown on a solid medium
Compatible solute a molecule that is accumulated in the cytoplasm of a cell for adjustment of water activity but that does not inhibit biochemical processes
Complex medium a culture medium of highly nutritious substances for which the exact composition is unknown
Culture medium a nutrient solution required for growing a laboratory culture of a specific microorganism
Decline phase the component of a microbial growth curve during which the rate of cell death exceeds the rate of cell growth
Defined medium a culture medium for which the exact composition is known
Disinfectant an antimicrobial agent used only on inanimate objects
Disinfection rendering a surface or object free of all pathogenic microorganisms
Exponential phase (exponential growth) a condition during which the cell numbers in a microbial population double at regular intervals
Extreme halophile a microorganism that requires very large amounts of NaCl, usually greater than 10% and in some cases near saturation, for growth
Facultative with respect to O_2, an organism that can grow in either its presence or absence
Generation time the time required for a population of microbial cells to double
Germicide (antiseptic) a chemical agent that kills or inhibits growth of microorganisms and is sufficiently nontoxic to be applied to living tissues
Growth curve the dynamics of microbial growth observed in batch culture, consisting of four phases: lag, exponential, stationary, and decline
Halophile a microorganism that requires NaCl for growth
Halotolerant a microorganism that does not require NaCl for growth but can grow

in the presence of NaCl, in some cases, substantial levels of NaCl

Hyperthermophile a species of *Bacteria* or *Archaea* whose growth temperature optimum is 80°C or greater

Hyphal growth a form of growth in which cells elongate only at the tip of a growing filament

Lag phase the component of a microbial growth curve prior to the onset of growth

Mesophile an organism that grows best at temperatures between 20 and 40°C

Microaerophile an aerobic organism that can grow only when O_2 tensions are reduced from that present in air

Minimum inhibitory concentration (MIC) The smallest amount of an antimicrobial substance that prevents growth of the target microorganism

Neutrophile an organism that grows best at neutral pH, between pH 5.5 and 8

Obligate anaerobe an organism that cannot grow in the presence of O_2

Osmophile an organism that grows best in the presence of high levels of solute, typically a sugar

Pasteurization the heat treatment of milk or another liquid to reduce its total number of microorganisms

pH the negative logarithm of the hydrogen ion (H^+) concentration of a solution

Plate count a method for counting viable cells; the number of colonies on a plate is used as a measure of cell numbers

Psychrophile an organism with a growth temperature optimum of 15°C or lower and a maximum growth temperature below 20°C

Psychrotolerant capable of growing at low temperatures but having an optimum above 20°C

Sanitizer an agent that reduces microorganisms to a safe level, but may not eliminate them

Stationary phase the component of a microbial growth curve during which the rate of growth and death are approximately equal

Sterilant a chemical agent that destroys all forms of microbial life

Sterilization the killing or removal of all living organisms and viruses

Thermophile an organism whose growth temperature optimum lies between 45 and 80°C

Viable capable of reproducing

Viable count a measurement of the number of live cells in a population

Water activity the ratio of the vapor pressure of air in equilibrium with a solution to the vapor pressure of pure water

Xerophile an organism that is able to live, or that lives best, in very dry environments

5 Viruses and Their Multiplication

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II Overview of the Viral Replication Cycle 191

MICROBIOLOGYNOW

When Antibiotics Fail, Bacteriophage Therapy to the Rescue

Acquiring an antibiotic-resistant infection or "superbug" is one of medicine's biggest nightmares. What can medical practitioners do to treat the patient? Besides drugs, viruses known as bacteriophages have been recruited to specifically target and kill bacteria.

Despite microbiologists' tinkering with using bacteriophages as antimicrobials for decades, their actual application in medicine has been minimal. However, the emergence of antibiotic resistance has led to renewed focus on using these tiny microbes as therapeutic agents. The photo above shows Ella Balasa (right side of photo), a microbiologist who has cystic fibrosis. Cystic fibrosis is a genetic disease that results in a buildup of thick mucus in the lungs. This mucus allows bacteria to flourish in the lungs, which results in infections and subsequent lung damage that can be fatal. Ella had been treated numerous times with strong antibiotics specific for a respiratory infection caused by the bacterial pathogen *Pseudomonas aeruginosa,* but the microbial cells had become unresponsive to the drugs. At the time of this photo, the recurrent infection had decreased her lung function to the point where she required constant supplemental oxygen.

As an alternative treatment route, Dr. Benjamin Chan (on the left) took mucus from Ella's lungs infected with *P. aeruginosa* and isolated a bacteriophage that specifically killed the pathogen (see zones of clearing on Petri plate). This bacteriophage was propagated and then poured into a device so that Ella could inhale the therapy. The result of her treatment? Amazingly, the bacteriophage therapy along with a mixture of antibiotics resulted in the infection clearing a few weeks later!

While bacteriophage therapy is limited by bacterial host specificity and the ability of the pathogenic bacteria to become resistant to viral infection, this trial and other recent success stories illustrate the future promise of phage therapy when all other options fail.

Source: Kortright, K.E., B.K. Chan, J.L. Koff, and P.E. Turner. 2019. Phage therapy: A renewed approach to combat antibiotic-resistant bacteria. *Cell Host Microbe 25*(2): 219.

Thus far in Unit 1 we have focused on the fundamentals of the three phylogenetic groups of cells—*Bacteria*, *Archaea*, and microbial *Eukarya*—by highlighting their structure, growth, and overall impact on the biosphere. Here we shift gears and discuss the most abundant microbes on Earth—viruses. With the population of viruses estimated to outnumber bacterial and archaeal cells by a factor of 10, viruses have a huge impact on the biosphere and have a unique biology reminiscent of, yet distinct from, that of the cellular world.

The study of viruses is the science of *virology*, and this chapter covers the basic principles needed to understand their biology. In Chapter 11 we consider the genomic, replication, and diversity aspects of viruses in detail, and in Unit 7 we consider pathogenic viruses and the extensive role they play in governing human health and disease.

I • The Nature of Viruses

Viruses are noncellular microbes that show many of the properties of cells except that viruses absolutely require a growing host cell in order to multiply. Viral diversity in nature is enormous, virus densities can be quantified, and cells in all domains of life are susceptible to viral infection.

5.1 What Is a Virus?

A **virus** is a genetic element that can multiply only inside a living cell, called the **host cell**. Not considered living entities, viruses are not found on the tree of life (◀ Figure 1.41*b*). Viruses rely on the host cell for energy, metabolic intermediates, and protein synthesis, and so they are *obligate intracellular parasites*. However, viruses possess their own nucleic acid genomes and in this sense are independent of the host's genome. Viruses infect cells in all three domains of life and are responsible for many infectious diseases of humans, plants, and other organisms.

Although viruses are not cells, their genomes encode those functions needed to multiply and they have a structurally intricate extracellular form, called the **virion** (Figure 5.1); the virion allows the virus to travel from one host cell to another. Viruses cannot reproduce unless the virion itself, or in some cases its genome only, has gained entry into a suitable growing host cell, a process called *infection*.

Viral Components and Activities

Virions are composed of a protein shell, called the **capsid**, and the virus genome that the capsid contains. Most bacterial and plant viruses are *naked*, with no further layers, whereas many other types of viruses, especially animal viruses, have an outer layer. If the layer is composed of a phospholipid bilayer taken from the host cell membrane, it is called the *envelope* (Figure 5.2). In enveloped viruses, the inner structure of nucleic acid plus capsid protein is called the **nucleocapsid**. The virion protects the viral genome when the virus is outside the host cell, and proteins on the virion surface are important in attaching it to its host cell. The virion may also contain one

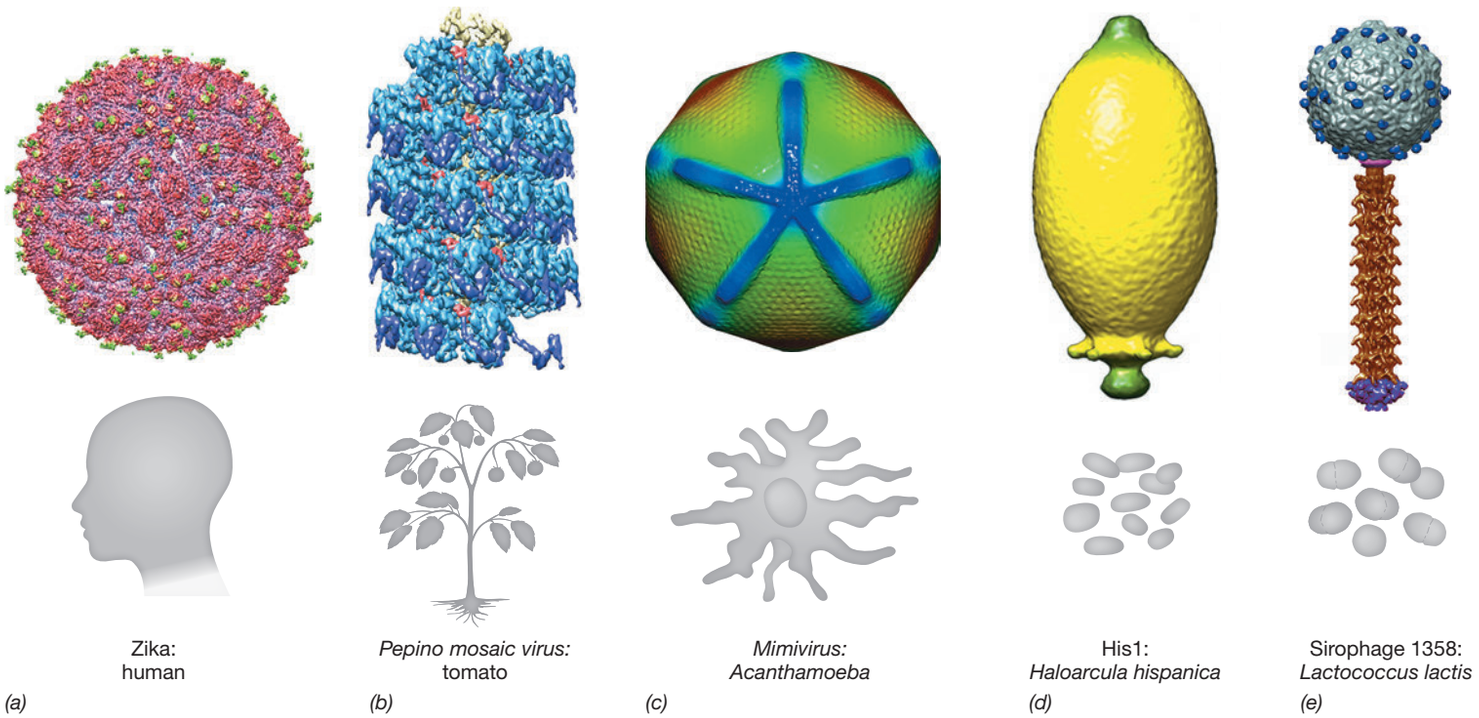


Figure 5.1 Virion morphology and their hosts. Cryo-electron micrograph (◀ Section 1.10) reconstructions of the following virions: *(a)* Zika virus (40 nm) that infects humans. *(b) Pepino mosaic virus* (12.5 × 510 nm) that infects tomatoes. *(c) Mimivirus* (400 nm) that infects *Acanthamoeba*, a protozoan. *(d)* His1 virus (74 × 44 nm) that infects *Haloarcula hispanica* (*Archaea*). *(e)* Sirophage 1358 (54 × 103 nm) that infects *Lactococcus lactis* (*Bacteria*). Credits for micrographs: *(a)* Sun, L., Sirohi, D., Chen, Z., Klose, T., Pierson, T.C., Rossmann, M.G., and Kuhn, R.J. *(b)* Agirrezabala, X., Méndez-López, E., Lasso, G., Sánchez-Pina, M.A., Aranda, M. and Valle, M. 2015. *eLife 4*: e11795. *(c)* Xiao, C., Kuznetsov, Y.G., Sun, S., Hafenstein, S.L., Kostyuchenko, V.A., Chipman, P.R., Suzan-Monti, M., Raoult, D., McPherson, A, and Rossmann, M.G. EMDataBank ID: 5039. *(d)* Hong, C., Pietila, M.K, Fu, C., Schmid, M.F., Bamford, D.H., and Chiu W. EMDataBank ID: 6221. *(e)* Christian Cambillau.

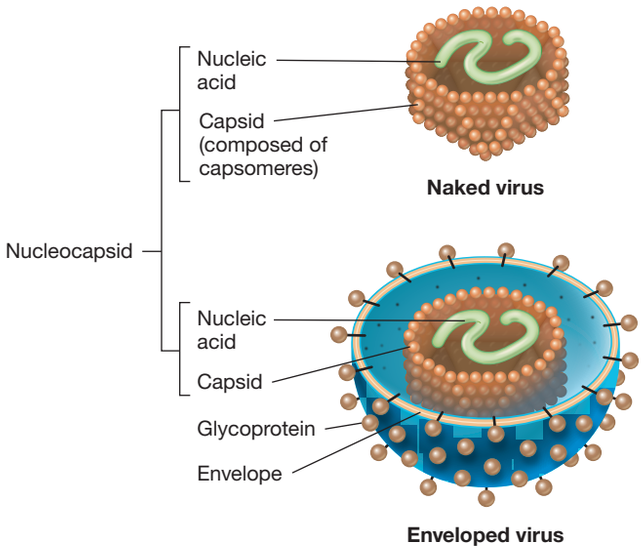


Figure 5.2 Comparison of naked and enveloped virus particles. The envelope originates from host cytoplasmic membrane.

or more virus-specific enzymes that play a role during infection and replication, as discussed later.

Once inside the host cell, a viral genome can orchestrate one of two quite different events. The virus may replicate and destroy the host in a *virulent infection* via a **lytic pathway**. In a lytic infection, the virus redirects the host cell's metabolism from growth to support virus multiplication and the assembly of new virions. Eventually, new virions are released, and the process can repeat itself within new host cells. Alternatively, some viruses can cause a *lysogenic infection*; in this case, the host cell is not destroyed and the viral genome becomes part of the host genome. These types of infection are discussed in detail later in the chapter (Section 5.6).

Viral Diversity and Hosts

While all cells contain double-stranded DNA genomes, viruses often break this rule by having genomes that consist of either DNA *or* RNA and are further subdivided based on whether the genome is *single-stranded* or *double-stranded*. As one might expect, this interesting feature of viral genomes requires special processes for genetic information flow (Chapter 11). Also, while viruses are generally considered to be smaller in both size and gene content than cells, some viruses do not fit into this paradigm. Giant viruses of protozoa are known that possess genomes larger than those of some bacteria. This is in sharp contrast to a common virus such as influenza A virus, which contains only seven genes, and some viruses have even fewer genes than this.

Viruses can be classified on the basis of the hosts they infect as well as by their genome structure (▶ Section 11.1). Thus, we have bacterial viruses, archaeal viruses, animal viruses, plant viruses, protozoan viruses, and so on (Figure 5.1). Bacterial viruses are called **bacteriophages** (or simply *phage* for short) and have been intensively studied as model systems for the molecular biology and genetics of virus replication. In this chapter we will use bacteriophages many times to illustrate basic viral principles. Indeed, many of the key tenets of virology were discovered in studies of bacteriophages and subsequently applied to viruses of higher organisms.

Because of their frequent medical and agricultural importance, disease-causing viruses have been extensively studied. However, not all viruses have negative effects on their hosts, and in some cases viruses are even beneficial to their hosts! For example, when *Arabidopsis* plants are infected with plum pox virus, the plant's tolerance to drought increases due to virally induced production of the plant growth factor salicylic acid (**Figure 5.3**). In the rosy apple aphid, infection by an insect densovirus results in both decreased aphid size and offspring numbers. However, despite these negative effects, the infection also results in the formation of wings, structures not produced in uninfected aphids. Wing formation benefits the virus-infected aphid as it allows it to fly to new apple trees to feed on. And finally, in humans, although hepatitis G virus rarely causes symptoms in healthy adults, humans coinfected with both HIV (human immunodeficiency virus) and hepatitis G virus benefit from the hepatitis G infection because it decreases the replication rate and infectivity of HIV.

With viruses being the most abundant entity on Earth, numerous other examples of positive host–virus interactions likely exist in nature. But coupled with these, of course, are the countless negative

(a) **Watered plants**

(b) **Drought conditions**

Figure 5.3 Virus-infected *Arabidopsis thaliana* plants and response to drought. The plants on the left are uninfected, while the plants on the right are infected with plum pox virus. *(a)* In well-watered plants, the virus affects the plant negatively. *(b)* Under drought conditions, the virus affects the plant positively. Images adapted from Aguilar, E., Cutrona, C., Del Toro, F.J., Vallarino, J.G., Osorio, S., Perez-Bueno, M.L., Baron, M., Chung, B.M., Canto, T., and Tenllado, F. 2017. *Plant, Cell Environ. 40*: 2909.

interactions brought about by viral infection, and examples of these will unfold in many places throughout this book.

Check Your Understanding

- How does a virus differ from a cell?
- Why does a virus need a host cell?
- Compared with cells, what is unusual about viral genomes?

5.2 Structure of the Virion

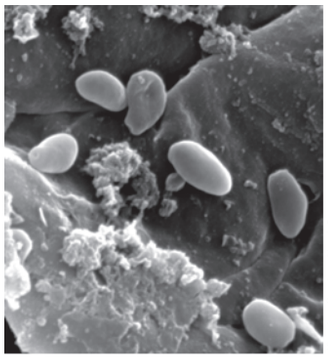
Virions come in many shapes and sizes (Figure 5.1). Most viruses are smaller than prokaryotic cells, ranging in size from 0.02 to 0.3 μm (20–300 nanometers, nm). While the human smallpox virus is about the size of the smallest known bacterial cells (200 nm in diameter, ◀ Section 1.3), giant viruses exist. Viruses such as the *Pandoravirus*, which has been isolated from protozoa, can rival the size of bacterial cells at over 1 μm in length (**Figure 5.4**). Poliovirus, one of the smallest viruses at only 28 nm in diameter, is on the opposite end of the size spectrum. This tiny virus is about the size of a ribosome, the cell's protein-synthesizing machine (◀ Section 1.2).

Virion Structure

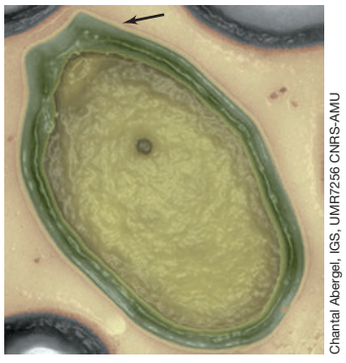
The structures of virions are quite diverse, varying widely in size, shape, and chemical composition. The nucleic acid of a virion is always surrounded by its capsid (Figure 5.2). The capsid is composed of a number of individual protein molecules called **capsomeres** that are often arranged in a precise and highly repetitive pattern around the nucleic acid.

The small size of most viral genomes restricts the number of distinct viral proteins that can be encoded. As a consequence, a few viruses have only a single kind of protein in their capsid. An example is the well-studied tobacco mosaic virus (TMV), which causes disease in tobacco, tomato, and related plants. TMV is a virus in which the 2130 copies of the capsomere protein are arranged in a helix with dimensions of 18 × 300 nm (**Figure 5.5**).

The information required for the proper folding and assembly of viral proteins into capsomeres and subsequently into capsids is often embedded within the amino acid sequence of the viral proteins themselves. When this is the case, virion assembly is a spontaneous process and is called *self-assembly*. However, some virus proteins and structures require assistance from the host cell's folding proteins for proper folding and assembly. For example, the capsid protein of bacteriophage lambda (Section 5.6) requires assistance from an *Escherichia coli* host cell's chaperone protein in order to fold into its active conformation.

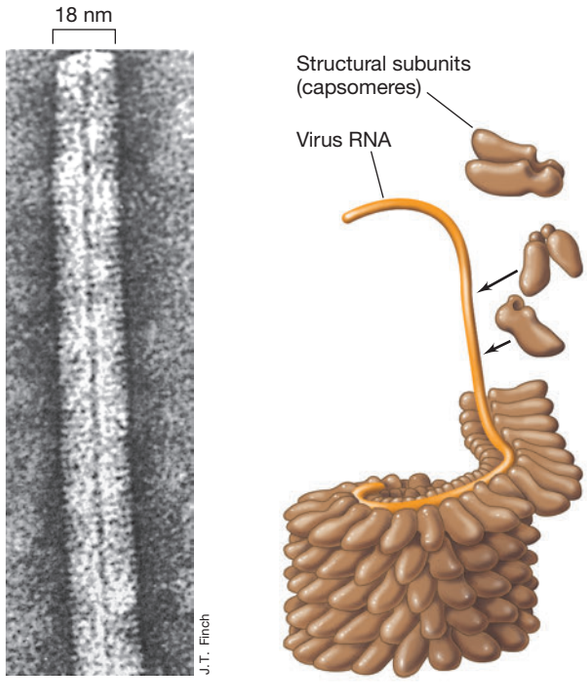


(a)



(b)

Figure 5.4 The giant and asymmetrical *Pandoravirus*. *(a)* Scanning electron micrograph of *Pandoravirus* in a sewage sample from Mergulhão, Brazil. *(b)* Color-enhanced transmission electron micrograph of *Pandoravirus*. The virion is ~1.2 μm long and the arrow indicates apical pore.



(a) (b)

Figure 5.5 The arrangement of RNA and protein coat in tobacco mosaic virus, a simple naked virus. *(a)* High-resolution electron micrograph of a portion of the tobacco mosaic virus particle. *(b)* Cutaway showing structure of the virion. The RNA forms a helix surrounded by the protein subunits (capsomeres). The center of the virus particle is hollow.

Virus Symmetry

Many virions are highly symmetric structures. When a symmetric structure is rotated around an axis, the same form is seen again after a certain number of degrees of rotation. Two kinds of symmetry are recognized in viruses, which correspond to the two primary viral shapes, rod and spherical. Rod-shaped viruses have *helical* symmetry while spherical viruses have *icosahedral* symmetry. A typical virus with helical symmetry is TMV (Figure 5.5). The lengths of helical viruses are determined by the length of the nucleic acid, and the width of the helical virion is determined by the size and packaging of the capsomeres.

Viruses with icosahedral symmetry contain 20 triangular faces and 12 vertices and are roughly spherical in shape (**Figure 5.6*a***). Axes of symmetry divide the icosahedron into 5, 3, or 2 segments of identical size and shape (Figure 5.6*b*). Icosahedral symmetry is the most efficient arrangement of subunits in a closed shell because it requires the smallest number of capsomeres to build the shell. The simplest arrangement of capsomeres is 3 per triangular face, for a total of 60 capsomeres per virion. However, most viruses have more nucleic

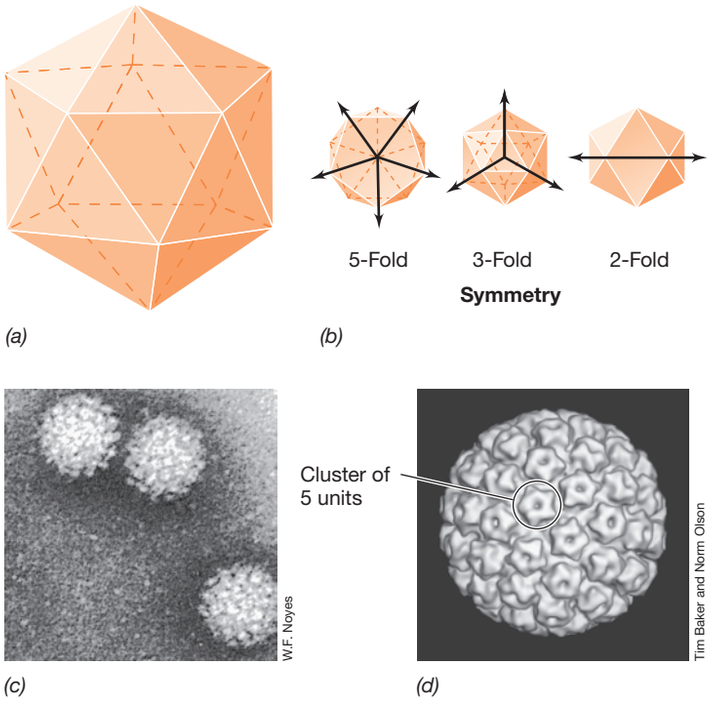


Figure 5.6 Icosahedral symmetry. *(a)* Model of an icosahedron. *(b)* Three views of an icosahedron showing 5-, 3-, or 2-fold symmetry. *(c)* Electron micrograph of human papillomavirus, a virus with 5-fold icosahedral symmetry. The virion is about 55 nm in diameter. *(d)* Three-dimensional reconstruction of human papillomavirus; a virion contains 360 units arranged in 72 pentacapsomeres, each consisting of 2 separate proteins.

acid than can be packed into a shell made from 60 capsomeres and so viruses with some multiple of 60 capsomeres, such as 180, 240, or 360, are more common. The capsid of the human papillomavirus (Figure 5.6*c*), for example, consists of 360 capsomeres, with the capsomeres arranged into 72 clusters of 5 each (Figure 5.6*d*).

The structure of some viruses is highly complex, with the virion consisting of several parts each displaying its own shape and symmetry. The most structurally complex of all viruses are the head-plus-tail bacteriophages such as phage T4 that infects *Escherichia coli*. A T4 virion consists of an icosahedral head plus a helical tail (**Figure 5.7**). Some large viruses that infect eukaryotes, especially the giant viruses of the protozoan *Acanthamoeba*, are also structurally complex. Quite distinct from the head-plus-tail bacteriophages are *Pandoravirus* and *Mimivirus*. *Pandoravirus* possesses an ovoid morphology and an apical pore (Figure 5.4), while *Mimivirus* has a five-pronged star feature called *stargate* (Figure 5.1 and see Figure 5.19*b*).

Enveloped Viruses

Enveloped viruses have a lipoprotein envelope surrounding the nucleocapsid (Figure 5.2). Most enveloped viruses (for example, Ebola, **Figure 5.8*a, b***; see also Figure 5.21) use proteins on the virion's outer surface to attach to the cytoplasmic membrane and subsequently infect the cell. In contrast to the cells of humans and other animals, plant and bacterial cells are surrounded by a cell wall outside the cytoplasmic membrane, and thus few examples of enveloped viruses are known to infect these organisms. Typically, the *entire virion* enters an animal cell during infection, with the envelope, if present, assisting in the infection process by fusing with the

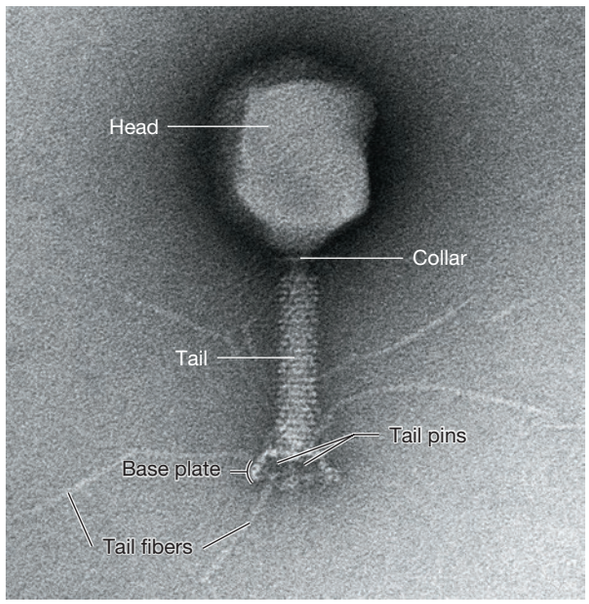


Figure 5.7 Structure of T4, a complex bacteriophage. Transmission electron micrograph of bacteriophage T4 of *Escherichia coli*. The tail components function in attachment of the virion to the host and injection of the nucleic acid (see Figure 5.15). The T4 head is about 85 nm in diameter.

host membrane. Enveloped viruses also exit more easily from animal cells. As they pass out of the host cell, they are draped in cytoplasmic membrane material. The viral envelope consists primarily of host cytoplasmic membrane, but some viral surface proteins (Figure 5.8*a*, *b*) also become embedded in the envelope as the virus passes out of the cell.

The viral envelope is important in infection, as it is the component of the virion that makes contact with the host cell. The specificity of infection by enveloped viruses and some aspects of their penetration are thus controlled in part by the biochemistry of their envelopes. The virus-specific envelope proteins are critical for both attachment of the virion to the host cell during infection and for release of the virion from the host cell after replication.

Besides an envelope, some viruses possess other structures important for infection. The giant *Mimivirus* and *Tupanvirus* that infect *Acanthamoeba* possess hairlike structures called *fibrils* on their capsid (**Figure 5.9**). These fibrils are made of a polymer similar to peptidoglycan (◀ Section 2.3) and are believed to increase infection rates by attracting amoeba hosts that have fed on bacterial cells. Also facilitating infection are the apical pore and stargate features of *Pandoravirus* and *Mimivirus* mentioned earlier (Figures 5.4*b*, 5.1*c*, and see Figure 5.19*b*). These unique features act as portals, which open to release the viral genome once the virus is inside the host cell.

Enzymes inside Virions

Viruses do not carry out metabolic processes and are thus metabolically inert. Nonetheless, some viruses carry enzymes in their virions that play important roles in infection. For example, viruses that infect *Bacteria* and *Archaea* often only insert their genome into their host. Thus some bacteriophages contain an enzyme that resembles lysozyme (◀ Section 2.3), which is used to make a small hole in the

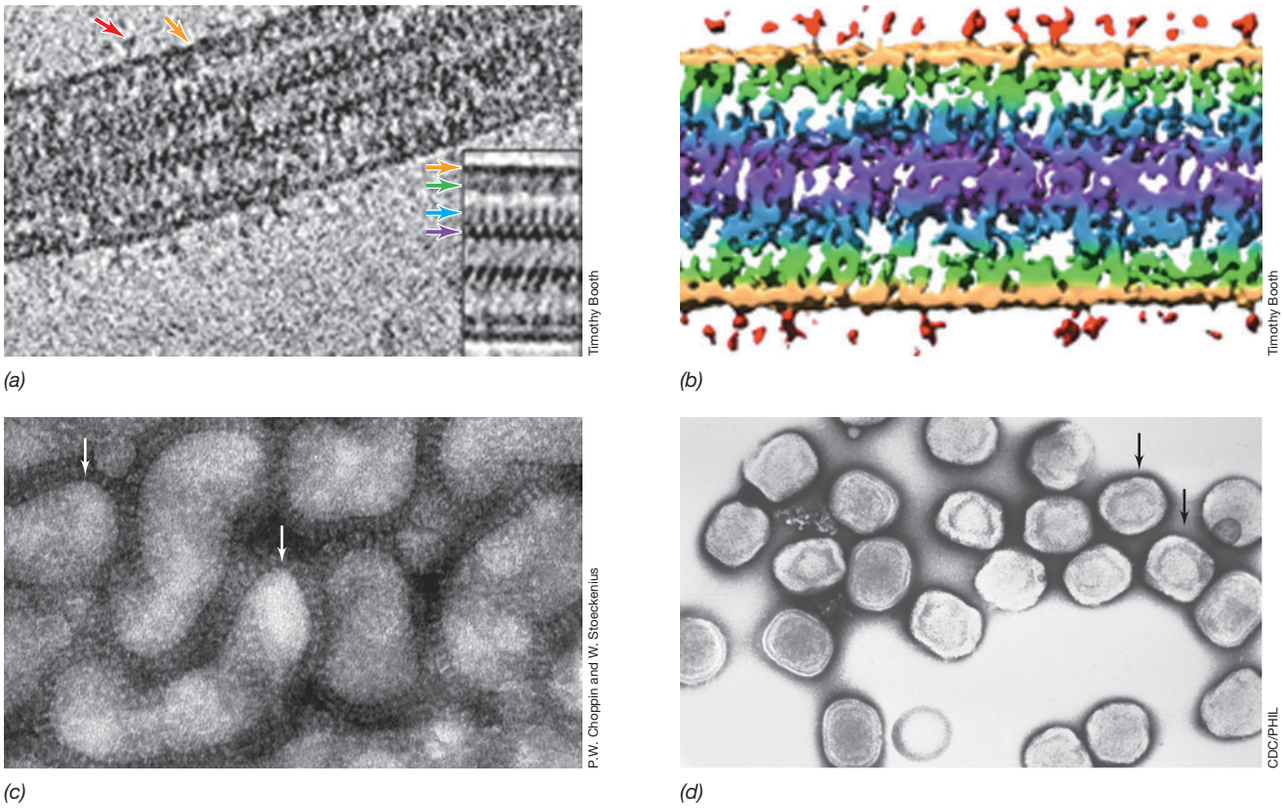


Figure 5.8 Enveloped viruses. *(a)* Cryo-electron micrograph (◀ Section 1.10) of an Ebola virion. The virions are helical with a diameter of 80 nm. *(b)* Three-dimensional surface representation of an Ebola tomograph. Color-coding (also applies to arrows in part *a*) is as follows: red, spikes of envelope surface glycoproteins; orange, lipid envelope; green, membrane-associated proteins; blue/purple, nucleocapsid proteins. *(c)* Electron micrograph of influenza virus. The virions are about 80 nm in diameter and can have many shapes. *(d)* Electron micrograph of vaccinia virus, an enveloped icosahedral pox virus about 350 nm wide. The arrows in both *c* and *d* point to the envelopes surrounding the nucleocapsids. Parts *a* and *b* modified from Beniac, D.R., Melito, P.L., deVarennes, S.L., Hiebert, S.L., Rabb, M.J., Lamboo, L.L., Jones, S.M., Booth, T.F. 2012. *PLoS One* 7(1): e29608.

peptidoglycan layer of the bacterial cell to allow nucleic acid from the virion to get into the host cytoplasm. A similar protein is produced in the later stages of infection to lyse the host cell and release new virions. Some animal viruses also contain enzymes that aid in their release from the host. For example, influenza virus (Figure 5.8*c*) has envelope proteins called *neuraminidases* that destroy glycoproteins and glycolipids of animal cell connective tissue, thus liberating the virions (▶ Section 11.9).

Viruses with RNA genomes carry enzymes (RNA-dependent RNA polymerases called *RNA replicases*) in their virion that function to replicate and express the viral RNA genome. Such enzymes are necessary because host cells lack enzymes of any type that can make RNA from an RNA template. **Retroviruses**, such as HIV, are unusual animal viruses that convert their RNA genome to a DNA intermediate. Because this is another process that host cells cannot do, retroviral virions contain enzymes called *reverse transcriptase* (▶ Section 11.11).

Although we will see that most viruses do not need to carry special enzymes in their virions, those that do absolutely require them for successful infection and replication. The genes that encode these special enzymes are carried by the virus genome and are expressed in the infection cycle at just the right time to facilitate host infectivity and successful replication of the virus.

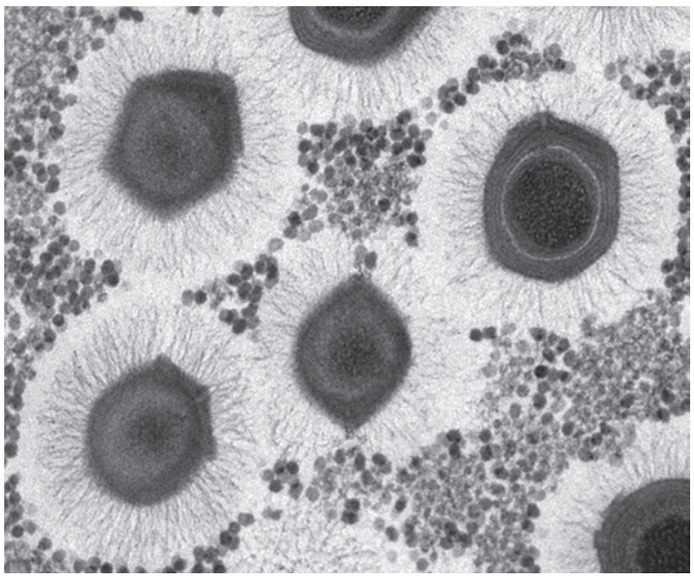
Check Your Understanding

- Distinguish between a capsid and a capsomere. What is a common symmetry for spherical viruses?
- What is the difference between a naked virus and an enveloped virus?
- What kinds of enzymes can be found within the virions of RNA viruses? Why are they there?

5.3 Culturing, Detecting, and Counting Viruses

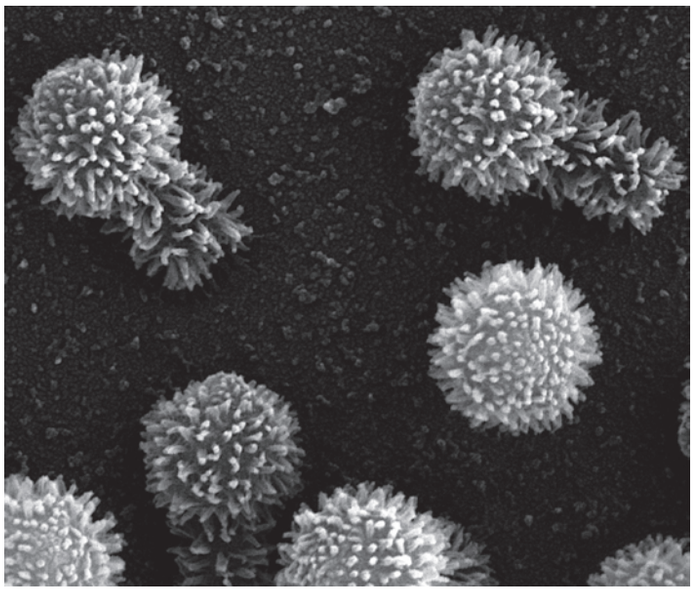
Host cells need to be growing in order for viruses to multiply in them; dead cells cannot produce viruses. While not all viruses kill their host cell, this section focuses on lytic bacteriophages because the culturing and detection of these viruses are easier to describe and visualize.

To culture a bacteriophage such as T4 of *Escherichia coli*, pure cultures of the bacterial host are either inoculated in liquid medium or spread as "lawns" on the surface of agar plates and then inoculated with a virus suspension. By contrast, animal and plant viruses are cultivated in *tissue cultures*. For animal viruses, the cells are obtained from an animal organ and grown in sterile glass or plastic vessels containing an appropriate culture medium (see Figure 5.11). Animal



Jônatas Santos Abrahão

(a)



Jônatas Santos Abrahão

(b)

Figure 5.9 **Giant *Mimiviridae* viruses and their surface fibrils.** *(a)* Transmission electron micrograph of thin sections of the Samba virus. A virion is about 0.57 μm in diameter. Modified from Campos, R.K., et al. 2014. *Virol. J. 11*: 95. (*b*) Scanning electron micrograph of Tupanvirus. A virion is about 1.2 μm in length. Both Samba virus and Tupanvirus infect species of amoebae of the genus *Acanthamoeba* found in Brazil.

tissue culture media are often highly complex, containing a wide assortment of nutrients including blood serum and other highly nutritious substances to feed the animal cells as well as antimicrobial agents to prevent bacterial contamination. For plant tissue cultures, a hairy root-based system is often used to easily propagate viruses, as the infected roots can grow in a liquid medium.

Mastering Microbiology
Art Activity: Figure 5.10 Quantification of bacterial virus by plaque assay

Detecting and Counting Viruses: The Plaque Assay

A viral suspension can be quantified to estimate the number of infectious virions present per volume of fluid, a quantity called the **titer**. This is typically done using a *plaque assay*. When a lytic virus infects host cells growing on a flat surface, a zone of cell lysis called a **plaque** forms and appears as a clear area in the lawn of host cells (**Figure 5.10**).

With bacteriophages, plaques may be obtained when virions are mixed into a small volume of molten agar containing host bacteria that is spread on the surface of an agar medium (Figure 5.10*a*). During incubation, the bacteria grow and form a turbid layer (lawn) that is visible to the naked eye. However, wherever a successful viral infection has occurred, cells are lysed, forming a plaque (Figure 5.10*b*). By counting the number of plaques, one can calculate the titer of the virus sample. The titer is typically expressed as the number of "plaque-forming units" per milliliter rather than an absolute viral number because of variations in plating efficiency, to be described next. However, when considering the plaque assay as a method, one should note that quantifying viruses by obtaining their titer is analogous to the counting of bacterial colonies that have formed on the surface of an agar plate from spreading a bacterial cell suspension ("plate count methods," ◀ Section 4.4).

In order to replicate animal viruses that kill their host cell, a tissue culture is grown, and a diluted virus suspension is overlaid on it. As for bacterial viruses, plaques are revealed as cleared zones in the tissue culture cell layer, and from the number of plaques produced, an estimate of the animal virus titer can be made (**Figure 5.11**). Viral titer is much harder to determine in plants, as plaques do not form in plant tissue culture. Instead viruses must be purified from the plant tissue and enumerated microscopically or through serological or molecular methods that specifically target viral proteins (Chapter 29).

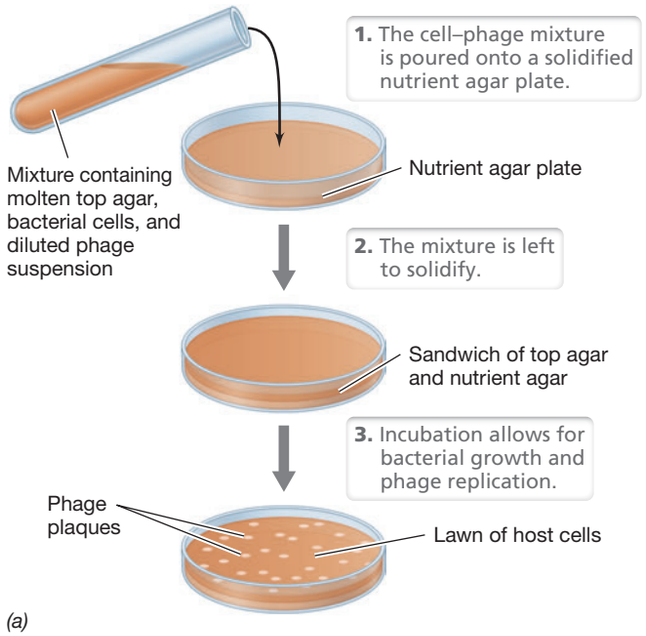
Plating Efficiency in Estimates of Viral Titers

The concept of *plating efficiency* is important in quantitative virology for all virus types. In any given viral preparation, the number of plaque-forming units is always lower than actual counts of viral particles made microscopically (using an electron microscope). This is because the efficiency with which virions infect host cells is rarely 100% and may often be considerably less. Virions that fail to infect may have assembled incompletely during the maturation process, may contain defective genomes, or may have suffered a spontaneous mutation that prevents them from attaching or otherwise properly replicating. Alternatively, a low plating efficiency may mean that viral growth conditions were not optimal, host viability was low, or that some virions were damaged by handling or storage conditions.

Although plating efficiencies of bacterial viruses can often be higher than 50%, with many animal viruses it may be much lower, 0.1% or 1%. Knowledge of plating efficiency is useful in cultivating viruses because it allows the investigator to estimate what a titer needs to be to yield a certain number of plaques. If the titer is extremely low, the viral suspension may need to be concentrated by centrifugation or filtration before being used to infect host cells. This is especially true of animal viruses, as the costs of growing and maintaining tissue cultures can be significant.

Check Your Understanding

- What is meant by a viral titer?
- What is a plaque-forming unit?
- What is meant by the term plating efficiency?



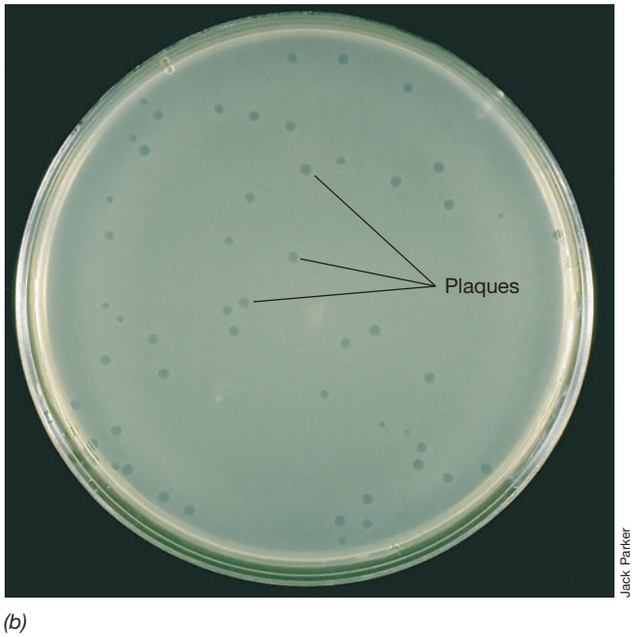


Figure 5.10 **Quantification of bacterial virus by plaque assay.** *(a)* "Top agar" (a dilute agar solution) containing a dilution of virions mixed with permissive host bacteria is poured over a plate of "bottom agar" (a more concentrated and thus stiffer agar). Infected cells begin to grow but then are lysed, forming plaques in the lawn. *(b)* Plaques (about 1–2 mm in diameter) formed by bacteriophage T4.

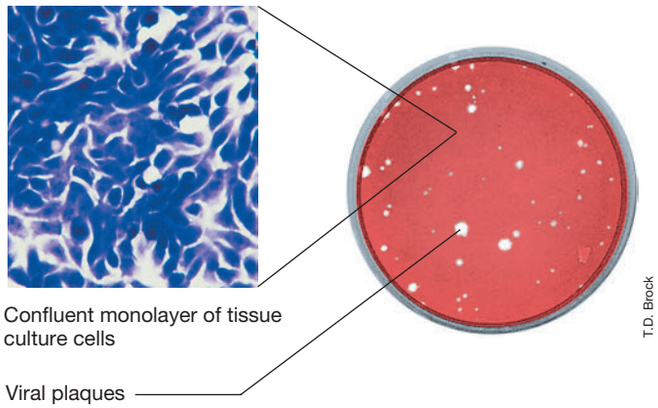


Figure 5.11 **Animal cell cultures and viral plaques.** The stained monolayer of animal cells support replication of the virus, and lysed cells result in plaques.

II • Overview of the Viral Replication Cycle

Following viral infection of a host cell, new virions are produced by the host as directed by the viral genome. Some aspects of viral infection differ between prokaryotic and eukaryotic cells, but in both cell types, lytic and long-term relationships are possible depending on the virus.

For a virus to replicate, it must induce a living host cell to synthesize all the essential components needed to make new virions. Because of these biosynthetic and energy requirements, dead host cells cannot replicate viruses. During an active viral infection, viral components are assembled into new virions that are released from the cell. Although replication steps are similar in most viruses, a major difference between viral infection of a *prokaryotic* cell and viral infection of a *eukaryotic* cell surrounds the initial step in infection. In cells of *Bacteria* and those *Archaea* in which the infection process has been studied, only the viral nucleic acid enters the host cell. By contrast, in plant and animal cells the entire virion is taken up. Despite this key difference, the replication of bacterial viruses has been extremely well studied and so we use them here as a model of viral replication events.

5.4 Steps in the Replication Cycle

A cell that supports the complete replication cycle of a virus is said to be *permissive* for that virus. In a permissive host, the viral replication cycle can be divided into five steps (**Figure 5.12**):

1. *Attachment* (adsorption) of the virion to the host cell
2. *Penetration* (entry, injection) of the virion nucleic acid into the host cell
3. *Synthesis* of virus nucleic acid and protein by host cell machinery as redirected by the virus
4. *Assembly* of capsids and *packaging* of viral genomes into new virions
5. *Release* of new virions from the cell

The growth response during virus replication is graphically illustrated in **Figure 5.13**. The response takes the form of a *one-step growth curve*, so named because a time course of virion numbers in the culture medium shows essentially no increase during the replication cycle until cells burst and release their newly synthesized virions. In the first few minutes after infection, the virus enters the *eclipse* phase, during

Mastering Microbiology
Art Activity: Figure 5.13 One-step growth curve of virus replication

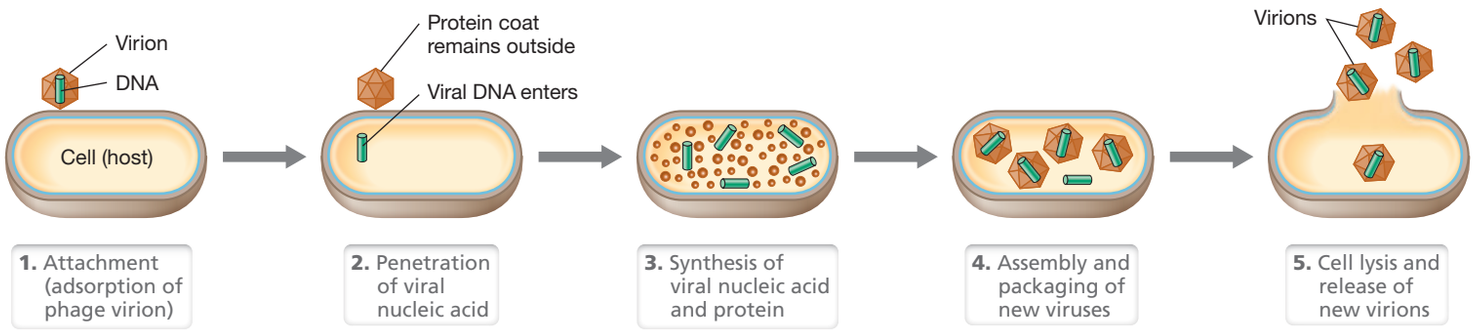


Figure 5.12 The replication cycle of a lytic bacterial virus. The virions and cells are not drawn to scale. The burst size can be a hundred or more virions per cell depending on the virus and the host cell.

which the viral genome and proteins will be replicated and produced, respectively. Once attached to a permissive host cell, a virion is no longer available to infect another cell. This is followed by the entry of viral nucleic acid into the host cell (Figure 5.12). If the infected cell breaks open at this point, the virion no longer exists as an infectious entity since the viral genome is no longer inside its capsid.

The *maturation* phase (Figure 5.13) begins as newly synthesized viral genomes become packaged inside their capsids. During the maturation phase, the number of infectious virions inside the host cell rises dramatically. However, the new virions still cannot be detected in a plaque assay unless the cells are artificially lysed to release them. Because newly assembled virions are not yet present outside the cell, the eclipse and early maturation periods together comprise the *latent period* of viral infection (Figure 5.13).

At the end of maturation, mature virions are released, either as a result of cell lysis or by budding or excretion, depending on the virus. The number of virions released per cell, called the *burst size*, varies with the particular virus and the particular host cell, and can range from a few to a few thousand. The duration of the virus replication cycle also varies, from 20–60 min (in many bacterial viruses) to 8–40 h (in most animal viruses).

In Sections 5.5 and 5.6 we use a specific example to revisit these stages of the virus replication cycle and examine each in more detail.

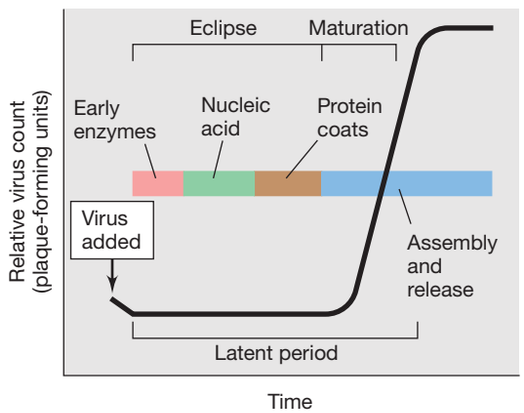


Figure 5.13 One-step growth curve of virus replication. Following adsorption, infectious virions cannot be detected in the growth medium, a phenomenon called *eclipse*. During the latent period, which includes the eclipse and early maturation phases, viral nucleic acid replicates and protein synthesis occurs. During the maturation period, virus nucleic acid and protein are assembled into mature virions and then released.

Check Your Understanding

- What is packaged into capsids during maturation?
- Explain the term burst size.
- Why is the latent period so named?

5.5 Bacteriophage T4: A Model Lytic Virus

Much of our understanding of *lytic* virus replication comes from the study of bacteriophages that infect *Escherichia coli*. Here we focus on one, bacteriophage T4 (Figure 5.7), as our model for reviewing the individual stages of the lytic virus life cycle in more detail. The study of bacteriophage T4 has contributed to many of the paradigms of modern genetics and molecular biology such as gene structure, the nature of the genetic code, and the discovery of mRNA (Chapter 6).

Attachment and Entry of Bacteriophage T4

The early steps in the life cycle of any bacteriophage are attachment to the surface of its host cell followed by penetration of the host cell outer layer(s) and entry of the viral genome into the cell (Figure 5.12).

A major factor in host specificity of a virus is *attachment*. The virion itself has one or more proteins on its external surface that interact with specific components called *receptors* on the host cell surface. In the absence of its specific receptor, the virus cannot attach to the cell and hence cannot infect. Moreover, if the receptor is altered, for example by mutation, the host may become resistant to virus infection. The host range of a given virus is thus to a major extent determined by the presence of a suitable receptor that the virus can recognize and attach to.

Viral receptors are surface components of the host, such as proteins, carbohydrates, glycoproteins, lipids, or lipoproteins, or cell structures made from these macromolecules (**Figure 5.14**). The receptors carry out normal functions for the cell; for example, the receptor for phage T1 is an iron-uptake protein (Figure 5.14) and that for bacteriophage lambda functions in maltose uptake. Carbohydrates specific to the lipopolysaccharide (LPS) outer membrane of *Escherichia coli* (◀ Section 2.4) are the receptors recognized by bacteriophage T4 (Figure 5.14). Appendages that project from the cell surface, such as flagella and pili, are also common receptors for bacterial viruses. Small icosahedral viruses often bind to the side of these structures (◀ Figure 2.18), whereas filamentous bacteriophages typically bind at the tip, such as on the pilus (Figure 5.14).

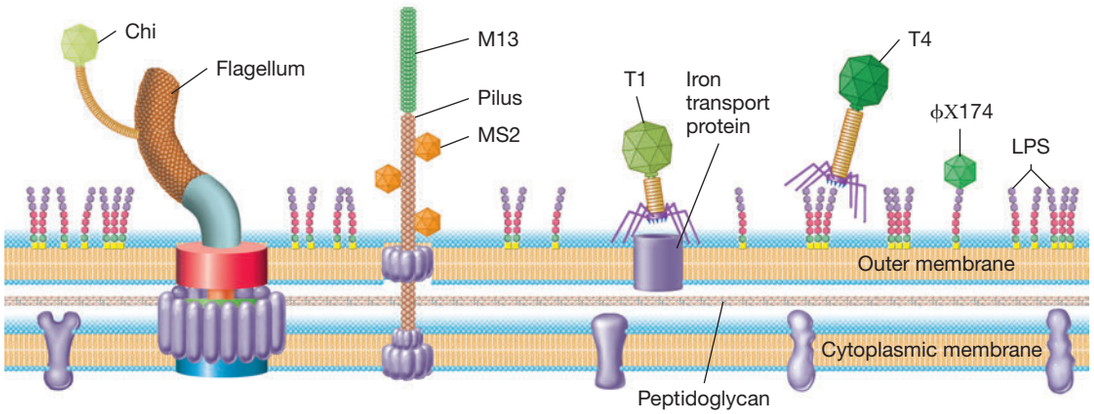


Figure 5.14 **Bacteriophage receptors.** Examples of the cell receptor sites used by different bacteriophages that infect *Escherichia coli.*

Regardless of the receptor used, however, once attachment has occurred, the stage is set for viral infection.

Penetration

Attachment of a virus to its host cell causes changes to both the virus and the host cell surface that result in penetration. Bacteriophages abandon their capsid outside the cell and only the viral genome reaches the cytoplasm. However, entry of the viral genome into a host cell only results in virus replication if the viral genome can be read. Consequently, for the replication of some viruses, for example RNA viruses, specific viral proteins must enter the host cell along with the viral genome (Section 5.2).

The most intricate viral penetration mechanisms belong to the tailed bacteriophages. Bacteriophage T4 consists of an icosahedral head, within which the viral linear double-stranded DNA is folded, and a long, complex tail, which ends in a series of tail fibers and tail pins that contact the cell surface (Figure 5.7). Phage T4 virions first attach to *Escherichia coli* cells using their tail fibers (**Figure 5.15**). The ends of the tail fibers interact specifically with polysaccharides in the cell's LPS layer and then the tail fibers retract, allowing the tail itself to contact the cell wall via the tail pins. The activity of T4 lysozyme then forms a small pore in the peptidoglycan layer and the tail sheath contracts. When this occurs, the T4 genome enters the cytoplasm of the cell through a tail tube in a fashion resembling that of injection by a syringe. By contrast, the T4 capsid remains outside the cell (Figure 5.15). DNA inside bacteriophage heads is under high pressure, and because the interior of a bacterial cell is also under pressure from osmotic forces, the phage DNA injection process takes several minutes to complete.

Once a bacteriophage injects its genome into a host cell, a productive infection is not absolutely ensured. Although they lack the immune systems of animals (Chapters 26 and 27), *Bacteria* and *Archaea* possess several weapons to protect against viral attack. Toxin–antitoxin modules (▶ Section 8.12) and an antiviral system called CRISPR (▶ Section 9.12) are two of these mechanisms. Additionally, *Bacteria* and *Archaea* can destroy double-stranded viral DNA through the activity of *restriction endonucleases,* enzymes that cleave foreign DNA at specific sites (▶ Section 12.2). Although host restriction systems confer significant protection from viral attack, some DNA viruses have overcome host restriction by modifying their own genome so it is no longer subject to restriction enzyme attack.

Production of T4 Virions and Release

Once the T4 genome has successfully entered and been retained in a permissive host cell, the process of new virion synthesis begins. In less than half an hour, the process culminates in the release of new virions from the lysed cell. The major events are summarized in **Figure 5.16**.

Mastering Microbiology
Art Activity: Figure 5.16 Time course of events in phage T4 infection

Within a minute after the T4 genome enters the host cytoplasm, the synthesis of host-specific proteins ceases and production of phage-specific proteins begins, thus inhibiting normal host growth. The T4 genome encodes three major sets of proteins called **early proteins**, **middle proteins**, and **late proteins**, the terms referring to the general order of their

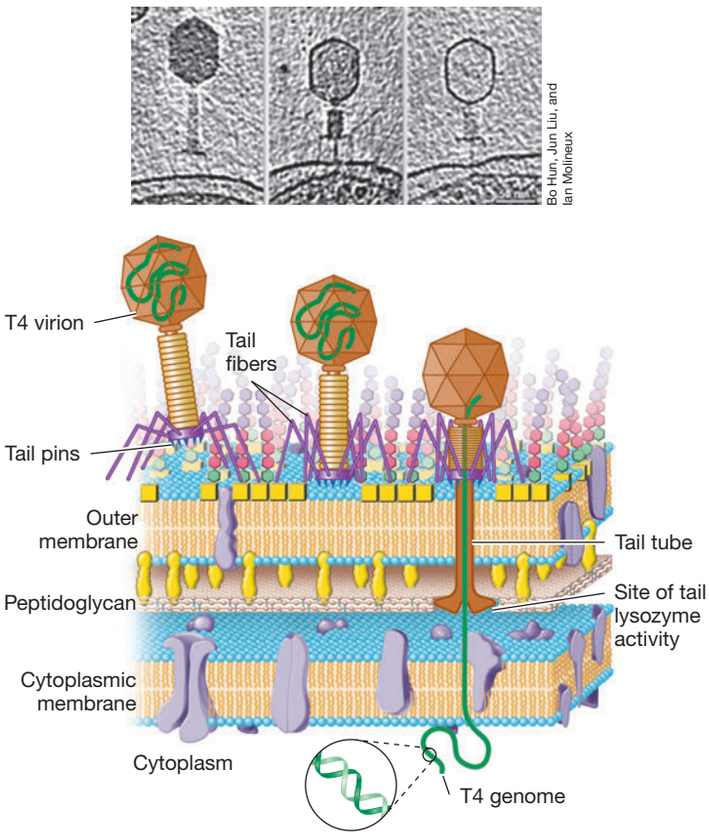


Figure 5.15 **Attachment and infection of an *Escherichia coli* cell by bacteriophage T4.** The three transmission electron tomographs and the art beneath each depict (left to right) the initial attachment of a T4 virion to the cell outer membrane by tail fiber interactions with lipopolysaccharide (LPS); contact of the cell wall by the tail pins; and contraction of the tail sheath and injection of the T4 genome. The tail tube penetrates the outer membrane, and T4 lysozyme digests a small opening through the *E. coli* cell peptidoglycan layer.

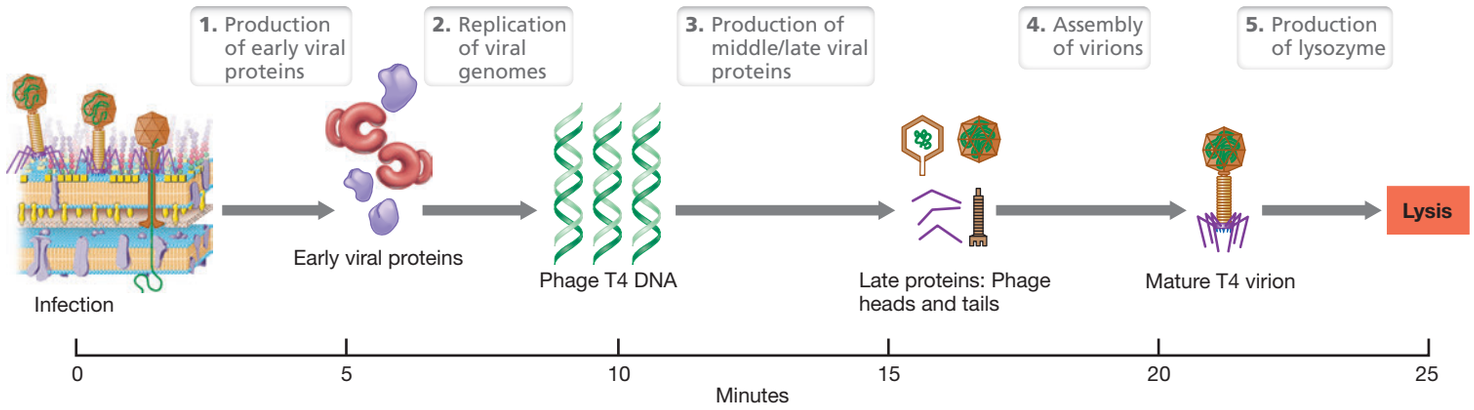
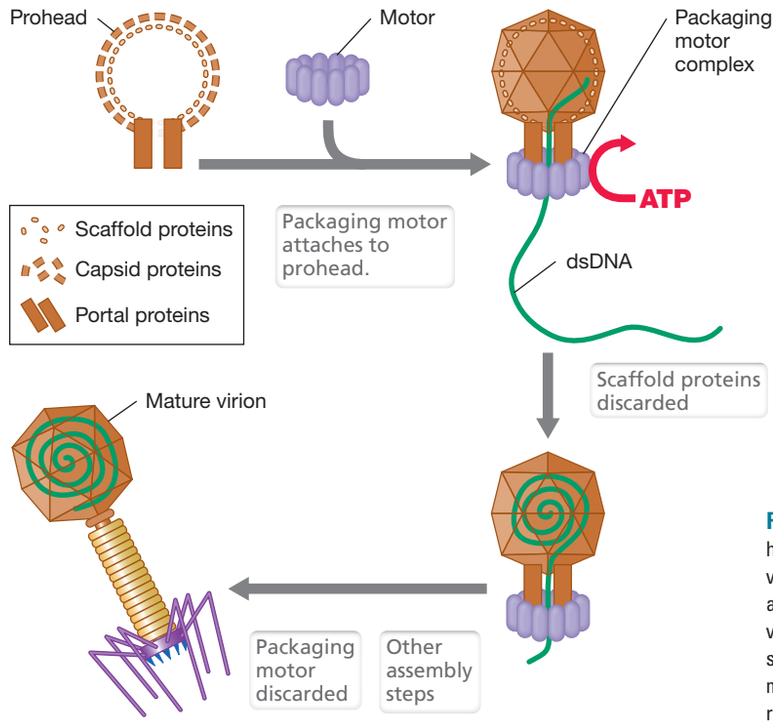


Figure 5.16 **Time course of events in phage T4 infection.** Following injection of DNA, early and middle mRNAs are produced that encode proteins needed for DNA replication. Following injection of DNA, early and middle proteins needed for DNA replication are produced. Late proteins are virion structural proteins and T4 lysozyme, which is needed to lyse the cell and release new virions.

appearance in the cell. Early proteins include enzymes for the production of T4 genome copies and proteins that modify host enzymes to express viral genes. This viral takeover of the host cell results in replication of the 170-kilobase T4 genome within 4 minutes of infection (we save the details of T4 genome replication for Chapter 11). By contrast, middle and late proteins include additional proteins that modify host enzymes, and virion structural and release proteins. These include, in particular, viral head and tail proteins and the enzymes required to liberate new virions from the cell (Figure 5.16).

Mastering Microbiology
Art Activity: Figure 5.17*a* Packaging of DNA into a T4 phage head

Once viral head proteins are made, the bacteriophage T4 DNA genome is forcibly pumped into a preassembled capsid using an energy-linked packaging motor. The motor components are encoded by viral genes, but host cell metabolism is needed to produce the proteins and supply the ATP required for the pumping process. The packaging process can be divided into three stages (**Figure 5.17*a***). First, precursors of the bacteriophage head called *proheads* are assembled but remain empty of DNA. Proheads contain temporary "scaffolding proteins" as well as head structural proteins. Second, a packaging motor is assembled at the opening to the prohead (Figure 5.17*b*). The T4 DNA genome is then pumped into the prohead under pressure using ATP as the driving force. The prohead expands when pressurized by the entering DNA, and the scaffolding proteins are simultaneously discarded. Third, the packaging motor itself is discarded and the capsid head is sealed.



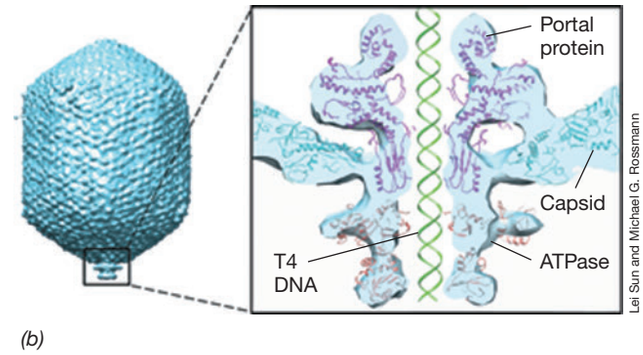


Figure 5.17 **Packaging of DNA into a T4 phage head.** A T4 virion consists of a head, tail, collar, and various attachment structures (Figure 5.7). Assembly of the T4 virion requires the packaging of viral DNA into the phage head. *(a)* Proheads are assembled from capsid and portal proteins, both of which remain in the mature virion. As the head fills with DNA (driven by the energy released from ATP hydrolysis), it expands and becomes more angular. Once the head is filled, the packaging motor detaches and the tail components are added. *(b)* Cryo-electron micrograph reconstruction of the T4 prohead and packaging motor. The blowup shows proteins within the motor and the prohead and their contact with DNA.

After the head has been filled, the T4 tail, tail fibers, and the other components of the virion are added, primarily by spontaneous reactions or self-assembly (Figure 5.16). The phage genome encodes a pair of very late enzymes that combine to breach the two major barriers to virion release: the host cytoplasmic membrane and peptidoglycan layer. Once these structures are compromised, the cell breaks open by osmotic lysis and the newly synthesized virions are released. After each replication cycle, which takes only about 25 min (Figure 5.16), over 100 new virions are released from each host cell (the *burst size*, Section 5.4), and these are now free to infect neighboring host cells.

Check Your Understanding

- How does attachment contribute to virus–host specificity?
- Why does phage T4 need a lysozyme-like protein in order to infect its host, and what part of T4 enters the host cytoplasm?
- What is required to package the T4 genome into its phage head?

5.6 Temperate Bacteriophages and Lysogeny

Bacteriophage T4 is a **virulent virus** and once infection begins, it proceeds to kill its host through lysis. However, some double-stranded DNA bacterial viruses, although capable of a virulent cycle, can also infect their host and establish a long-term stable relationship. Such a virus is called a **temperate virus**.

Temperate viruses can enter into a state called **lysogeny**. In this state, very few viral proteins are produced; instead, the virus genome is replicated in synchrony with the host chromosome and passed to daughter cells at cell division. A cell that harbors a temperate virus is therefore called a **lysogen**. While growth of a lysogen is controlled by its local environment and nutritional profile, the lysogenic state may confer new genetic properties—a condition called *lysogenic conversion*. We will see several examples in later chapters of pathogenic bacteria whose virulence (ability to cause disease) is at least in part linked to a lysogenic bacteriophage.

Two well-characterized temperate bacteriophages are lambda and P1. The life cycle of a temperate bacteriophage is shown in **Figure 5.18**. During lysogeny, the temperate virus genome is often integrated into the bacterial chromosome. The viral DNA, now called a **prophage**, replicates along with the host cell as long as the genes that activate the phage virulent pathway are repressed.

Maintenance of the lysogenic state is due to a phage-encoded *repressor protein*. Normally, this repressor is maintained at a low level in the host cell. However, if the phage repressor is inactivated or if its synthesis is in some way prevented, the prophage can be induced to switch to the lytic stage. This process is called *induction*. When induction occurs, the viral DNA is excised and phage early, middle, and late proteins are produced similarly to the process described for T4 phage (Section 5.5 and Figure 5.16). New virions are then produced, and the host cell is lysed (Figure 5.18). Various cell stress conditions, especially damage to host cell DNA, can induce a prophage to enter the lytic pathway. In contrast to this process, the viral "decision" to proceed to lysogeny or to the lytic pathway upon initial viral infection is another matter altogether, and has been particularly well studied in bacteriophage lambda. We explore this rather intricate story in Chapter 11.

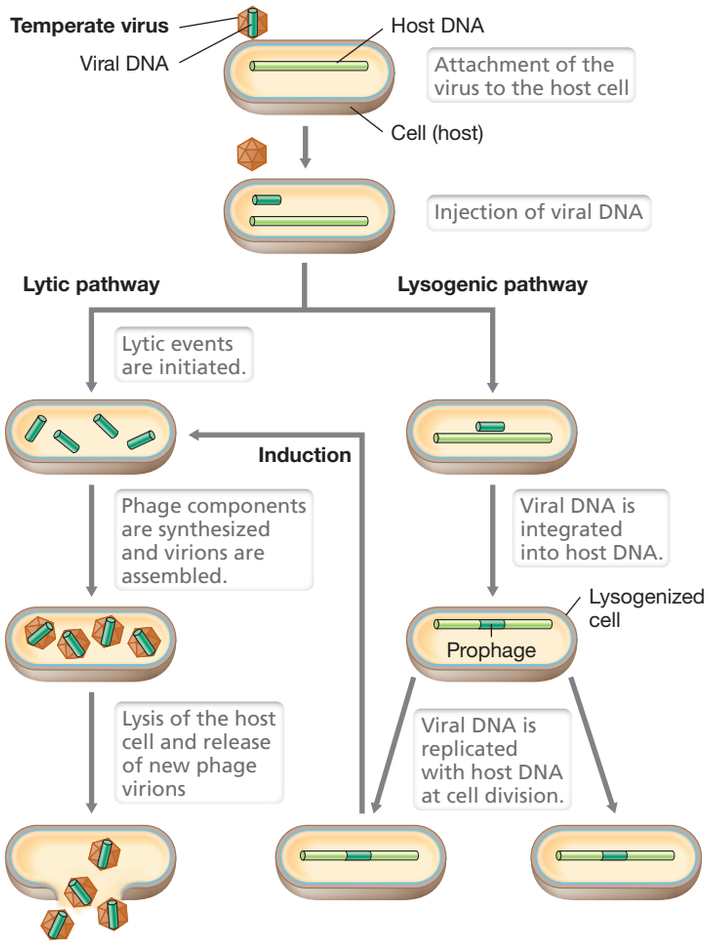


Figure 5.18 Consequences of infection by a temperate bacteriophage. The alternatives upon infection are replication and release of mature virions (lysis) or lysogeny, often by integration of the virus DNA into the host DNA, as shown here. The lysogen can be induced to produce mature virions and lyse.

Mastering Microbiology
Art Activity: Figure 5.18 Consequences of infection by a temperate bacteriophage

We finish our introduction to the viral world by briefly considering viruses that infect eukaryotic hosts, both plant and animal. As we will see in Chapters 29–33, many of the most notorious scourges of humankind are of viral origin (see Table 5.1 for some examples).

Check Your Understanding

- What is a lysogen and what is a prophage?
- How do temperate viruses differ from virulent (lytic) viruses?
- When considering lysogeny, what occurs during the process of induction?

5.7 An Overview of Viruses of Eukaryotes

The major tenets of virology—presence of a capsid to carry the viral DNA or RNA genome, infection and takeover of host metabolic processes, and assembly and release from the cell—are universal, regardless of the nature of the host. Like bacterial viruses, eukaryotic viruses are classified by their genomes (**Table 5.1**). However, three key differences between bacterial and eukaryotic viruses are that (1) the entire virion of eukaryotic viruses (rather than just the nucleic acid)

TABLE 5.1 Representative viral diseases of humans and plants

Disease or host	Virus	Genome DNA or RNA[a]	Size[b]
Human viruses			
Cold sores/genital herpes	Herpes simplex	dsDNA	152,000
Smallpox	Variola major	dsDNA	190,000
Influenza	Influenza A virus	ssRNA	13,600
Ebola hemorrhagic fever	Ebola virus	ssRNA	19,000
Severe acute respiratory syndrome (SARS)	SARS virus	ssRNA	29,800
Infant diarrhea	Rotavirus	dsRNA	18,600
Acquired immunodeficiency syndrome (AIDS)	Human immunodeficiency virus (HIV)	ssRNA/dsDNA (a retrovirus)	9,700
Plant viruses			
Cauliflower, turnip, cabbage	Cauliflower mosaic virus	dsDNA	8,000
Cassava	African cassava virus	ssDNA	5,500
Strawberry	Strawberry crinkle virus	ssRNA	14,500
>900 plant species	Tomato spotted wilt virus	ssRNA	16,600
Tobacco, tomato, pepper	Tobacco mosaic virus	ssRNA	6,500
>1200 plant species	Cucumber mosaic virus	ssRNA	8,600
Rice, maize, wheat, barley	Rice black streaked dwarf virus	dsRNA	29,100

[a]ss, single-stranded; ds, double-stranded.
[b]In bases (ss genomes) or base pairs (ds genomes). These viral genomes have been sequenced and thus their lengths are known precisely. However, the sequence and length often vary slightly among different isolates of the same virus. Hence, the genome sizes listed here have been rounded off in all cases.

enters the host cell, (2) eukaryotic cells contain a nucleus, where many (but not all) viruses replicate, and (3) in some eukaryotic cells, membrane-bound "viral factories," also known as *viroplasms*, form to increase the rate of virion assembly and protect the process from host defenses (**Figure 5.19**). We explore some aspects of animal and plant viruses here.

Viral Infection of Animal Cells

While a large number of animal viruses exist (Chapter 11), most animal viruses that have been studied in detail are those that can replicate in cell cultures (Section 5.3 and Figure 5.11). To initiate infection, animal viruses also bind to specific host cell receptors. These virus receptors are typically animal cell surface proteins used in cell–cell contact or that function in the immune system. For example, the receptors for poliovirus and for HIV (the virus that causes AIDS) are normally used in intercellular communication between human cells. In multicellular organisms, cells in different tissues or organs often express different proteins on their cell surfaces. Consequently, viruses that infect animals often infect only certain tissues. For example, viruses that cause the common cold infect only cells of the upper respiratory tract.

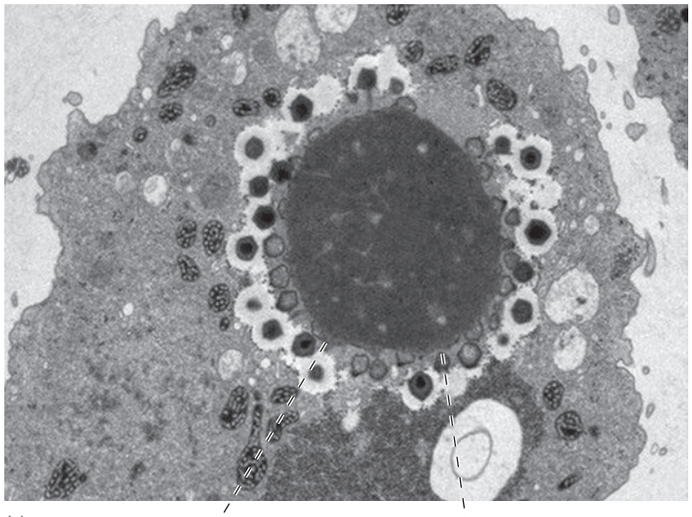
Mastering Microbiology
Art Activity: Figure 5.22 Possible effects that animal viruses may have on cells they infect

Once an animal virus is bound to a receptor, its entry into a host cell generally occurs by fusion with the cytoplasmic membrane or by endocytosis (**Figure 5.20**). After entry into the cell, animal viruses must eventually lose their outer coat to deliver their genomes to the cytosol. Some enveloped animal viruses are uncoated at the host cytoplasmic membrane, releasing the nucleocapsid into the cytoplasm, while naked and enveloped animal viruses that enter via endocytosis are uncoated in the host cytoplasm. If the viral genome is DNA, the genome passes through the nuclear membrane to the nucleus for replication. Conversely, the genomes of most RNA-based viruses are replicated or converted to DNA by viral enzymes within the nucleocapsid. We will focus on the unique mode of replication of retroviruses, a special class of highly unusual RNA animal viruses with significant medical implications, in Chapter 11.

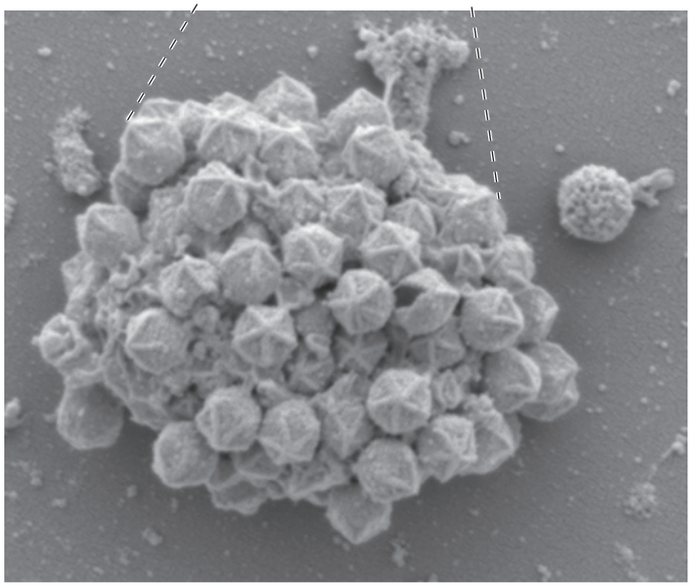
Animal Virion Assembly and Infection Outcomes

Virion assembly and morphogenesis occurs once viral nucleocapsids have been produced by the host's machinery. After viral genome copies are packaged within their outer coat, many animal viruses must be enveloped. This typically occurs when the virion exits the animal cell through cell lysis or a process called *budding* (**Figure 5.21**). During this process the virus may pick up part of the cell's cytoplasmic membrane as it exits the cell and use it as part of the viral envelope.

Unlike a bacteriophage infection, in which one of only *two* outcomes—lysis or lysogeny—is possible depending on the virus, other events are possible in an animal virus infection. If an animal virus initially evades the immune system, animal viruses can catalyze at least four different outcomes (**Figure 5.22**). A *virulent infection* (or lytic infection) results in lysis of the host cell; this is the most common outcome. By contrast, in a *latent infection*, the viral DNA exists in the host's genome as a **provirus** from a process similar to that described for lysogeny (Section 5.6) and virions are not produced; this leaves the host cell unharmed unless and until an event triggers the virulent pathway. With some enveloped animal viruses, release of virions may be slow, and the host cell may not be lysed (and thus not killed); instead the host cell continues to grow and produce more virions. Such infections are called *persistent infections*. Finally, certain animal viruses can convert a normal cell into a tumor cell, a process called *transformation* (Figure 5.22). Some human cancer viruses are capable of such activity (▶ Section 11.7), although the vast majority of cancers are not of viral origin.



(a)



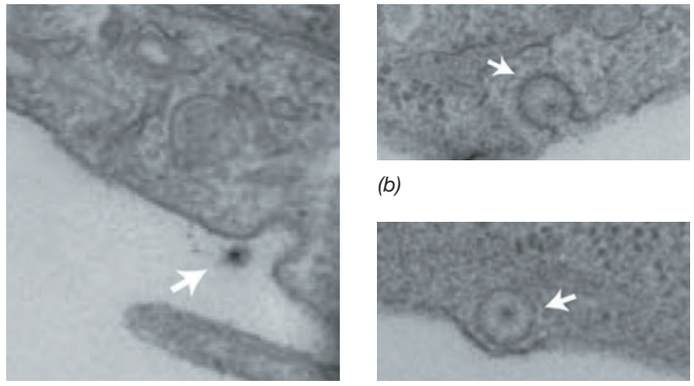
Jônatas Santos Abrahão

(b)

Figure 5.19 ***Mimivirus*** **viral factory.** *(a)* Electron micrograph of the cytoplasm of *Acanthamoeba castellanii* infected with *Acanthamoeba polyphaga mimivirus* (APMV). Dashed leaders indicate the viral factory or viroplasm where APMV is being replicated and assembled. Modified from Campos, R.K., et al. 2014. *Virol. J. 11*: 95. *(b)* Scanning electron micrograph of a viral factory with APMV. Note prominent stargate feature (Figure 5.1*c*).

Viral Infection of Plant Cells

Plant viruses share many traits with animal viruses; for example, most have RNA genomes, the complete virion enters the host cell, and viral factories form in infected cells (Figure 5.19). However, three major differences between animal and plant viruses are that (1) many plant viruses have a wide host range (Table 5.1), with viruses such as the cucumber mosaic virus able to infect over 1200 species of plants; (2) most plant viruses are not enveloped; and (3) plant virus transmission to host cells occurs in a different manner due to both the immobility of plants and the rigidity of the cell wall, a structure that is absent from animal cells.



(a)

(b)

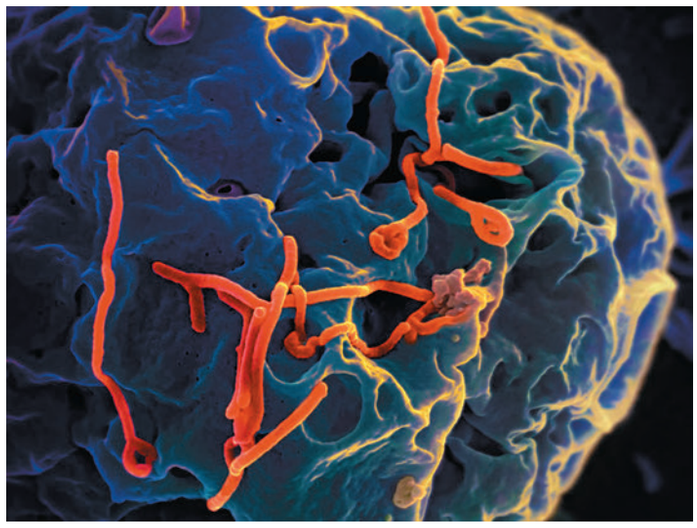
(c)

Stephen C. Harrison

Figure 5.20 **Rotavirus cell entry.** Electron micrographs of rotavirus virions illustrating the stages of cell entry. The arrows point to the virion. *(a)* Virion bound to the cell surface. *(b)* Engulfment of virion by the cytoplasmic membrane. *(c)* Compartmentalization of the virion. Modified from Abdelhakim, A.H., Salgado, E.N., Fu, X., Pasham, M., Nicastro, D., Kirchhausen, T., and Harrison, S.C. 2014. *PLoS Pathog. 10*: e1004355.

While animal viruses often enter host cells by endocytosis, plant cells contain a protective cell wall preventing such entry. Thus, viruses enter new plant hosts through wounds in the plant tissue or through penetration of the plant cell wall by insects, nematodes, or fungi. In fact, many plant viruses can attach to the mouthparts of plant pests or even propagate in the guts of such pests. Pests that are able to transfer viruses to other types of host cells are called *vectors*.

Once the virion has successfully entered a plant cell, the capsid is removed, the genome is replicated in the nucleus or viral factory, and new virions are assembled (**Figure 5.23**). For infection of other cells within the plant to occur, the viral genome often encodes special movement proteins that help viruses travel through small channels, called plasmodesmata, that connect plant cells. This movement can lead to the viruses entering the vascular system and infecting the



CDC/PHIL 17778/National Institutes of Allergy and Infectious Disease

Figure 5.21 **Budding of Ebola virus.** Scanning electron micrograph depicting filamentous Ebola virus particles (red) budding from the surface of an infected African monkey kidney epithelial cell (blue-gray).

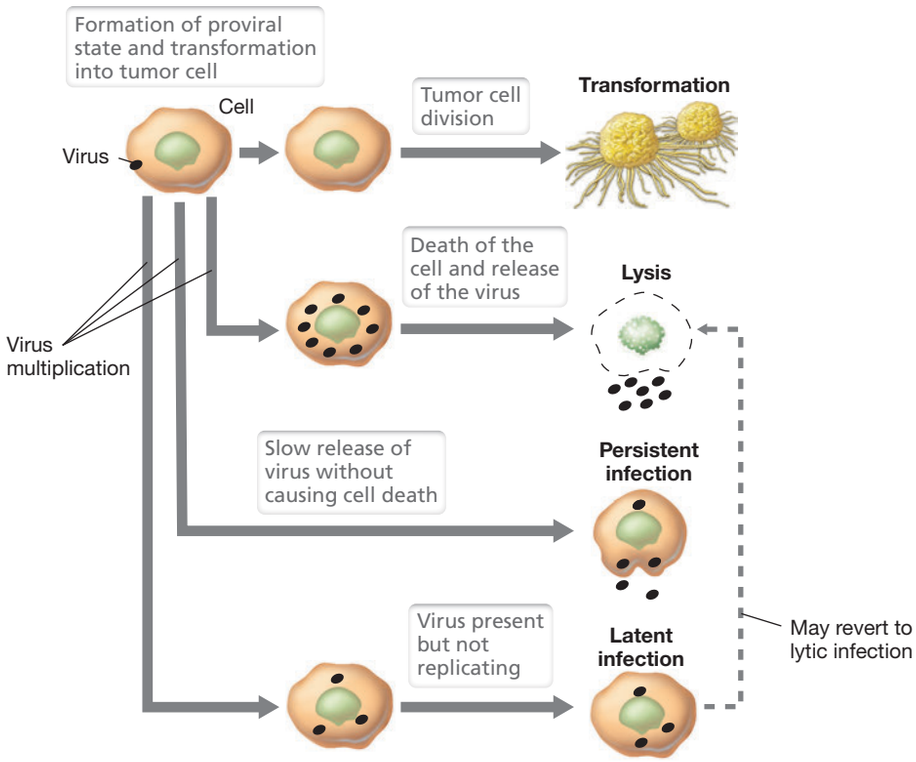


Figure 5.22 **Possible effects of animal virus infection of host cells.** Most animal viruses are lytic, and only a very few are known to cause cells to transform and become cancerous.

entire plant (Figure 5.23). Vectors that feed on this infected plant can then transfer the virus to healthy plants, repeating the infection cycle and causing widespread crop damage.

As exemplified by the viruses we have considered in this chapter, viruses are truly fascinating microbes. On the one hand, the multiplication of any virus is completely dependent on the growth and activities of its host cell; on the other hand, viruses can control the growth, genetic properties, and very survival of their hosts. We revisit the viruses in Chapter 11 with a focus on viral genomics as the criterion by which we reveal the enormous genetic diversity of these important microbes. Although our discussion of viruses in this chapter has covered some of their remarkable characteristics, we have only scratched the surface.

Check Your Understanding

- Contrast the ways in which animal and bacterial viruses enter their hosts.
- What is the difference between a persistent and a latent viral infection?
- How does viral infection of plants differ from viral infection of animals?

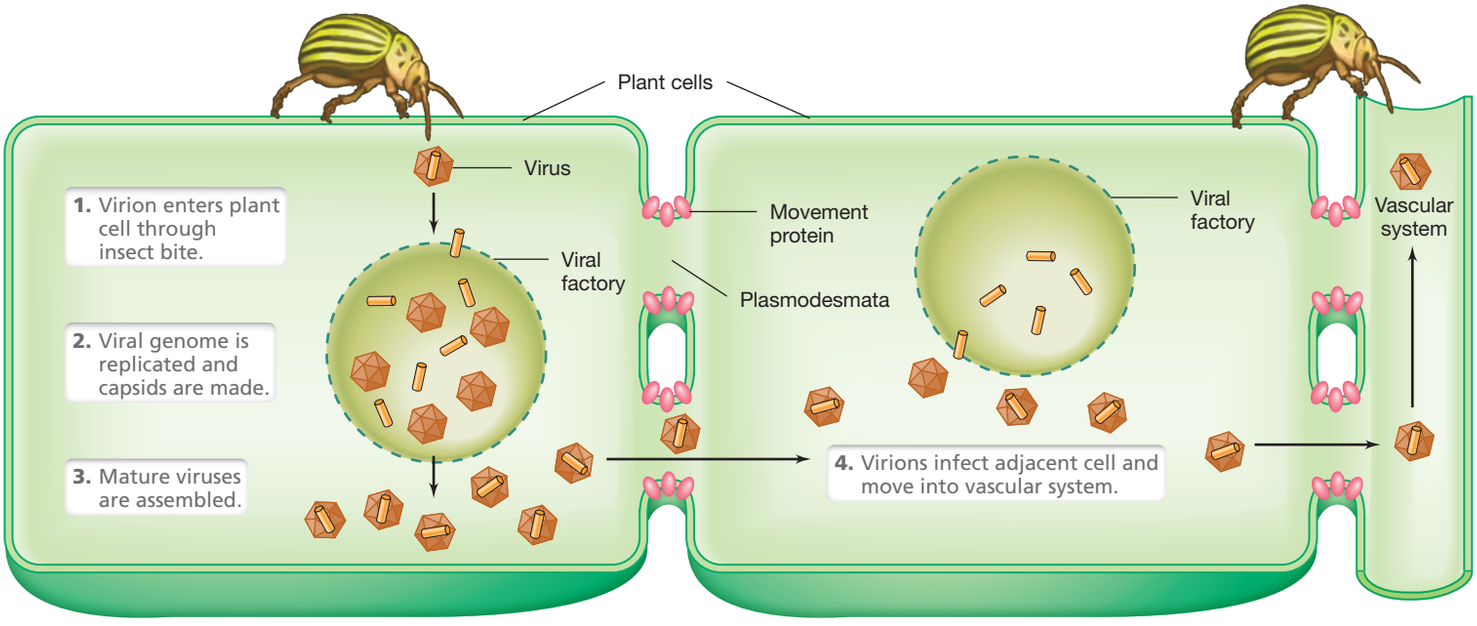


Figure 5.23 **Viral infection of plants.** Unlike infecting an animal, where viruses can readily attach and fuse with the animal cell's cytoplasmic membrane, to infect a plant, viruses have to traverse the plant cell wall that may be thick. Virions rely on some form of surface damage to the plant, entering a plant cell through a wound or insect bite. Infection leads to viral multiplication and the production of viral-encoded movement proteins, which allow for transfer of viruses to adjacent plant cells through plasmodesmata. The entire plant can be infected once the virus enters the vascular system.

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CHAPTER REVIEW

I • The Nature of Viruses

5.1 A virus is an obligate intracellular parasite that requires a suitable host cell for replication. A virion is the extracellular form of a virus and contains either an RNA or a DNA genome inside a protein shell. Once the virus is inside the cell, the viral nucleic acid, and sometimes viral enzymes, redirects host metabolism to support virus multiplication. Viruses are classified by the characteristics of their genome and hosts. Bacteriophages infect bacterial cells and are model systems for studying viruses.

Q Briefly describe the two approaches a bacteriophage can take to trigger an infection once inside its host cell.

5.2 In the virion of a naked virus, only nucleic acid and protein are present; the entire unit is called the nucleocapsid. Enveloped viruses have one or more lipoprotein layers surrounding the nucleocapsid. The nucleocapsid is often arranged in a symmetric fashion, with the icosahedron being a common morphology. Some viruses also possess structures that assist with the infection process. Although virus particles are metabolically inert, some viruses have one or more key enzymes within their virion that carry out processes that the host cannot.

Q Where does the envelope surrounding animal viruses originate?

5.3 Viruses can replicate only in their correct host cells. Bacterial viruses have proved useful as model systems because their host cells are easy to grow and manipulate in culture. Many animal viruses can be grown in cultured animal cells. Viruses can be quantified (titered) by a plaque assay. Plaques are clearings that develop on lawns of host cells, and in analogy to bacterial colonies, they arise from the viral infection of a single cell.

Q Draw a diagram of the one-step growth curve of virus replication, and briefly explain why the growth curve differs from a typical bacterial growth curve.

II • Overview of the Viral Replication Cycle

5.4 The virus replication cycle can be divided into five major stages: attachment (adsorption), penetration (uptake of the entire virion or injection of the nucleic acid only), protein and nucleic acid synthesis, assembly and packaging, and virion release.

Q Why does the viral one-step growth curve differ in shape from that of a bacterial growth curve?

5.5 The attachment of a virion to a host cell is a highly specific process. Recognition proteins on the virus recognize specific receptors on the host cell. Sometimes the entire virion enters the host cell, whereas in other cases, as with most bacteriophages, only the viral genome enters. After a T4 virion penetrates a host cell, its genome enters the cytoplasm and viral genes are expressed to redirect the host synthetic machinery to make viral nucleic acid and protein. Early viral genes encode viral genome replication events; middle and late viral genes encode structural proteins and capsid assembly. Once T4 components have been synthesized, new virions are assembled and released after lysis of the host cell.

Q What is required for a bacteriophage T4 virion to attach to an *Escherichia coli* cell? What host barriers must be broken before release of T4 virions from the host cell?

5.6 Some bacteriophages are temperate, meaning that they can initiate lytic events or integrate into the host genome as a prophage. Integration initiates a state called lysogeny in which the virus does not destroy the cell. If the lysogenic state goes through the process of induction, the viral infection switches to virulent (lytic). A well-studied lysogenic virus of *Escherichia coli* is phage lambda.

Q What kind of protein is required for bacteriophage lambda to maintain a lysogenic state?

5.7 There are eukaryotic viruses with all known modes of viral genome replication. This replication can occur in the nucleus or a compartment called a viroplasm. Many animal viruses are enveloped, picking up portions of host membrane as they leave the cell. Viral infection of animal host cells can result in cell lysis, but latent or persistent infections are also common, and a few animal viruses can cause cancer. In contrast, plant viruses are rarely enveloped and can often infect a wide range of plant, insect, nematode, and fungal species. These pests transfer the virion from one plant to another by puncturing through the rigid plant cell wall.

Q How do virions spread throughout the cells of a plant host? Why do plant viruses require host damage for infectivity?

APPLICATION QUESTIONS

1. What causes the viral plaques that appear on a bacterial lawn to stop growing larger?
2. What would happen if a prophage was not able to undergo the process of induction? How would this affect the virus and its host cell?

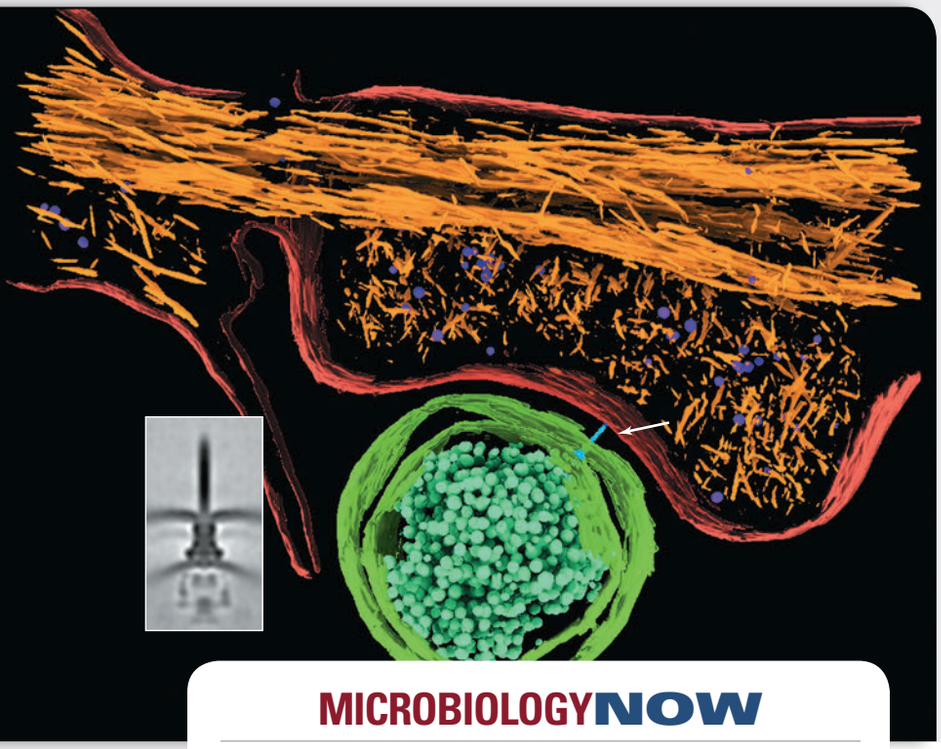
3. Under some conditions, it is possible to obtain nucleic acid–free protein coats (capsids) of certain viruses. Under the electron microscope, these capsids look very similar to complete virions. What does this tell you about the role of the virus nucleic acid in the virus assembly process? Would you expect such particles to be infectious?

CHAPTER GLOSSARY

Bacteriophage a virus that infects bacterial cells
Capsid the protein shell that surrounds the genome of a virus particle
Capsomere the subunit of a virus capsid
Early protein a protein synthesized soon after viral infection and before replication of the viral genome
Enveloped in reference to a virus, having a lipoprotein membrane surrounding the virion
Host cell a cell inside which a virus replicates
Late protein a protein, typically a structural protein, synthesized late in viral infection
Lysogen a bacterium containing a prophage
Lysogeny a state in which the viral genome is replicated in step with the genome of the host
Lytic pathway the type of viral infection that leads to virus replication and destruction of the host cell
Middle protein a protein with either a structural or a catalytic function synthesized after the early proteins in a virus infection
Nucleocapsid the complex of nucleic acid and capsid (shell) proteins of a virus
Plaque a zone of lysis or growth inhibition caused by viral infection of a bacterial lawn or other culture of sensitive host cells
Prophage the lysogenic form of a bacteriophage (see *provirus*)
Provirus the genome of a temperate, or latent, animal virus when it is replicating in step with the host chromosome
Retrovirus a virus whose RNA genome is replicated via a DNA intermediate
Temperate virus a virus whose genome can replicate along with that of its host without causing cell death, in a state called lysogeny (bacterial viruses) or latency (animal viruses)
Titer the number of individual particles of a substance, such as antibodies or infectious virions, per unit volume of a solution
Virion the infectious virus particle; the viral genome surrounded by a protein coat and sometimes other layers
Virulent virus a virus that lyses, or kills, the host cell after infection
Virus a genetic element that contains either RNA or DNA surrounded by a protein capsid and that replicates only inside host cells

Molecular Information Flow and Protein Processing

6



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MICROBIOLOGY**NOW**

Injectisomes: *Salmonella*'s Mode of Attack

Salmonella is a genus of gram-negative *Bacteria* that causes salmonellosis in humans. Once *Salmonella* colonizes the intestines, the pathogen produces proteins called effectors to manipulate host epithelial cells and trigger disease symptoms. In fact, this pathogen delivers over 40 different effectors, many of which are toxins, directly into host epithelial cells during infection. How does *Salmonella* accomplish such an aggressive feat?

Salmonella possesses a secretion system known as type III that moves effectors all the way through its membranes and cell wall into a host cell. The inset in the photo here shows a cryo-electron tomograph of a type III secretion system. The machinery can be divided into three parts: bottom—a platform that resides inside the bacterial cytoplasm; middle—a basal body within the periplasm and an outer membrane–anchored base; and top—a protruding needle filament. Based on this syringe-like morphology, type III secretion systems have been termed "injectisomes."

The actual injection of effector proteins into host cells requires an orchestrated series of events that is triggered when the injectisome tip makes contact with the host cell, and the colored tomograph illustrates this cell-to-cell contact. Bacterial cell membranes and ribosomes are green, and a blue injectisome (white arrow) is shown making contact with the host cell membrane (red). Host cell actin filaments and ribosomes are depicted in orange and purple, respectively.

Injectisome contact does not actually stab a hole into the host cell as one might expect. Instead, the contact leads to the formation of a protein channel (not shown) that allows for the passage of the toxin proteins from one domain of life to the other. Injectisomes are deployed by several other pathogens including *Yersinia* (plague), *Shigella* (gastrointestinal distress), *Pseudomonas* (various infections), and *Chlamydia* (sexually transmitted infections), and thus the success of this structure in facilitating disease symptoms is readily apparent.

Source: Park, D., et al. 2018. Visualization of the type III secretion mediated *Salmonella*–host cell interface using cryo-electron tomography. *eLife* 7: e39514.

In this chapter we take a brief detour from our coverage of microbiology per se to consider some basic principles of molecular biology, principles that govern the activities of all cells regardless of domain. The goal is to review how an organism orchestrates the series of macromolecular events that precede cell division and place these events in the context of modern molecular microbiology.

I • Molecular Biology and Genetic Elements

Genetic information flow is the hallmark of all cells, as the central dogma of cell biology is that DNA begets RNA begets protein. These processes are invariant in cells of all domains of life and can be initiated from many different DNA genetic elements, the chromosome being chief among them.

In Part I we examine the structure of DNA and the basic processes in genetic information flow and then move on to consider the various types of genetic elements. Part I lays the groundwork for a detailed consideration of DNA replication in prokaryotic cells (Part II) and the mechanisms behind RNA and protein synthesis (Parts III and IV).

6.1 DNA and Genetic Information Flow

An overview of the major macromolecules and processes of molecular biology is shown in **Figure 6.1*a***. In addition, the functional unit of genetic information is the **gene**, and genes make up parts of chromosomes or other large molecules known collectively as **genetic elements**; the total complement of genetic elements is the **genome**. Genetic information is embedded in the sequence of nucleotides in the nucleic acids **DNA** and **RNA**. DNA carries the cell's genetic blueprint while RNA, produced in transcription, carries a copy of this blueprint. One form of RNA called messenger RNA is converted by translation into defined amino acid sequences in proteins. Collectively, nucleic acids and proteins are called **informational macromolecules** (Figure 6.1*a*).

The monomers of nucleic acids are called **nucleotides** and so DNA and RNA are **polynucleotides**. A nucleotide has three components: a pentose sugar (either ribose in RNA or deoxyribose in DNA), a nitrogenous base, and a molecule of phosphate, PO_4^{3-} (Figure 6.1*b*). A **nucleoside** has a pentose sugar and a nitrogenous base but does not include a phosphate group. The nitrogenous bases in nucleic acids are either **pyrimidines** or **purines**. The purines guanine and adenine and the pyrimidine cytosine are present in both DNA and RNA, whereas the pyrimidines thymine and uracil are only present (with minor exceptions) in DNA and RNA, respectively (Figure 6.1*c*).

Properties of the Double Helix

The nucleic acid backbone is a polymer of alternating sugar and phosphate molecules, and nucleotides are linked by phosphate between the 3′-carbon of one sugar and the 5′-carbon of the next

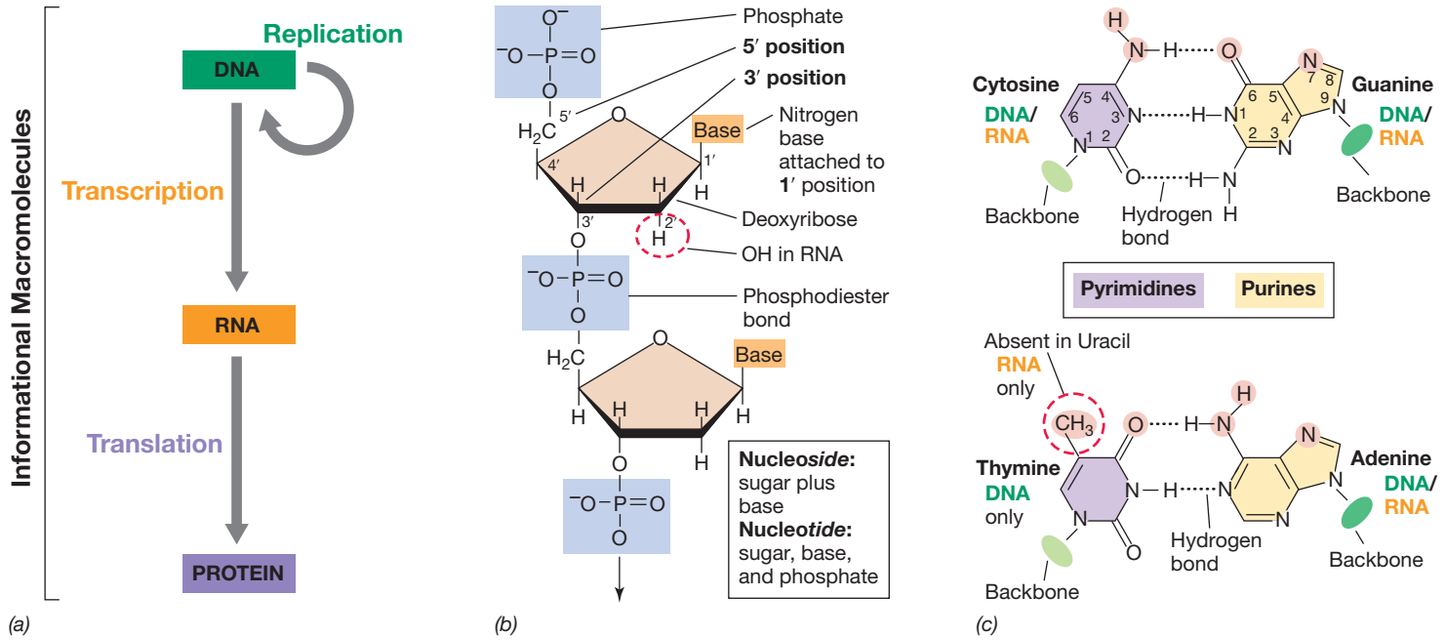


Figure 6.1 Genetic information flow and the components of the nucleic acids. *(a)* An overview of the types of informational macromolecules. *(b)* Part of a DNA chain. The numbers on the sugar of the nucleotide contain a prime (′) to differentiate them from the numbering on the rings of the nitrogen bases. In DNA, a hydrogen is present on the 2′-carbon of the pentose sugar. In RNA, an OH group occupies this position (indicated by the red dashed circle). The nucleotides are linked by a phosphodiester bond. *(c)* The nitrogen bases of DNA and RNA and the specific pairing between cytosine (C) and guanine (G) and between thymine (T) and adenine (A) via hydrogen bonds. Uracil (U) instead of thymine is present in RNA as indicated by the red dashed circle. Note the numbering system of the rings in that a pyrimidine base bonds through N-1 to the sugar–phosphate backbone and that a purine base bonds through N-9. Atoms that are found in the major groove of the double helix (see Figure 6.3) and that interact with proteins are highlighted in pink.

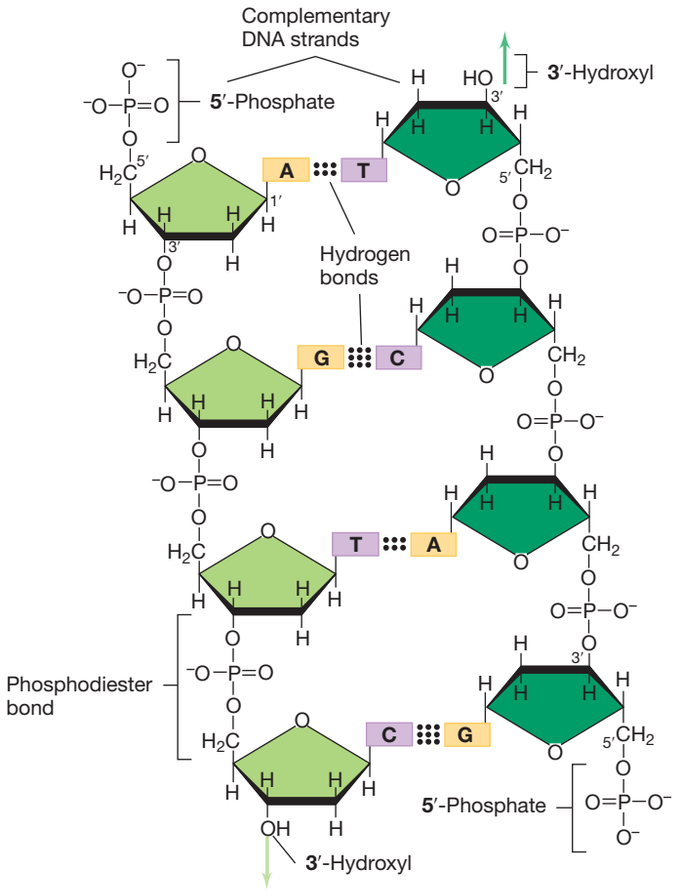


Figure 6.2 DNA structure. Complementary and antiparallel nature of DNA. Note that one chain ends in a 5′-phosphate group, whereas the other ends in a 3′-hydroxyl. The purple bases represent the pyrimidines cytosine (C) and thymine (T), and the yellow bases represent the purines adenine (A) and guanine (G).

sugar, the **phosphodiester bond** (Figure 6.1*b*, **Figure 6.2**). The *sequence* of nucleotides in a DNA or RNA molecule is its **primary structure** and encodes the genetic information. In cells, DNA is *double-stranded*, the strands being held together by hydrogen bonds between the bases in the two strands (Figure 6.1*c*). Specific base pairing, A with T and G with C, ensures that the two strands of DNA are **complementary** in base sequence, and this complementarity is essential for the faithful replication of the molecule. The two strands of the DNA molecule are also arranged in an **antiparallel** fashion; one strand runs 5′ to 3′ (top to bottom), whereas its complement runs 5′ to 3′ (bottom to top) (Figure 6.2).

The complementary and antiparallel strands of DNA are wrapped around each other to form a double helix (**Figure 6.3**). The helix naturally forms two distinct grooves, the *major groove* and the *minor groove*. Most proteins that interact specifically with DNA bind in the major groove, where space is abundant. Because the double helix is a regular structure, some atoms of each base are always exposed in the major groove (and some in the minor groove). These key regions of the DNA that are important in interactions with proteins can be seen in Figure 6.3, and the atoms of the major groove that interact with proteins are highlighted in pink in Figure 6.1*c*.

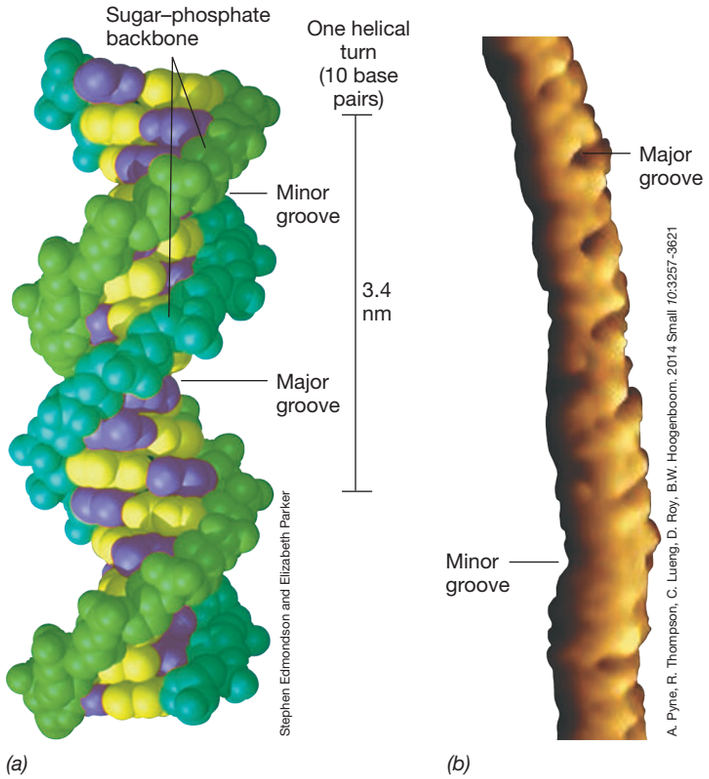


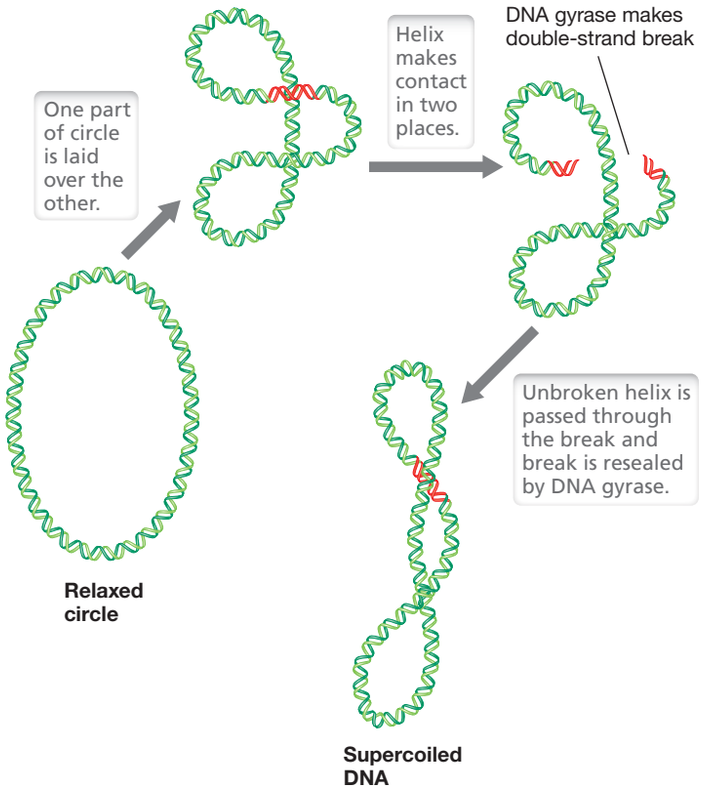
Figure 6.3 Arrangement of the DNA double helix. *(a)* A computer model of a short segment of DNA showing one of the sugar–phosphate backbones in blue-green and the other in light green. The pyrimidine bases are shown in purple and the purines in yellow. Note the locations of the major and minor grooves. One helical turn contains 10 base pairs. *(b)* Atomic force microscopy showing the biomolecular structure of a small piece of DNA. Note the locations of the major and minor grooves.

Size, Shape, and Supercoiling of DNA

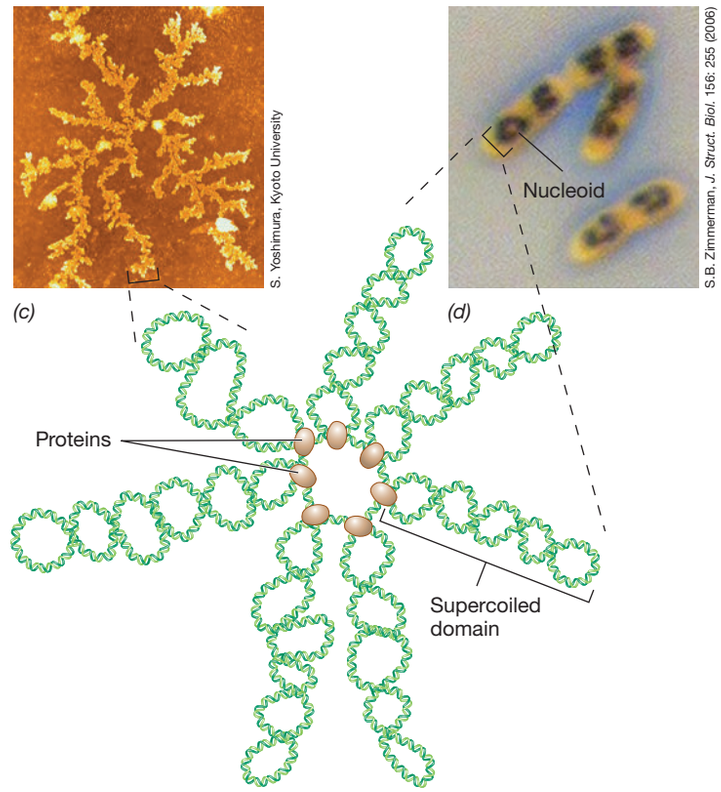
The size of a DNA molecule is expressed as its total number of nucleotide base pairs. Thus, a double-stranded DNA molecule consisting of 1000 bases is one kilobase pair (kbp) of DNA. The bacterium *Escherichia coli* has about 4640 kbp (4.64 megabase pairs, Mbp) of DNA in its genome. If this molecule were extended linearly it would be several hundred times longer than the cell itself. To accommodate their genome, cells of *Bacteria* and *Archaea* must compact the DNA, and this is done by the process of *supercoiling* (**Figure 6.4**).

Supercoils are inserted or removed in DNA by enzymes called *topoisomerases*. The activity of supercoiling puts the DNA molecule under torsion (Figure 6.4), and DNA can be supercoiled in either a positive or a negative manner. Negative supercoiling results when the DNA is twisted about its axis in the opposite sense from the right-handed double helix and is the form found in most cells. In the *E. coli* chromosome, more than 100 supercoiled domains exist, each stabilized by specific proteins bound to the DNA. Inserting supercoils into DNA requires energy from ATP, whereas releasing supercoils does not. In *Bacteria* and most *Archaea*, the topoisomerase **DNA gyrase** inserts negative supercoils into DNA by making double-strand breaks (Figure 6.4*a*). As we see in Chapters 4 and 17, some

Mastering Microbiology
Art Activity: Figure 6.4 DNA gyrase



(a) **Supercoiling process in circular DNA**



(b) **Chromosomal DNA with supercoiled domains**

Figure 6.4 Supercoiled DNA and DNA gyrase. *(a)* Schematic showing the introduction of negative supercoiling into circular DNA by the activity of DNA gyrase (topoisomerase II), which makes double-strand breaks. *(b)* The double-stranded DNA in the bacterial chromosome is arranged not in one supercoil but in several supercoiled domains, as shown here. *(c)* Atomic force microscopy of the *Escherichia coli* nucleoid. *(d)* Simultaneous phase-contrast and fluorescence image of *E. coli* illustrating the location of the nucleoid within growing cells. Cells were treated with a fluorescent dye specific for DNA and the color was inverted to show the nucleoids as black.

Archaea live at very high temperatures—above the boiling point in some cases. These species have chromosomes that are *positively* (instead of negatively) supercoiled, and this genomic feature helps to maintain DNA structure (that is, it prevents the two strands from melting apart) at such high temperatures (▶ Section 17.13). Supercoiling is not a feature of eukaryotes since their genomic DNA is linear rather than circular. However, eukaryotic DNA must still be compacted, and this occurs when the DNA is highly wound around histone proteins.

Genes and the Steps in Biological Information Flow

Genetic information flow is a fundamental process in all cells and is the *central dogma of molecular biology* (Figure 6.1*a* and **Figure 6.5**). When genes are *expressed*, the genetic information encoded in DNA is transferred to ribonucleic acid (RNA). While several classes of RNA exist in cells, three main classes of RNA participate in protein synthesis. **Messenger RNAs (mRNAs)** are single-stranded molecules that carry the genetic information from DNA to the ribosome. **Transfer RNAs (tRNAs)** help convert the genetic information in the nucleotide sequences of RNA into a defined sequence of amino acids in proteins. **Ribosomal RNAs (rRNAs)** are important catalytic and structural components of the ribosome. The molecular processes of genetic information flow can be divided into three stages (Figure 6.5):

1. **Replication**. During replication, the DNA double helix is duplicated. Replication is catalyzed by the enzyme *DNA polymerase*.
2. **Transcription**. The transfer of genetic information from DNA to RNA is called transcription. Transcription is catalyzed by the enzyme *RNA polymerase*.
3. **Translation**. The formation of a polypeptide using the genetic information transferred to mRNA by DNA is a process that occurs on the ribosome.

Many different RNA molecules can be transcribed from a relatively short region of the long DNA molecule. In eukaryotes, each gene is transcribed to yield a single mRNA, whereas a single mRNA molecule may encode several different proteins in *Bacteria* and *Archaea*. However, a linear correspondence exists between the base sequence of a gene and the amino acid sequence of a polypeptide, and as we will see, each group of *three bases* on an mRNA molecule encodes a single amino acid (Section 6.9).

Eukaryotes differ from *Bacteria* and *Archaea* in that the first two steps of the central dogma, replication and transcription (Figures 6.1 and 6.5), occur in the nucleus. Because ribosomes are not present in

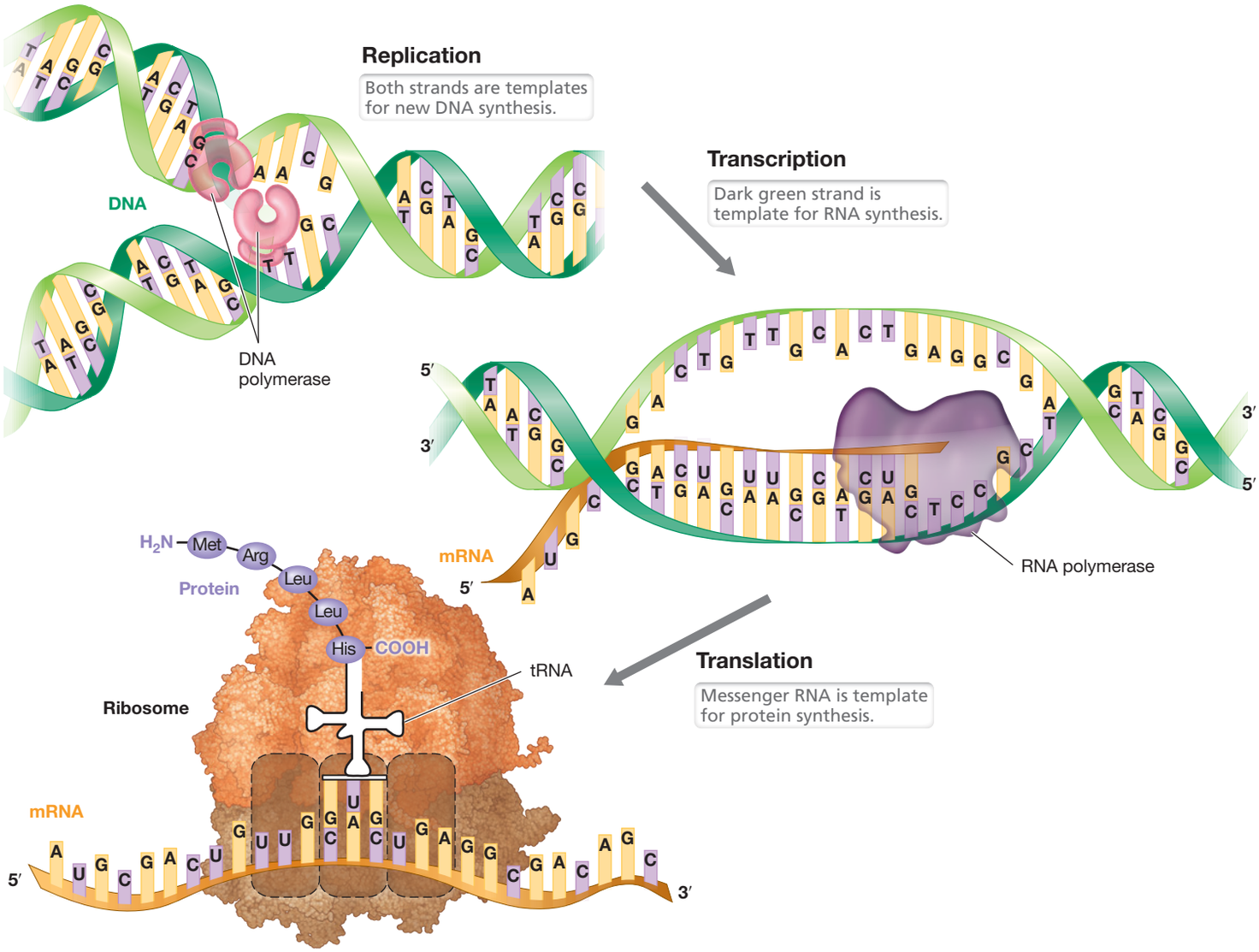


Figure 6.5 Synthesis of the three types of informational macromolecules in the processes of replication (DNA → DNA), transcription (DNA → RNA), and translation (RNA → protein). Note that for any particular gene only one of the two strands of the DNA double helix is transcribed.

the nucleus, mRNAs as well as other RNAs must be transported outside of the nucleus for translation. By contrast, in prokaryotic cells, mRNAs do not have to be exported from an organelle to be translated. Because of this fundamental difference, transcription and translation in *Bacteria* and *Archaea* can occur simultaneously in a process known as *coupled transcription and translation* (**Figure 6.6**). During this process, a ribosome initiates translation of an mRNA before RNA polymerase has finished synthesizing it. This allows rapidly growing cells to produce proteins at a maximal rate and also allows the cell to rapidly adapt to changes in growth conditions by quickly expressing the new protein sets required.

While the central dogma of molecular biology (Figures 6.1*a* and 6.5) is invariant in *cells*, we will see later that some *viruses* (which are not cells, ◀ Section 1.4) violate the dogma in many interesting ways (Chapter 11). But for now we move on to consider the different genetic elements present in prokaryotic cells.

Check Your Understanding

- What is a genome and what is it composed of? What is the central dogma of molecular biology?
- Define the terms complementary and antiparallel as they pertain to DNA.
- Why is supercoiling essential to a bacterial cell? What enzyme facilitates this process?

6.2 Genetic Elements: Chromosomes and Plasmids

Structures containing genetic material (DNA in cells but RNA in some viruses) are called *genetic elements*, and the main genetic element in prokaryotic cells is the **chromosome**. However, other genetic elements play important roles in microbes and these include

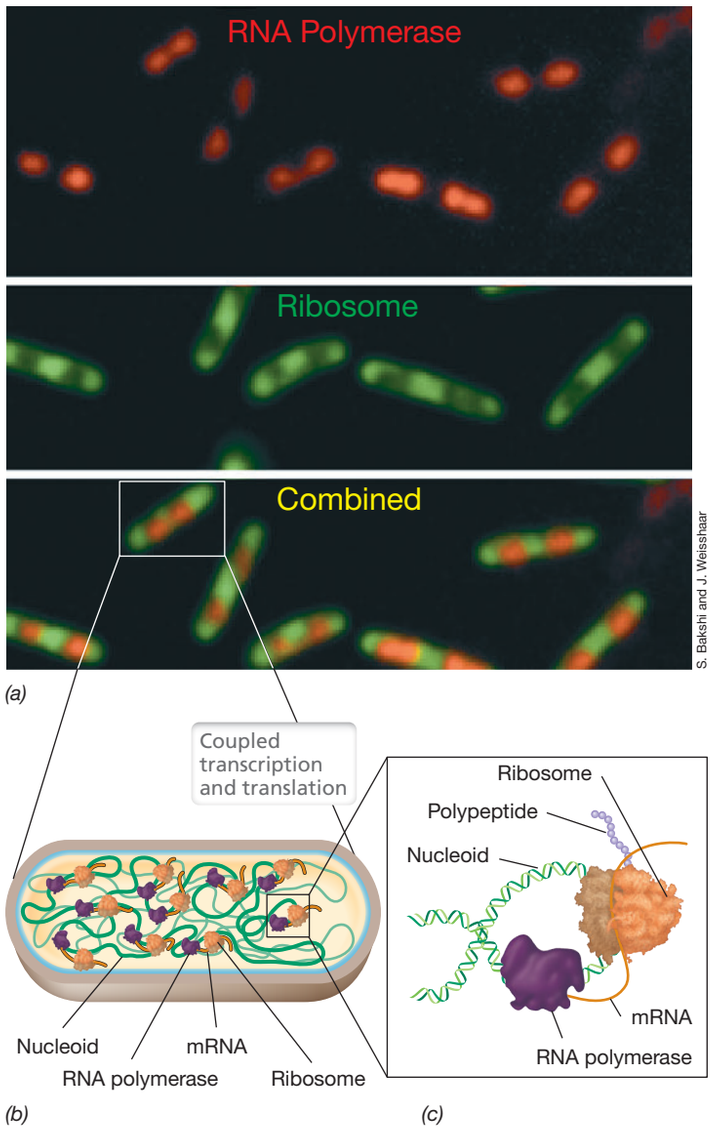


Figure 6.6 Coupled transcription and translation in prokaryotic cells. *(a)* Fluorescence microscopy and protein tagging of actively growing *Escherichia coli* cells illustrating the position of RNA polymerases and ribosomes performing transcription and translation, respectively. The combined photo (bottom image) shows that transcription and translation are occurring concurrently in the cell. *(b)* Location of the nucleoid, RNA polymerases, mRNA, and ribosomes in the cell during coupled transcription and translation. *(c)* Schematic illustration in a single cell of a ribosome actively translating an mRNA as it is being synthesized by RNA polymerase.

virus genomes (discussed in Chapters 5 and 11), *plasmids*, *organellar genomes*, and *transposable elements* (**Table 6.1**). Most *Bacteria* and *Archaea* contain a single circular chromosome containing all (or most) of the organism's genes. Although a single chromosome is the rule in prokaryotic cells, there are exceptions, as a few contain two or even three chromosomes. Eukaryotic genomes, by contrast, are composed of two or more chromosomes containing linear DNA. The genomes of viruses consist of *either* DNA or RNA and can be single- or double-stranded and either linear or circular.

Plasmids are circular or linear double-stranded DNA molecules that replicate separately from the chromosome and are typically much smaller than chromosomes. **Transposable elements** are sequences of DNA that are inserted into other DNA molecules but can move from one site on the DNA molecule to another, either within the same molecule or on a different DNA molecule. Chromosomes, plasmids, virus genomes, and any other type of DNA molecule may host a transposable element. Transposable elements are found in both prokaryotic and eukaryotic cells and play important roles in genetic variation (▶ Section 9.11).

Chromosomal Gene Arrangements

Thousands of genomes from species of *Bacteria* and *Archaea* have been completely sequenced, thus revealing the number and location (the genetic map) of the genes they possess. The genetic map of the 4,639,675-bp (4.639 Mb) chromosome of a widely studied strain of *Escherichia coli* is presented in **Figure 6.7**, with only a few of the organism's several thousand genes depicted. Analysis of the *E. coli* genome has revealed 4288 possible protein-encoding genes that account for 88% of the *E. coli* genome. Approximately 1% of the genome encodes tRNAs and rRNAs, and the remaining genes include regulatory sequences that may or may not be transcribed (but are not translated) and sequences that have other functions. The compact genomes of *Bacteria* and *Archaea* stand in contrast to the genomes of eukaryotes, which typically contain much more DNA than is needed to encode all the proteins required for cell function. This "extra" DNA in eukaryotes is present as intervening DNA between coding sequences (the intervening sequences are removed before translation) or as repetitive sequences, some of which are repeated hundreds or thousands of times (Chapter 10).

Genetic mapping of the genes encoding the enzymes that function in steps of the same biochemical pathway in *E. coli* has shown that these genes are sometimes clustered. On the genetic map in Figure 6.7, a few such clusters are shown (for example, the *gal*, *trp*, and *his* clusters); each of these groups is called an *operon*, which we will discuss in Section 6.5. In contrast to these, the genes for many other

TABLE 6.1 Kinds of genetic elements

Organism	Element	Type of nucleic acid	Description
Virus	Virus genome	Single- or double-stranded DNA or RNA	Relatively short, circular or linear
Bacteria, Archaea	Chromosome	Double-stranded DNA	Extremely long, usually circular
Eukarya	Chromosome	Double-stranded DNA	Extremely long, linear
Mitochondrion or chloroplast	Organellar genome	Double-stranded DNA	Medium length, usually circular
All organisms	Plasmid[a]	Double-stranded DNA	Relatively short circular or linear, extrachromosomal
All organisms	Transposable element	Double-stranded DNA	Always found inserted into another DNA molecule

[a]Plasmids are uncommon in eukaryotes.

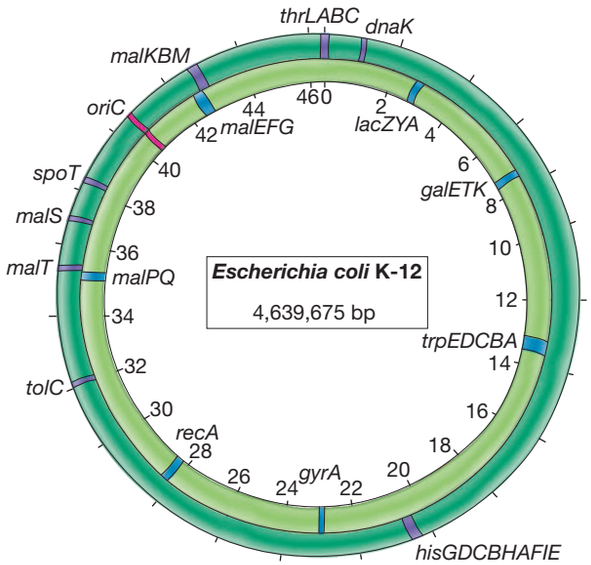


Figure 6.7 The chromosome of *Escherichia coli* strain K-12. Map distances are given in 100 kilobases of DNA. The chromosome contains 4,639,675 base pairs and 4288 open reading frames (genes). Depending on the DNA strand, the locations of a few genes and operons are indicated. Replication (Figure 6.5 and see Figures 6.15 and 6.16) proceeds in both directions from the origin of DNA replication, *oriC*, indicated in red.

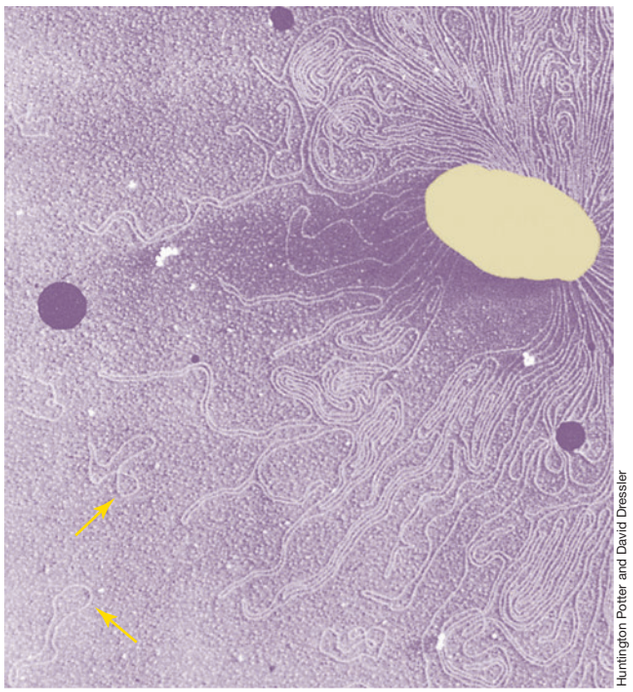


Figure 6.8 The bacterial chromosome and bacterial plasmids, as seen in the electron microscope. The plasmids (arrows) are the circular structures and are much smaller than the main chromosomal DNA. The cell (large, tan structure) was broken gently so the DNA would remain intact.

biochemical pathways in *E. coli* are not clustered. For example, genes for maltose degradation (*mal* genes, Figure 6.7) are scattered throughout the chromosome. In fact, analysis of the *E. coli* chromosome has shown that over 70% of the predicted or known transcriptional units are of only a single gene and that only 6% of operons have four or more genes. Thus operons, as efficient an arrangement of genes as they may be, appear to be the exception rather than the rule.

Plasmids

Many *Bacteria* and *Archaea* contain plasmids in addition to their chromosome(s). Most plasmids are nonessential since with rare exception they do not contain genes required for growth under all conditions. Thousands of different plasmids are known, and over 300 different plasmids have been isolated from strains of *E. coli* alone. Virtually all plasmids consist of double-stranded DNA and exist in the cytoplasm as free DNA. Most plasmids are circular, but many are linear and vary in size from approximately 1 kbp to more than 1 Mbp.

Typical plasmids are less than 5% of the size of the chromosome (Figure 6.8), and some bacteria contain several different plasmids. Moreover, different plasmids may be present in different *copy number*. For example, some plasmids may be present in only one or a few copies per cell, whereas others may be present in over 100 copies. Enzymes that replicate chromosomal DNA also replicate plasmids. Some of the genes encoded on a plasmid function to direct the initiation of plasmid replication and to partition replicated plasmids between daughter cells.

Although by definition plasmids do not encode functions essential to the host, plasmids may carry genes that profoundly influence host cell physiology; for example, plasmid genes may encode enzymes for some special metabolism that ensures survival under certain conditions. Among the most widespread and well-studied groups of plasmids are the resistance plasmids, called *R plasmids*, which confer resistance to antibiotics or other growth inhibitors. The resistance genes encode proteins that either inactivate the antibiotic or protect the cell in some other way (▶ Section 8.11), and several antibiotic resistance genes can be encoded on a single R plasmid. Plasmid R100 (Figure 6.9), for example, encodes resistance to sulfonamides, streptomycin, spectinomycin, fusidic acid, chloramphenicol, and tetracycline,

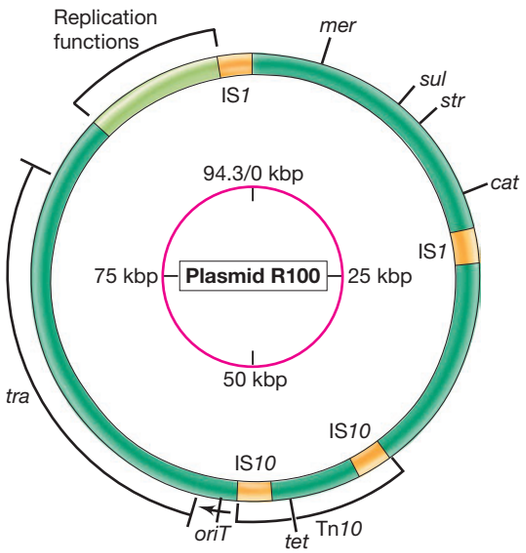


Figure 6.9 Genetic map of the resistance plasmid R100. The inner circle shows the size in kilobase pairs. The outer circle shows the location of major antibiotic resistance genes and other key functions: *mer*, mercuric ion resistance; *sul*, sulfonamide resistance; *str*, streptomycin resistance; *cat*, chloramphenicol resistance; *tet*, tetracycline resistance; *oriT*, origin of conjugative transfer; *tra*, transfer functions. The locations of insertion sequences (IS) and the transposon Tn*10* are also shown. Genes for plasmid replication are found in the region from 88 to 92 kbp.

as well as the toxic heavy metal mercury. Pathogenic bacteria resistant to antibiotics are of considerable medical significance, and their increasing incidence is correlated with the increasing use of antibiotics for treating infectious diseases in humans and animals (Chapter 28).

Pathogenic bacteria express a variety of plasmid-encoded *virulence factors* that assist them in establishing infections. For example, the ability of a pathogen to attach to and colonize specific host tissues and to produce toxins, enzymes, and other invasive molecules that damage the host are sometimes plasmid encoded. Some bacteria also produce proteins called **bacteriocins** that inhibit or kill closely related species of bacteria (or even different strains of the same species of bacteria), and the genes encoding these bacteriocins and other proteins that protect the producing organism are typically found on plasmids.

In a few cases plasmids encode properties that are fundamental to the ecology of the bacterium. For example, the ability of the soil bacterium *Rhizobium* to form nitrogen-fixing nodules on the roots of plants (▶ Section 23.4) requires certain functions encoded by plasmids. Other plasmids confer special metabolic properties. For example, the ability to degrade hydrocarbons or toxic pollutants, such as polychlorinated biphenyls (PCBs) and herbicides or other pesticides, is often plasmid encoded. In addition, plasmids play a crucial role in the horizontal gene transfer process called conjugation that we consider in detail later (Chapter 9).

Check Your Understanding

- Approximately how large is the *Escherichia coli* genome in base pairs? How many genes does it contain?
- What are viruses and plasmids?
- What properties does an R plasmid confer on its host cell?

II • Copying the Genetic Blueprint: DNA Replication

DNA replication must occur in a cell before it can divide. This process has a unique dimension in prokaryotic cells because their circular chromosome allows bidirectional DNA synthesis.

DNA replication is necessary for cells to divide, whether to reproduce new organisms, as in unicellular microorganisms, or to produce new cells as part of a multicellular organism. To successfully transmit genetic information from a mother cell to an identical daughter cell, DNA replication must be extremely accurate. We review the basic principles of DNA replication here as a prelude to focusing on the process as it o ccurs in prokaryotic cells.

6.3 Templates, Enzymes, and the Replication Fork

DNA exists in cells as a double helix of two complementary strands (Figures 6.2 and 6.5), and if the helix is opened up, a new strand can be synthesized as the complement of each parental strand. As shown in **Figure 6.10*a***, replication is thus a *semiconservative* process; in **semiconservative replication**, the two resulting double helices each consist of one new strand and one parental strand. The DNA strand that is used to make a complementary daughter strand is called the *template strand*, and in DNA replication, each parental strand is a template for one newly synthesized strand (Figure 6.10*a*). The precursor of each new nucleotide in the DNA strand is a deoxynucleoside 5′-triphosphate. During insertion of this molecule, the two terminal phosphates are removed and the remaining phosphate is bonded to a deoxyribose of the growing chain (Figure 6.10*b*). This addition of the incoming nucleotide requires the presence of a free hydroxyl group, which is available only at the 3′ end of the molecule. This leads to the important principle that DNA replication always proceeds *from the* 5′ *end to the* 3′ *end*, the 5′-phosphate of the incoming nucleotide being attached to the 3′-hydroxyl of the previously added nucleotide (Figure 6.10*b*).

Replication Enzymes

Enzymes that catalyze the polymerization of deoxynucleotides are called **DNA polymerases** (abbreviated DNA Pol), and there are five different enzymes in *Escherichia coli*, DNA Pol I–V. DNA Pol III is the primary enzyme for replicating chromosomal DNA, although DNA Pol I also plays a lesser role. The other DNA polymerases function to repair damaged DNA (▶ Section 9.4). DNA Pol enzymes are just some of the many enzymes that are required for DNA replication (**Table 6.2**).

TABLE 6.2 Major enzymes that participate in DNA replication in *Bacteria*

Enzyme	Encoding genes	Function
DNA gyrase	*gyrAB*	Replaces supercoils ahead of replisome
Origin-binding protein	*dnaA*	Binds origin of replication to open double helix
Helicase loader	*dnaC*	Loads helicase at origin
Helicase	*dnaB*	Unwinds double helix at replication fork
Single-strand binding protein	*ssb*	Prevents single strands from annealing
Primase	*dnaG*	Primes new strands of DNA
DNA polymerase III		Main polymerizing enzyme
Sliding clamp	*dnaN*	Holds Pol III on DNA
Clamp loader	*holA–E*	Loads Pol III onto sliding clamp
Dimerization subunit (Tau)	*dnaX*	Holds together the two core enzymes for the leading and lagging strands
Polymerase subunit	*dnaE*	Strand elongation
Proofreading subunit	*dnaQ*	Proofreading
DNA polymerase I	*polA*	Excises RNA primer and fills in gaps
DNA ligase	*ligA, ligB*	Seals nicks in DNA
Tus protein	*tus*	Binds terminus and blocks progress of the replication fork
Topoisomerase IV	*parCE*	Unlinking of interlocked circles

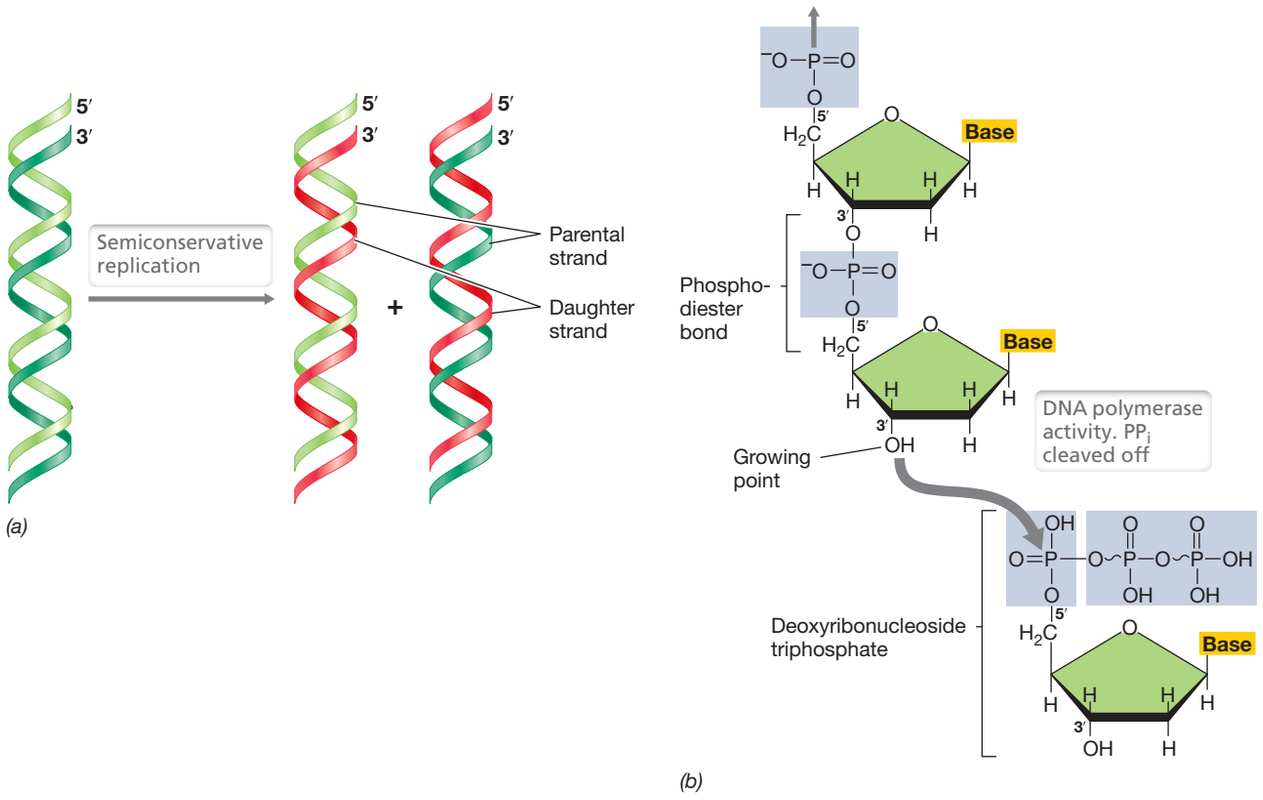


Figure 6.10 Overview of DNA replication. *(a)* In cells, DNA replication is a semiconservative process. Note that the new double helices each contain one new daughter strand (shown in red) and one parental strand. *(b)* Extension of a DNA chain by adding a deoxyribonucleoside triphosphate at the 3′ end. Growth proceeds from the 5′-phosphate to the 3′-hydroxyl end. DNA polymerase catalyzes the reaction. The four precursors are deoxythymidine triphosphate (dTTP), deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP). Upon nucleotide insertion, the two terminal phosphates of the triphosphate are split off as pyrophosphate (PP_i). Thus, two energy-rich phosphate bonds are consumed when adding each nucleotide.

All DNA polymerases synthesize DNA in the 5′ → 3′ direction, but none of them can initiate a new chain de novo; they can only add a nucleotide onto a preexisting 3′-OH group. Thus, in order to start a new DNA chain, a **primer**, a nucleic acid molecule to which DNA polymerase can attach the first nucleotide, is required, and this primer is a short stretch of *RNA* rather than DNA (**Figure 6.11**). When the DNA helix is first opened, the enzyme **primase** makes this RNA primer, synthesizing a short stretch (11–12 nucleotides) of RNA complementary in base pairing to the template-strand DNA. At the growing end of this RNA primer is a 3′-OH group to which DNA polymerase adds the first deoxyribonucleotide. Continued extension of the molecule thus occurs as DNA rather than RNA (Figure 6.11), and the primer is eventually removed and replaced with DNA (as described shortly).

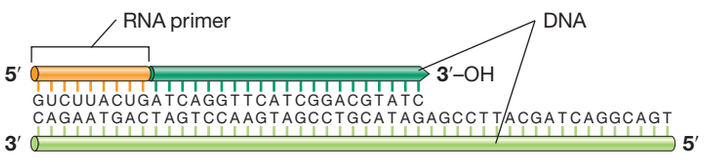


Figure 6.11 The RNA primer. Structure of the RNA–DNA hybrid formed during initiation of DNA synthesis. Orange depicts the RNA primer.

Initiation of DNA Synthesis

Before replication can begin, the double helix must be unwound to expose the template strands at the so-called **replication fork**. The enzyme **DNA helicase** unwinds the double helix (using energy from ATP) and exposes a short single-stranded region (**Figure 6.12**). Helicase moves along the DNA and separates the strands just in advance of the replication fork. The single-stranded region is immediately covered with copies of single-strand binding protein to stabilize the single-stranded DNA and prevent the double helix from re-forming. DNA synthesis begins at a single site on the chromosome, the *origin of replication* (*oriC*), where the protein DnaA (Table 6.2) binds and opens up the double helix. Next to assemble is helicase (DnaB), which is helped onto the DNA by a loader protein (DnaC) (Figure 6.12*b*). Finally, primase and DNA polymerase enzymes are loaded onto the DNA behind the helicase and initiation of DNA replication begins. As replication proceeds, the replication fork appears to move along the DNA (Figure 6.12*a*).

Mastering Microbiology
Microbiology Animation: DNA Replication: Forming the DNA Replication Fork

Leading and Lagging Strands and the Replication Process

Figure 6.13 depicts DNA replication at the replication fork. Recall that replication always proceeds from 5′ to 3′ (5′ → 3′), always adding a new nucleotide to the 3′-OH of the growing chain. On the strand

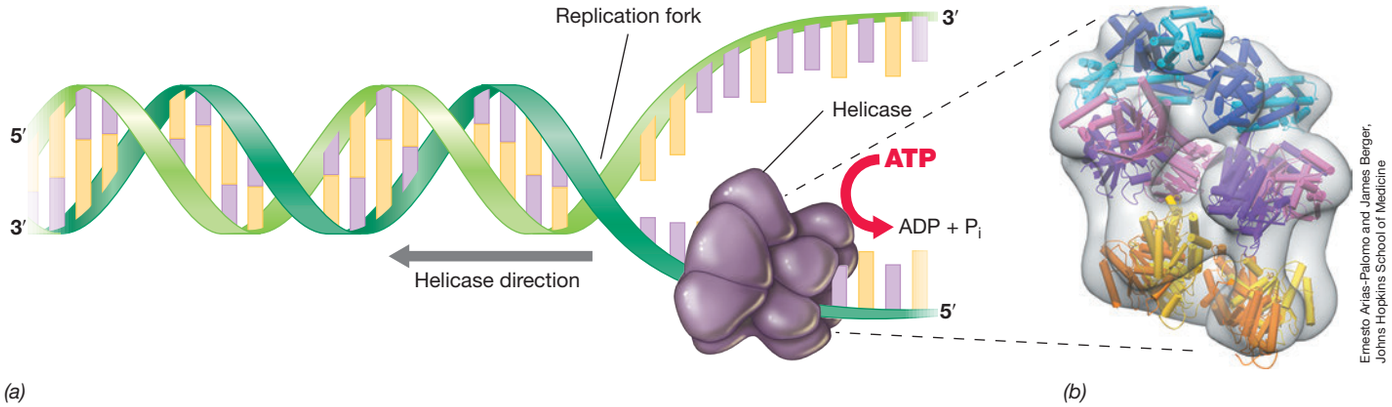


Figure 6.12 DNA helicase unwinding a double helix. *(a)* In this schematic, the helicase is seen pulling the two antiparallel strands of DNA apart beginning from the right and moving to the left. *(b)* A three-dimensional model of the helicase (DnaB) along with its loader protein (DnaC, yellow and orange) based on cryo-electron microscopy.

Mastering Microbiology
Microbiology Animation: DNA Replication: Replication Proteins

growing from the 5′-PO_4^{2-} to the 3′-OH, called the **leading strand**, DNA synthesis occurs *continuously* because there is always a free 3′-OH at the replication fork to which a new nucleotide can be added; the leading strand must therefore be primed only once. By contrast, on the opposite strand, called the **lagging strand**, DNA synthesis occurs *discontinuously* because there is no 3′-OH at the replication fork to which a new nucleotide can attach; on this strand, primase must synthesize multiple RNA primers in order to provide free 3′-OH groups for DNA Pol III (Figure 6.13). As a result, the lagging strand forms from several short DNA fragments that are combined later to yield a continuous strand of DNA.

After synthesizing the RNA primer, primase is replaced by DNA Pol III. This enzyme complex (Table 6.2) is held on the DNA by a "sliding clamp," which encircles and slides along the single template strands of DNA. Consequently, the replication fork contains two polymerase core enzymes and two sliding clamps, one set for each strand. After assembly on the lagging strand, the elongation activity of DNA Pol III adds deoxyribonucleotides sequentially until it reaches previously synthesized DNA (**Figure 6.14**); at this point, activity of DNA Pol III stops.

To complete DNA synthesis, DNA Pol I catalyzes two different reactions. Besides synthesizing DNA, Pol I has a 5′ → 3′ exonuclease activity that removes the RNA primer (Figure 6.14). When the primer has been excised and replaced with DNA, Pol I is released. The *very last* phosphodiester bond in replicating DNA is made by **DNA ligase**. This enzyme seals nicks in DNAs that have an adjacent 5′-PO_4^{2-} and 3′-OH (something that DNA Pol I and Pol III are unable to do), and along with DNA Pol I, it also participates in DNA repair. DNA ligase is also important for sealing genetically manipulated DNA during molecular cloning (▶ Section 12.2).

We now put DNA synthesis in the context of *Archaea* and *Bacteria* to see how replication events occur around the covalently closed and circular chromosomes typical of these organisms.

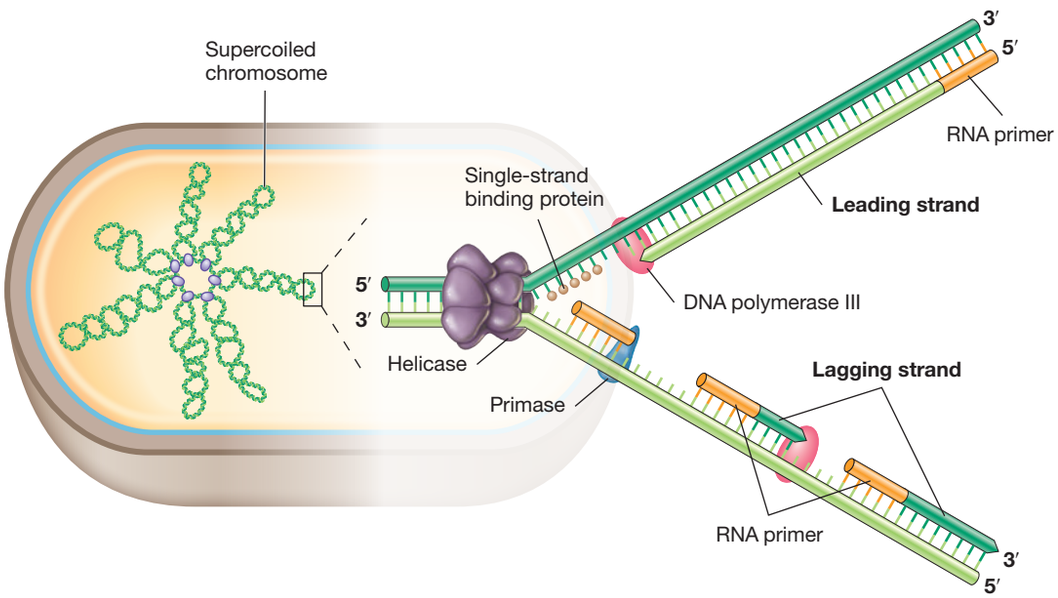


Figure 6.13 Events at the DNA replication fork on the nucleoid. Note the polarity and antiparallel nature of the DNA strands. Helicase unwinds the DNA while primase adds the RNA primer. For the steps in introducing and removing supercoils from DNA, see Figure 6.4. Further events in DNA synthesis including sealing replicated fragments are shown in Figure 6.14.

Check Your Understanding

- What is the difference between a template strand and a daughter strand of DNA?
- To which end (5′ or 3′) of a newly synthesized strand of DNA does DNA polymerase add a nucleotide?
- In DNA replication, what is the primer composed of and why are there leading and lagging strands?
- What are the functions of DNA Pol I and III, DNA helicase, and DNA ligase?

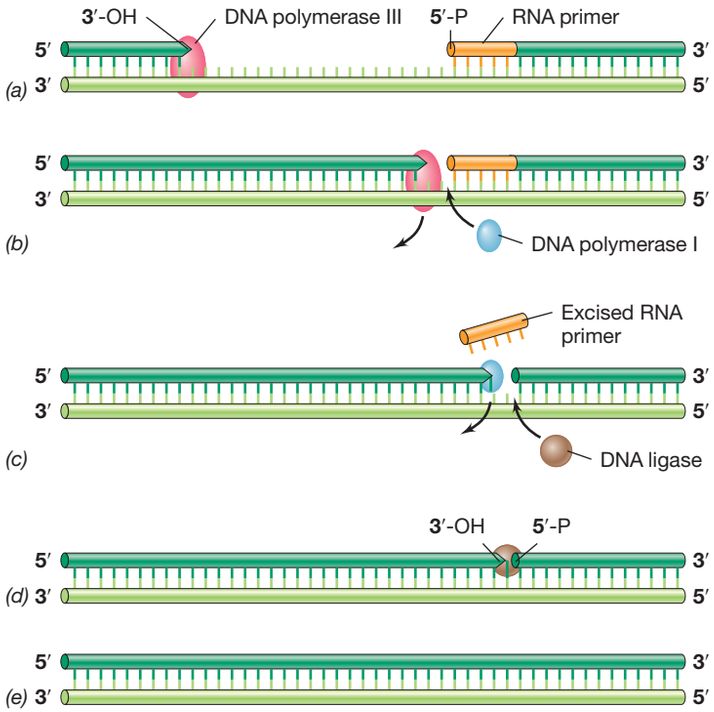


Figure 6.14 Sealing two fragments on the lagging strand. Unlike the leading strand, where synthesis occurs in a continuous fashion, on the lagging strand, DNA fragments need to be sealed to form the intact DNA strand. *(a)* DNA polymerase III is synthesizing DNA in the 5′ → 3′ direction toward the RNA primer of a previously synthesized fragment on the lagging strand. *(b)* On reaching the fragment, DNA polymerase III leaves and is replaced by DNA polymerase I. *(c)* DNA polymerase I continues synthesizing DNA while removing the RNA primer from the previous fragment, and DNA ligase replaces DNA polymerase I after the primer has been removed. *(d)* DNA ligase seals the two fragments together. *(e)* The final product, complementary and antiparallel double-stranded DNA.

6.4 Bidirectional Replication, the Replisome, and Proofreading

The circular nature of the bacterial and archaeal chromosome accelerates the genomic replication process. In *Escherichia coli*—and probably in all cells with circular chromosomes—DNA replication occurs *bidirectionally* from the origin of replication. There are thus *two* replication forks on each chromosome, each moving in opposite directions. In circular DNA, bidirectional replication leads to the formation in the replicating molecules of characteristic shapes (so-called "theta structures") as synthesis proceeds in both a leading and a lagging fashion on each template strand (Figure 6.15). In an actively growing cell of *E. coli*, DNA Pol III adds nucleotides at the rate of about 1000 per second; hence, replication of the entire chromosome takes about 40 min.

The Replisome

Figure 6.13 shows the enzymes that participate in replication, and from such a schematic it may appear that the enzymes are working independently. However, this is not the case. Instead, replication proteins aggregate to form a large replication complex called the **replisome** (Figure 6.16). The lagging strand of DNA actually loops out to allow the replisome to move smoothly along both strands, the complex literally pulling the DNA template through it as replication proceeds. In addition to the replisome, helicase and primase form their own subcomplex within the replisome called the *primosome*. This close association facilitates the sequential activities of these two enzymes during the replication process (Figure 6.16). Table 6.2 summarizes the functions of proteins essential for DNA replication in *Bacteria*.

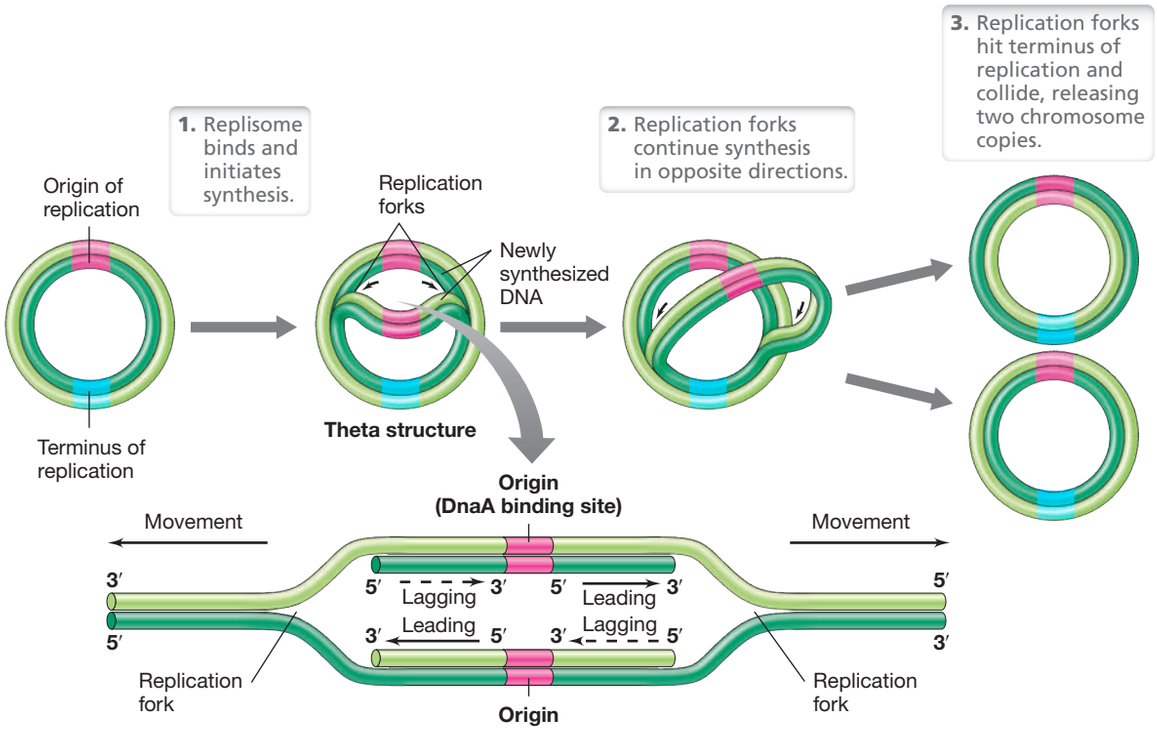


Figure 6.15 Replication of circular DNA: the theta structure. In circular DNA, bidirectional replication from an origin forms an intermediate structure resembling the Greek letter theta (θ). The blowup shows dual replication forks in the circular chromosome. In *Escherichia coli*, the origin of replication is recognized by the DnaA protein and the terminus of replication is recognized by the Tus protein. Note that DNA synthesis is occurring in both a leading and a lagging manner on each of the new daughter strands until the replication forks hit the terminus. Compare this figure with the illustration of the replisome in Figure 6.16.

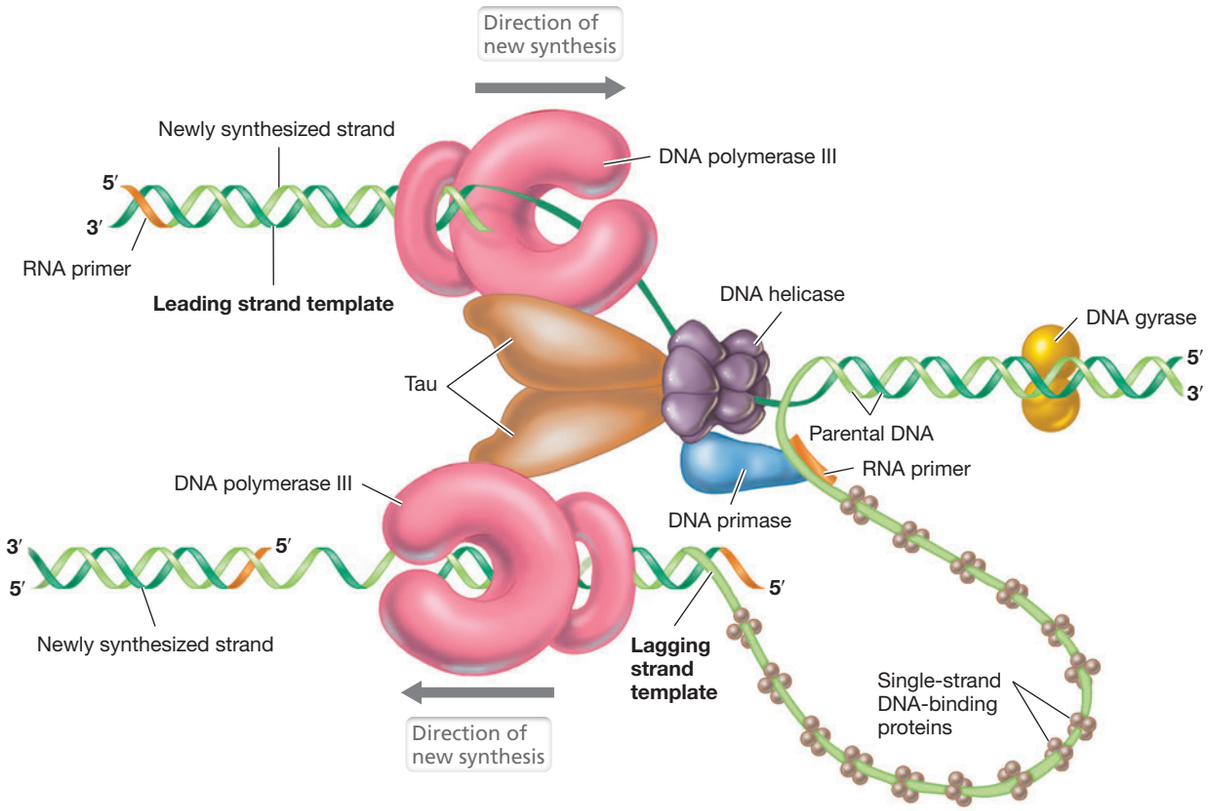


Figure 6.16 The replisome. The replisome consists of two copies of DNA polymerase III and DNA gyrase, plus helicase and primase (together forming the primosome), and many copies of single-strand DNA-binding protein. The Tau subunits hold the two DNA polymerase assemblies and helicase together. Just upstream of the rest of the replisome, DNA gyrase removes supercoils in the DNA to be replicated. Note that the two polymerases are replicating the two individual strands of DNA in opposite directions. Consequently, the lagging-strand template loops around so that the whole replisome moves in the same direction along the chromosome.

Eventually the activity of the replisome is finished, and this is signaled when the replication forks collide at the *terminus of replication*, a site located on the opposite side of the chromosome from the origin. In the terminus region are several DNA sequences called *Ter* sites that are recognized by a protein called Tus, whose function is to block progress of the replication forks. When replication of the circular chromosome is complete, the two circular molecules are linked together, much like the links of a chain. After replication, the DNA is partitioned so that each daughter cell receives a copy of the chromosome; DNA partitioning is facilitated by FtsZ, a protein that orchestrates several key events in the cell division process (Chapter 8).

Fidelity of DNA Replication: Proofreading

DNA replication occurs with a remarkably low error rate. Nevertheless, when errors do occur, a mechanism exists to detect and correct them. Errors in DNA replication introduce *mutations*, changes in DNA sequence. Mutation rates in cells are extremely low, between 10^{-8} and 10^{-11} errors per base pair inserted. This accuracy is achieved because DNA polymerases get *two* chances to incorporate the correct base at any given site. The first chance comes when DNA Pol III inserts bases according to the base-pairing rules (Figure 6.1*c*). The second chance comes when a process called *proofreading* takes place (**Figure 6.17**).

During replication, if an incorrect base has been inserted, a mismatch in base pairing (Figure 6.1*c*) occurs. Both DNA Pol I and Pol III possess a $3' \rightarrow 5'$ exonuclease activity that can remove such mismatched nucleotides. The polymerase detects the error because incorrect base pairing causes a slight distortion in the topology of the double helix. After the removal of a mismatched nucleotide, the polymerase then gets a second chance to insert the correct nucleotide (Figure 6.17; part *b* shows the polymerase working in "proofread mode"). With the extremely low error rate of DNA polymerases, the chance of inserting the wrong base at the same site twice is vanishingly small. Exonuclease proofreading occurs in *Bacteria* and *Archaea*, eukaryotes, and viral DNA replication systems.

We now move on from replicating genes to consider *gene expression* as a prelude to examining synthesis of the proteins encoded by the transcribed genes.

Check Your Understanding

- What is the replisome and what are its components?
- How are the activities of the replisome stopped?
- How are errors in DNA replication kept extremely low?

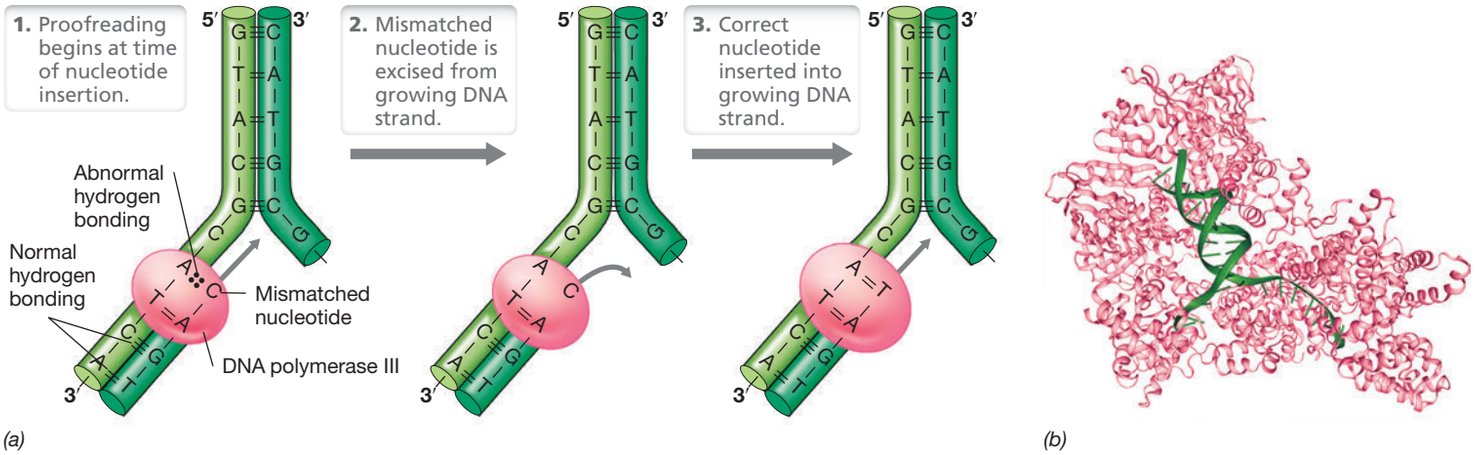


Figure 6.17 Proofreading by the 3′ → 5′ exonuclease activity of DNA polymerase III. *(a)* A mismatch in base pairing at the terminal base pair leads to a distortion in DNA topology that causes the polymerase to pause briefly. This signals the proofreading activity to cut out the mismatched nucleotide, after which the correct base is inserted by the polymerase activity. *(b)* Cryo-electron micrograph of *Escherichia coli* DNA polymerase III bound to DNA (green strands) in proofreading mode. Image credit: Fernandez-Leiro, R., Conrad, J., Scheres, S.H.W., and Lamers, M.H., EMDataBank: EMD-4141.

UNIT 2

III • RNA Synthesis: Transcription

Transcription transforms the genetic information inscribed in DNA into RNA, a form of nucleic acid that the cell's protein-synthesizing machinery can recognize and process into the many different types of proteins that a given cell needs.

Transcription—RNA synthesis off of a DNA template—yields three main forms of RNA: *messenger* (mRNA), *transfer* (tRNA), and *ribosomal* (rRNA) (Section 6.1). Several other minor classes of RNA exist, but most of these function in regulation (Chapter 7). RNA is both a genetic and a functional molecule. At the genetic level, mRNA encodes genetic information from the genome for the synthesis of proteins and carries this information to the ribosome. In contrast, rRNAs play both a structural and a functional role in ribosomes, while tRNAs function as the carriers of amino acids to the ribosome for protein synthesis.

There are two key differences in the chemistry of RNA and DNA: (1) RNA contains ribose instead of deoxyribose; and (2) RNA contains uracil instead of thymine. The change from deoxyribose to ribose dramatically affects the chemistry of a nucleic acid, and enzymes that act on DNA typically have no effect on RNA, and vice versa. However, the change from thymine to uracil does not affect base pairing, as these two bases pair with adenine equally well.

With the exception of a few viruses that contain double-stranded RNA genomes (Chapter 11), RNA is a single-stranded molecule. However, the primary structure (sequence of nucleotides) of some RNAs allows them to fold and exploit complementary base pairing. The term **secondary structure** refers to this folding, and the functional role an RNA plays in the cell may depend critically on its secondary structure. For example, messenger RNAs, which are typically unfolded, exist in *Bacteria* (and *Archaea*) for only a few minutes before enzymes called *ribonucleases* degrade them. By contrast, rRNAs and tRNAs (referred to as *stable RNAs*) are long-lived because their secondary structures prevent ribonuclease attack. The rapid turnover of mRNAs in *Bacteria* and *Archaea* allows them to quickly adapt to changing environmental conditions and halt the translation of mRNAs whose products are no longer needed.

We begin with transcription in *Bacteria* and contrast this in the following section with transcriptional events in *Archaea* and *Eukarya*.

6.5 Transcription in *Bacteria*

Transcription is catalyzed by the enzyme **RNA polymerase**. Like DNA polymerase, RNA polymerase forms phosphodiester bonds but between the *ribo*nucleotides rather than *deoxyribo*nucleotides (Figure 6.1*b*). Polymerization is driven by energy released from the hydrolysis of two energy-rich phosphate bonds of the incoming ribonucleoside triphosphates. The mechanism of RNA synthesis is thus quite similar to that of DNA synthesis (Figure 6.10*b*): During elongation of an RNA chain, ribonucleoside triphosphates are added to the 3′-OH of the ribose of the preceding nucleotide. Thus chain growth is 5′ → 3′ just as in DNA synthesis, and the newly synthesized strand of RNA runs antiparallel to the DNA template strand it was transcribed from. A summary of bacterial transcription is illustrated in **Figure 6.18**.

Mastering Microbiology
Art Activity: Figure 6.18*a* Transcription

RNA polymerase uses DNA as a template, but for any given gene, only one of the two strands is transcribed. Unlike DNA polymerase, RNA polymerase can initiate new RNA on its own; no priming is necessary as it is for DNA synthesis (Figure 6.11). Transcription continues until specific sequences called *transcription terminators* are reached, but unlike DNA replication, which copies the entire genome, transcription occurs on much smaller units of DNA, often as little as a single gene. This system allows the cell to transcribe different genes at different frequencies, depending on the needs of the cell for different proteins. Said a different way, gene expression is a *highly regulated* process. Transcriptional regulation can occur in bacterial and archaeal cells in many different ways, but the different mechanisms have a common outcome: Cell resources are conserved and cell fitness enhanced (Chapter 7).

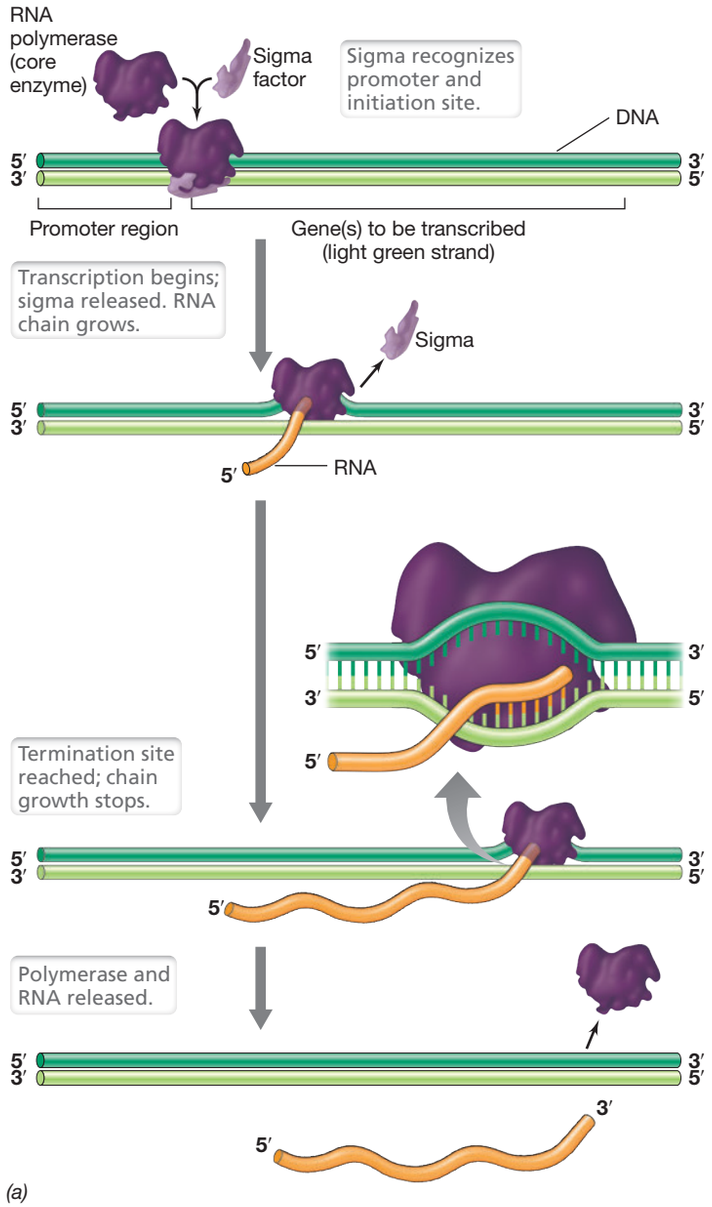


Figure 6.18 **Transcription.** *(a)* Steps in RNA synthesis. The initiation site (promoter) and termination site are specific nucleotide sequences on the DNA. Sigma binds to the promoter region and then RNA core polymerase binds to begin transcription. RNA polymerase moves down the DNA chain, temporarily opening the double helix (creating a transcription bubble) and transcribing one of the DNA strands. *(b)* Electron micrograph illustrates transcription by multiple polymerases along a gene on the *Escherichia coli* chromosome. Transcription is proceeding from left to right, with the shorter transcripts on the left becoming longer as transcription proceeds.

RNA Polymerases and the Promoter Sequence

The RNA polymerase from *Bacteria*, which has the simplest structure and about which most is known, consists of five different subunits, designated β, β′, α, ω (omega), and σ (sigma), with α present in two copies (**Figure 6.19**). The subunits form an enzyme complex called the *RNA polymerase holoenzyme*. Sigma is not as tightly bound as the other subunits and easily dissociates to yield the *RNA polymerase core enzyme*, $\alpha_2\beta\beta'\omega$. The core enzyme alone synthesizes RNA, and sigma functions only to recognize the appropriate site on the DNA to initiate transcription (sigma dissociates from the holoenzyme once a short sequence of RNA has been formed; Figure 6.18).

To begin transcription, RNA polymerase must first recognize initiation sites on the DNA; these sites are called **promoters**. In *Bacteria*, promoters are recognized by sigma (**Figure 6.20**). Once RNA polymerase has bound to a promoter, transcription can proceed (Figure 6.18). In this process, the DNA helix at the promoter site is opened up by RNA polymerase, and as the polymerase moves, it unwinds the DNA in short segments to expose template DNA, creating a *transcription bubble* (Figure 6.18). Thus, an enzyme with the activity of DNA helicase is not needed. Because some genes reside on one strand of DNA while other genes reside on the other strand of DNA, promoters are present on both strands; as a result, transcription occurs in *opposite directions* on the two different strands of DNA.

Sigma Factors, Consensus Sequences, and Transcriptional Termination

Promoters are specific DNA sequences; Figure 6.20*b* shows the sequence of several promoters from *Escherichia coli*. All of these sequences are recognized by the same *E. coli* sigma factor called σ^{70} (the superscript 70 indicates the size of this protein in kilodaltons, a measure of atomic mass). Although these sequences are not identical, sigma recognizes two highly conserved regions within the promoter. These conserved sequences are upstream of (prior to) the transcription start site. One is 10 bases upstream, the −10 region, or *Pribnow box*. Although promoter sequences differ slightly, comparison of many −10 regions gives a consensus sequence of TATAAT. The second conserved region is about 35 bases upstream of the start site and its consensus sequence is TTGACA (Figure 6.20). In *E. coli*, promoters that conform most closely to the consensus sequence are more effective in binding RNA polymerase. Such promoters are called *strong promoters* and are very useful in genetic engineering, where it is desirable to have only one or a restricted set of genes heavily transcribed in order to produce more of a particular protein product (Chapter 12).

While most genes in *E. coli* require σ^{70} for transcription, several alternative sigma factors exist that recognize different consensus sequences (**Table 6.3**). Each alternative sigma factor is specific for a group of genes required under special circumstances, and thus the presence or absence of a specific sigma factor is a mechanism for regulating gene expression (Chapter 7). That is, by changing the rate of either synthesis or degradation of a particular sigma factor, the cell can control the transcription of entire gene families.

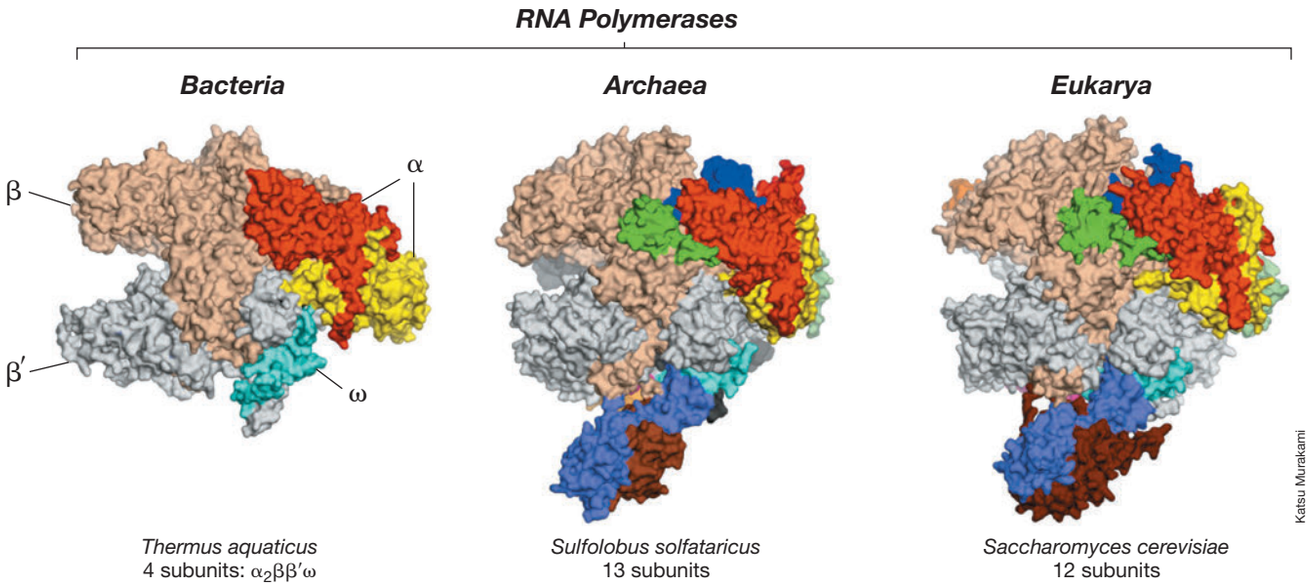
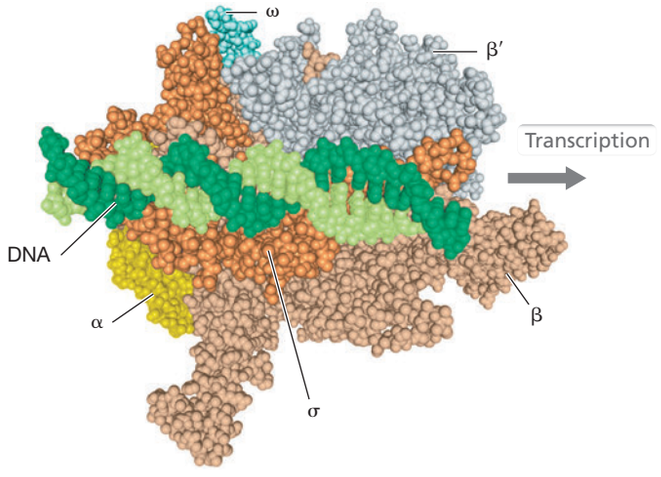


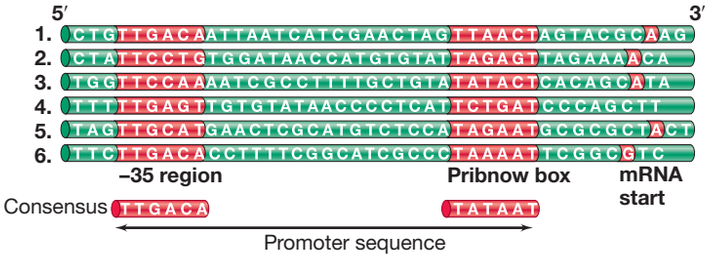
Figure 6.19 RNA polymerase from the three domains. Surface representation of multi-subunit cellular RNA polymerase structures from *Bacteria* (left, *Thermus aquaticus* core enzyme), *Archaea* (center, *Sulfolobus solfataricus*), and *Eukarya* (right, *Saccharomyces cerevisiae* RNA Pol II). Orthologous subunits are depicted by the same color. A unique subunit in the *S. solfataricus* RNA polymerase is not shown in this view.

Units of Transcription and Polycistronic mRNA

Genetic information is organized into *transcriptional units*, segments of DNA that are transcribed into a single RNA molecule bounded by their initiation and termination sites. Some transcriptional units contain RNA transcribed from a single gene, whereas others are formed from two or more genes (*cotranscribed genes*). Most genes encode proteins, but others encode nontranslated RNAs, such as ribosomal or transfer RNAs. For example, prokaryotic cells produce three size classes of rRNA: 16S, 23S, and 5S (the S refers to *Svedberg units*, a sedimentation coefficient that provides



(a)



(b)

Figure 6.20 The interaction of RNA polymerase with a bacterial promoter. *(a)* Cryo-electron micrograph of *Escherichia coli* RNA polymerase with sigma factor (together constituting the holoenzyme) bound to closed promoter region. Image credit: Glyde, R., Ye, F.Z., Darbari, V.C., Zhang, N., Buck, M., and Zhang, X.D. EMDataBank: EMD-3695. *(b)* Six different promoter sequences identified in the *E. coli* genome. The contacts of the RNA polymerase and sigma factor with the −35 region and the Pribnow box (−10 sequence) are shown. Transcription begins at a unique base just downstream from the Pribnow box. Below the actual sequences at the −35 and Pribnow box regions are consensus sequences derived from comparing many promoters. Although the consensus sequences are not identical, they constitute a sufficiently similar pattern recognized through contacts between the bases in the DNA and specific domains of the sigma factor. Note that although sigma recognizes the promoter sequences on the 5′ → 3′ (dark green) strand of DNA, the RNA polymerase core enzyme will actually transcribe the light green strand (that runs 3′ → 5′) because core enzyme synthesizes only in a 5′ → 3′ direction (Figure 6.13).

TABLE 6.3 Sigma factors in *Escherichia coli*

Name[a]	Upstream recognition sequence[b]	Function
σ^{70} RpoD	TTGACA	For most genes, major housekeeping sigma factor for normal growth
σ^{54} RpoN	TTGGCACA	Nitrogen assimilation
σ^{38} RpoS	CCGGCG	Stationary phase, plus oxidative and osmotic stress
σ^{32} RpoH	TNTCNCCTTGAA	Heat shock response
σ^{28} FliA	TAAA	For genes involved in flagella synthesis
σ^{24} RpoE	GAACTT	Response to misfolded proteins in periplasm
σ^{19} FecI	AAGGAAAAT	For certain genes in iron transport

[a]Superscript number indicates size of protein in kilodaltons. Many factors also have other names, for example, σ^{70} is also called σ^{D}. See Figure 6.20 for a description of how the recognition (consensus) sequence is recognized by the sigma factor.
[b]N = any nucleotide.

a measure of particle size based on sedimentation rate), and their genes are cotranscribed to form a single transcriptional unit that also includes a tRNA (Figure 6.21). This transcriptional unit is subsequently "processed" by proteins that cut them to form the individual rRNAs or tRNAs.

As we have previously considered, genes that encode several enzymes of a particular metabolic pathway in prokaryotic cells, for example the biosynthesis of a particular amino acid, are often

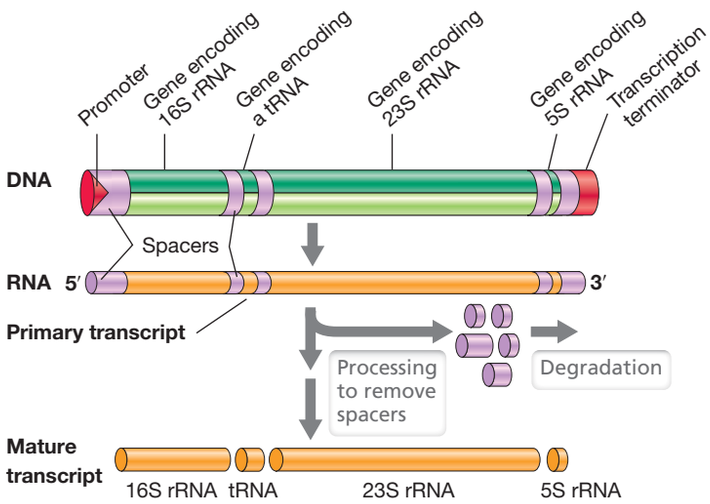


Figure 6.21 A ribosomal rRNA transcription unit from *Bacteria* and its subsequent processing. In *Bacteria*, all rRNA transcription units have the genes in the order 16S rRNA, 23S rRNA, and 5S rRNA (shown approximately to scale). In this particular transcriptional unit, the spacer between the 16S and 23S rRNA genes contains a tRNA gene. In other transcription units this region may contain more than one tRNA gene. Often one or more tRNA genes also follow the 5S rRNA gene and are cotranscribed. *Escherichia coli* contains seven rRNA transcription units.

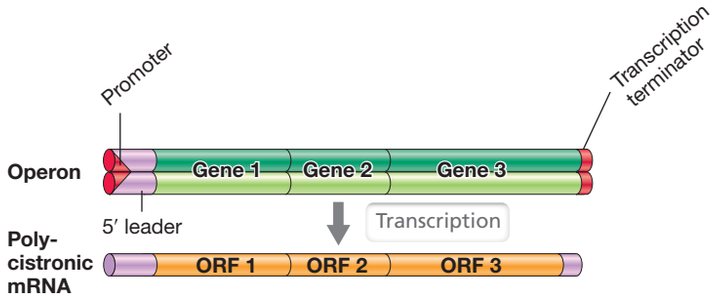


Figure 6.22 Operon and polycistronic mRNA structure. Note that a single promoter controls the three genes within the operon and that the polycistronic mRNA molecule contains an open reading frame (ORF) corresponding to each gene.

clustered together in an **operon** (Section 6.2). An operon is transcribed to form a single mRNA that encodes several different proteins and is regulated as a unit. Assembling genes for the same biochemical pathway or genes needed under the same conditions into an operon allows for their coordinate expression. During transcription, RNA polymerase proceeds through the operon and transcribes the entire set of genes into a single mRNA called a *polycistronic mRNA* (Figure 6.22). Polycistronic mRNAs contain multiple *open reading frames*, portions of the mRNA that actually encode amino acids (Section 6.9). When this mRNA is translated, several polypeptides are synthesized sequentially by the same ribosome. Bacterial and archaeal genomes can also possess *regulons*, which are multiple operons that encode genes whose products are needed under the same conditions. We discuss the regulation of operons and the coordinated expression of regulons in Chapter 7.

Termination of Transcription

In a growing bacterial cell, only those genes that need to be expressed are usually transcribed; therefore, it is critical that transcription end at the correct position. **Termination** of transcription is governed by specific base sequences on the DNA. In *Bacteria* a common termination signal is a GC-rich sequence containing an *inverted repeat* with a central nonrepeating segment. When such a DNA sequence is transcribed, the RNA forms a stem–loop structure by intra-strand base pairing (Figure 6.23). Coding sequences for stem–loops followed by a run of adenines in the DNA template (which yields a run of uridines in the RNA) are strong transcription terminators because a stretch of U–A base pairs are formed that hold the RNA and DNA together. However, this structure is very weak since U–A base pairs have only two hydrogen bonds rather than the three that form in G–C pairs (Figure 6.1*c*). Thus, RNA polymerase pauses at the stem–loop, and the DNA and RNA dissociate at the run of uridines, terminating transcription.

A second mechanism for terminating transcription is catalyzed by the terminator protein Rho. Rho does not bind to RNA polymerase or to the DNA but binds tightly to RNA and moves down the chain toward the RNA polymerase–DNA complex. Once RNA polymerase has paused at a Rho-dependent termination site (a specific nucleotide sequence on the DNA template), Rho causes both the RNA and

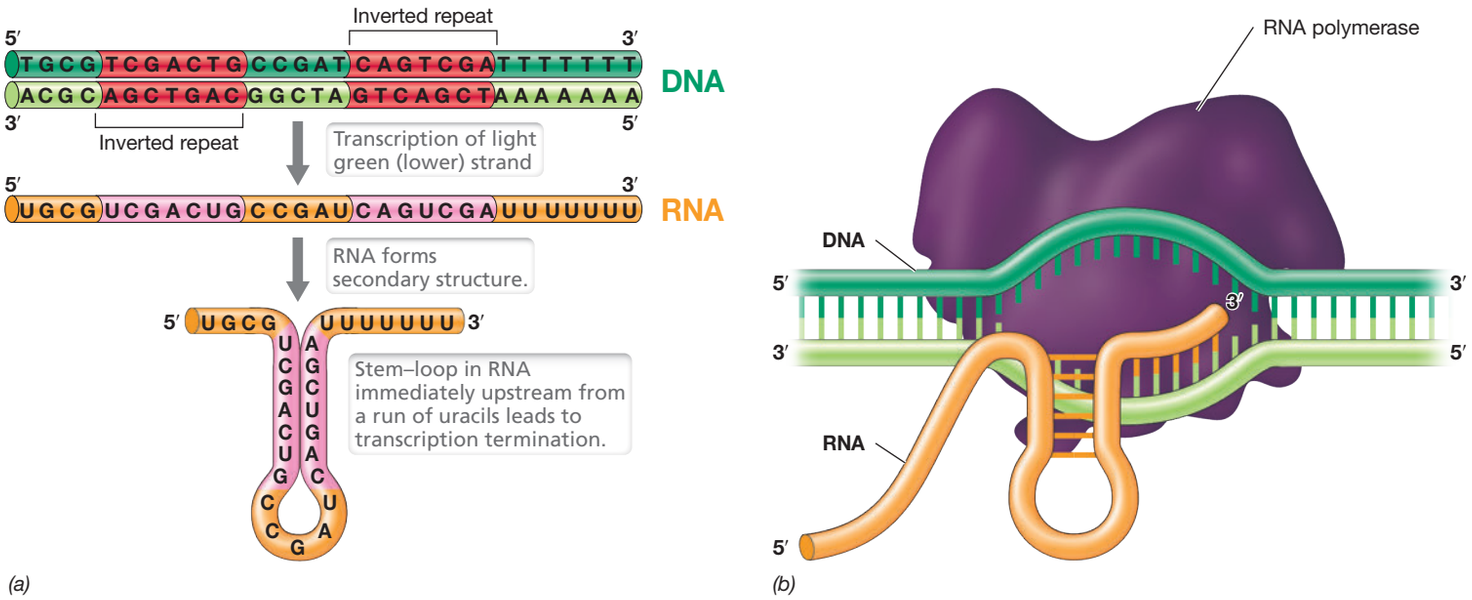


Figure 6.23 **Inverted repeats and transcription termination.** *(a)* Inverted repeats in transcribed DNA form a stem–loop structure in the RNA that terminates transcription when followed by a run of uracils. *(b)* Schematic indicating the formation of the terminator stem–loop in the RNA within the RNA polymerase.

RNA polymerase to be released from the DNA, thus terminating transcription.

Now that we have grasped the essentials of transcription in *Bacteria*, we turn our attention to this crucial cell process in *Archaea* and *Eukarya*, where the phylogenetic connection between these two domains (◀ Section 1.15) will be apparent in their mechanisms of transcription.

Check Your Understanding

- What enzyme catalyzes transcription? What is a promoter, and what protein recognizes promoters in *Bacteria*?
- What is the role of messenger RNA (mRNA)? What are the other two classes of RNA?
- How does polycistronic mRNA allow for gene families to be controlled as a group?
- What type of structures lead to transcription termination?

6.6 Transcription in *Archaea* and *Eukarya*

Here we discuss key elements of transcription in *Archaea* and *Eukarya* that differ from those of *Bacteria*. Although in both *Archaea* and *Eukarya* the overall flow of genetic information is the same as in *Bacteria*, some details differ, and in eukaryotic cells the presence of the nucleus complicates the routing of genetic information. Many of the details of transcription (and translation) in *Archaea* resemble those in *Eukarya* more closely than *Bacteria*. However, *Archaea* also share some transcriptional similarities with *Bacteria*, such as operons. We begin our discussion at center stage with a consideration of RNA polymerase.

Archaeal and Eukaryotic RNA Polymerases, Promoters, and Terminators

Archaeal and eukaryotic RNA polymerases are similar and more complex than those of *Bacteria*. *Archaea* contain only a single RNA polymerase while eukaryotes have three. The archaeal RNA polymerase most closely resembles eukaryotic RNA polymerase II and is composed of 11–13 subunits, depending on the species (eukaryotic RNA polymerase II has 12 or more subunits). These contrast with the comparatively simple four-subunit RNA polymerase core enzyme of *Bacteria* (Figure 6.19).

We learned the importance of the promoter and its recognition sequences to the overall process of transcription in Section 6.5. The most important recognition sequence in archaeal and eukaryotic promoters is the 6- to 8-base-pair "TATA" box, located 18–27 nucleotides upstream of the transcriptional start site (**Figure 6.24**). The TATA box is recognized by the *TATA-binding protein* (TBP), one of the many *transcription factors* present in *Archaea* and eukaryotes. Upstream of the TATA box is the *B recognition element* (BRE) sequence that is recognized by archaeal transcription factor B (TFB). In addition, a specific initiator element sequence is located at the start of transcription. Once TBP has bound to the TATA box and TFB has bound to the BRE, then archaeal RNA polymerase can bind and initiate transcription. This process is similar in eukaryotes except that several additional transcription factors are required.

Less is known about transcription termination in *Archaea* than in *Bacteria*, although some archaeal genes have inverted repeats followed by an AT-rich sequence similar to those present in many bacterial transcription terminators (Section 6.5). One other type of suspected transcription terminator lacks inverted repeats but contains repeated runs of thymines. While no Rho-like proteins (Section 6.5)

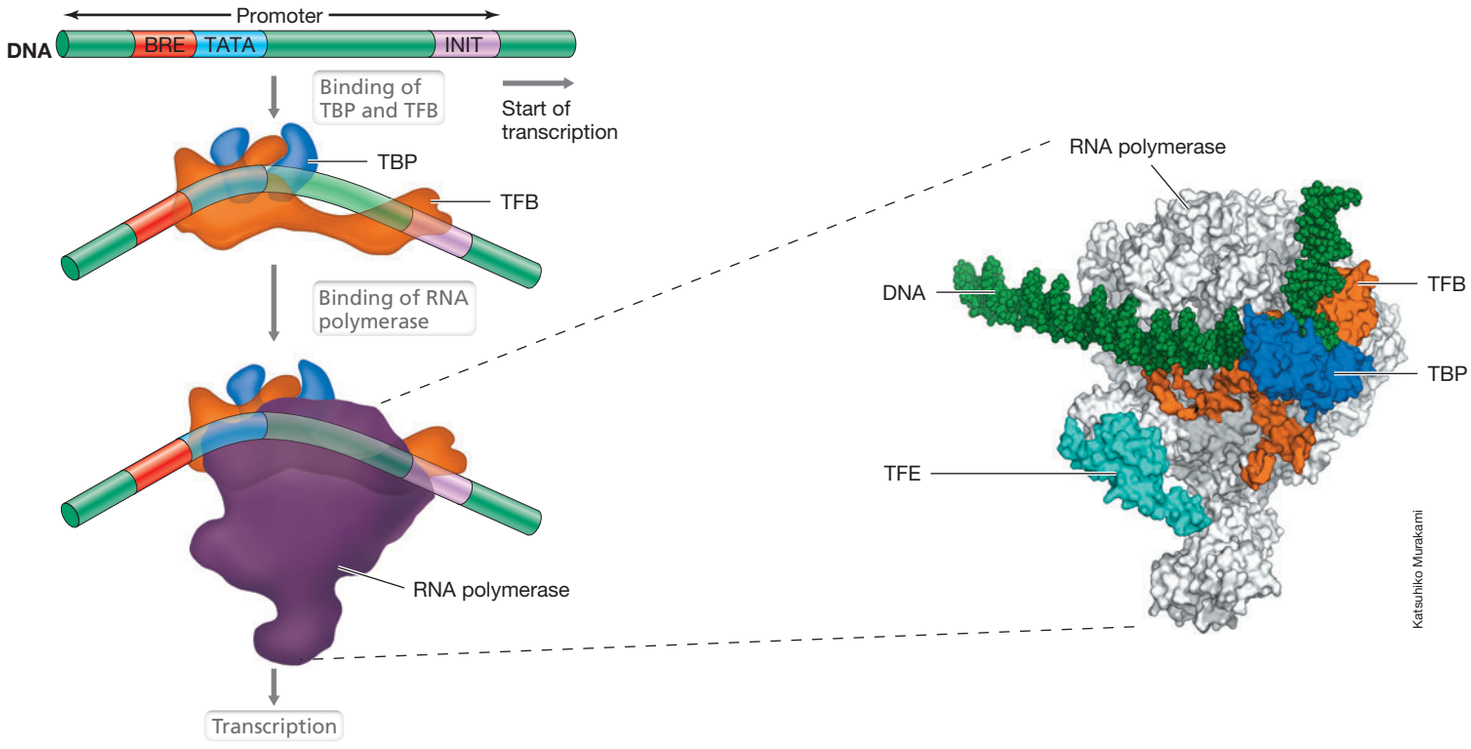


Figure 6.24 **Promoter architecture and transcription in *Archaea*.** *(a)* Three promoter elements are critical for promoter recognition in *Archaea*: the initiator element (INIT), the TATA box, and the B recognition element (BRE). The TATA-binding protein (TBP) binds the TATA box; transcription factor B (TFB) binds to both BRE and INIT. Once both TBP and TFB are in place, RNA polymerase binds. Note that the colors of the different transcription factors match those in the molecular model shown to the right. *(b)* Surface representation of the archaeal pre-initiation complex (with TBP and TFB) and including transcription factor E (TFE). TFE is an optional transcription factor frequently associated with the archaeal pre-initiation complex.

have been found in either *Archaea* or *Eukarya*, a separate transcriptional termination protein has been identified in *Euryarchaeota* (a major phylum of *Archaea*) called Eta. Eta binds to DNA upstream of the transcription bubble, and when it collides with the archaeal RNA polymerase, it bumps the polymerase off of the DNA. In eukaryotes, the termination process differs depending on the RNA polymerase and often requires a specific termination factor protein.

RNA Processing in Eukaryotes and Intervening Sequences in *Archaea*

In contrast to *Bacteria*, *Eukarya* contain many genes that are split into two or more coding regions separated by noncoding regions. The coding sequences are called **exons** while the intervening noncoding regions are called **introns**. Thus, the transcripts from exons typically require alterations—known as **RNA processing**—to form *mature RNAs* suitable for translation. The term **primary transcript** refers to the RNA molecule that is originally transcribed before the introns are removed to form the mature mRNA containing only exons. The process by which introns are removed and exons are joined is called *splicing* (Figure 6.25).

Mastering Microbiology
Art Activity: Figure 6.26 Processing of the primary transcript into mature mRNA in eukaryotes

RNA splicing occurs in the nucleus by the activity of a macromolecular complex containing both RNA and protein called the **spliceosome**. The proteins of the spliceosome excise the intron(s) from the primary transcript and link the flanking exons to form a contiguous protein-encoding mature mRNA (Figure 6.25). While intervening sequences in genes encoding proteins are extremely rare in *Archaea*, several archaeal tRNA- and rRNA-encoding genes contain introns that must be removed after transcription to generate the mature tRNA or rRNA. In analogy to the introns of eukaryotes, these intervening sequences are called "archaeal introns"; however, their processing is catalyzed by a special ribonuclease rather than by a spliceosome-type complex.

Two other steps in the processing of mRNA in *Eukarya* are unique to this domain, and both steps take place in the nucleus prior to splicing (Figure 6.26). The first step, called *capping*, occurs before transcription is complete. Capping is the addition of a methylated guanine nucleotide at the 5′-phosphate end of the mRNA (Figure 6.26). The cap is added in reverse orientation relative to the rest of the mRNA molecule and is needed to initiate translation. The second step, *polyadenylation*, consists of trimming the 3′ end of the primary transcript and adding 100–200 adenine residues, called the *poly(A) tail* (Figure 6.26). The poly(A) tail stabilizes mRNA against nuclease attack, and following translation, it must be removed before the mRNA can be degraded.

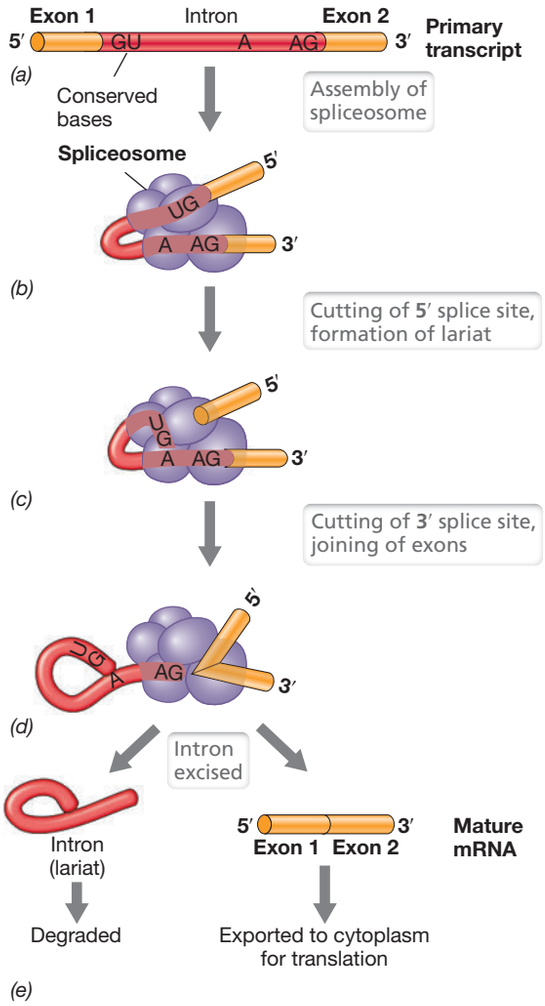


Figure 6.25 Activity of the spliceosome. Removal of an intron from the primary transcript of a protein-coding gene in a eukaryote. *(a)* A primary transcript containing a single intron. The sequence GU is conserved at the 5′ splice site, and AG is conserved at the 3′ splice site. There is also an interior A that serves as a branch point. *(b)* Several small ribonucleoprotein particles (shown in purple) assemble on the RNA to form a spliceosome. Each of these particles contains distinct small RNA molecules that take part in the splicing mechanism. *(c)* The 5′ splice site has been cut with the simultaneous formation of a branch point. *(d)* The 3′ splice site has been cut and the two exons have been joined. Note that overall, two phosphodiester bonds were broken, but two others were formed. *(e)* The final products are the joined exons (the mRNA) and the released intron.

We now consider the culmination of genetic information flow: protein synthesis. In Part IV we will see several events common to all cells with a few exceptions that once again link *Archaea* with *Eukarya*.

Check Your Understanding

- What three major components make up an archaeal promoter?
- What specific eukaryotic enzyme does the archaeal RNA polymerase resemble?
- What steps take place in the processing of eukaryotic RNA?

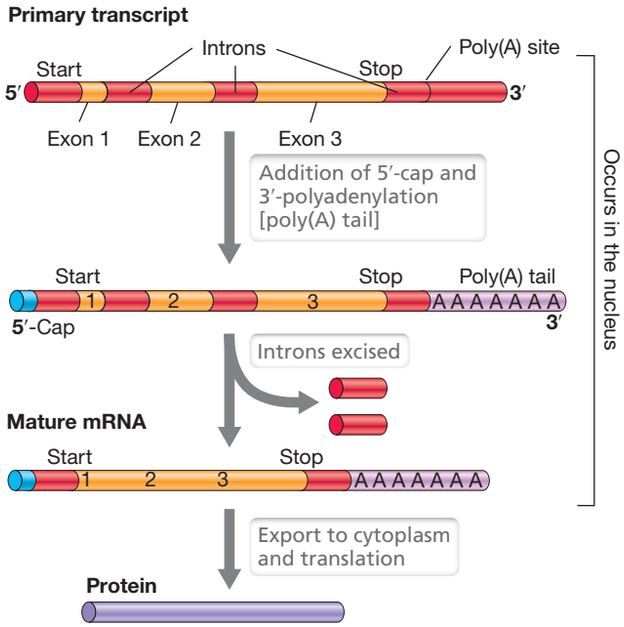


Figure 6.26 Processing of the primary transcript into mature mRNA in eukaryotes. The processing steps include adding a cap at the 5′ end, removing the introns, and clipping the 3′ end of the transcript while adding a poly(A) tail. All these steps are carried out in the nucleus. The location of the start and stop codons to be used during translation are indicated.

IV • Protein Synthesis: Translation

The culmination of replication and transcription is translation, the actual production of proteins by the cell's remarkable biomolecular machine, the ribosome.

Once transcription has occurred, the mRNAs are translated into protein. Translation requires many proteins and RNAs (in addition to mRNA) that combine to form a key cellular structure called the ribosome. How an mRNA and a ribosome interact to produce a cell's array of proteins is what we consider now, and we begin with a refresher section on the basic properties of proteins.

6.7 Amino Acids, Polypeptides, and Proteins

Proteins play major roles in cell function. Three major classes of cellular proteins are *catalytic proteins*, *structural proteins*, and *regulatory proteins*. *Enzymes* are the catalysts for chemical reactions that occur in cells (◀ Section 3.5). Structural proteins are parts of the major structures of the cell: membranes, the cell envelope, ribosomes, and so on (Chapter 2). Regulatory proteins control most cell processes by a variety of mechanisms, including binding to DNA and affecting transcription (Chapter 7). However, regardless of function, all proteins have certain basic features in common.

Composition

Proteins are polymers of **amino acids**, organic compounds that contain both an amino group ($-NH_2$) and a carboxylic acid group ($-COOH$) attached to the α-carbon (**Figure 6.27*a***). Bonds between

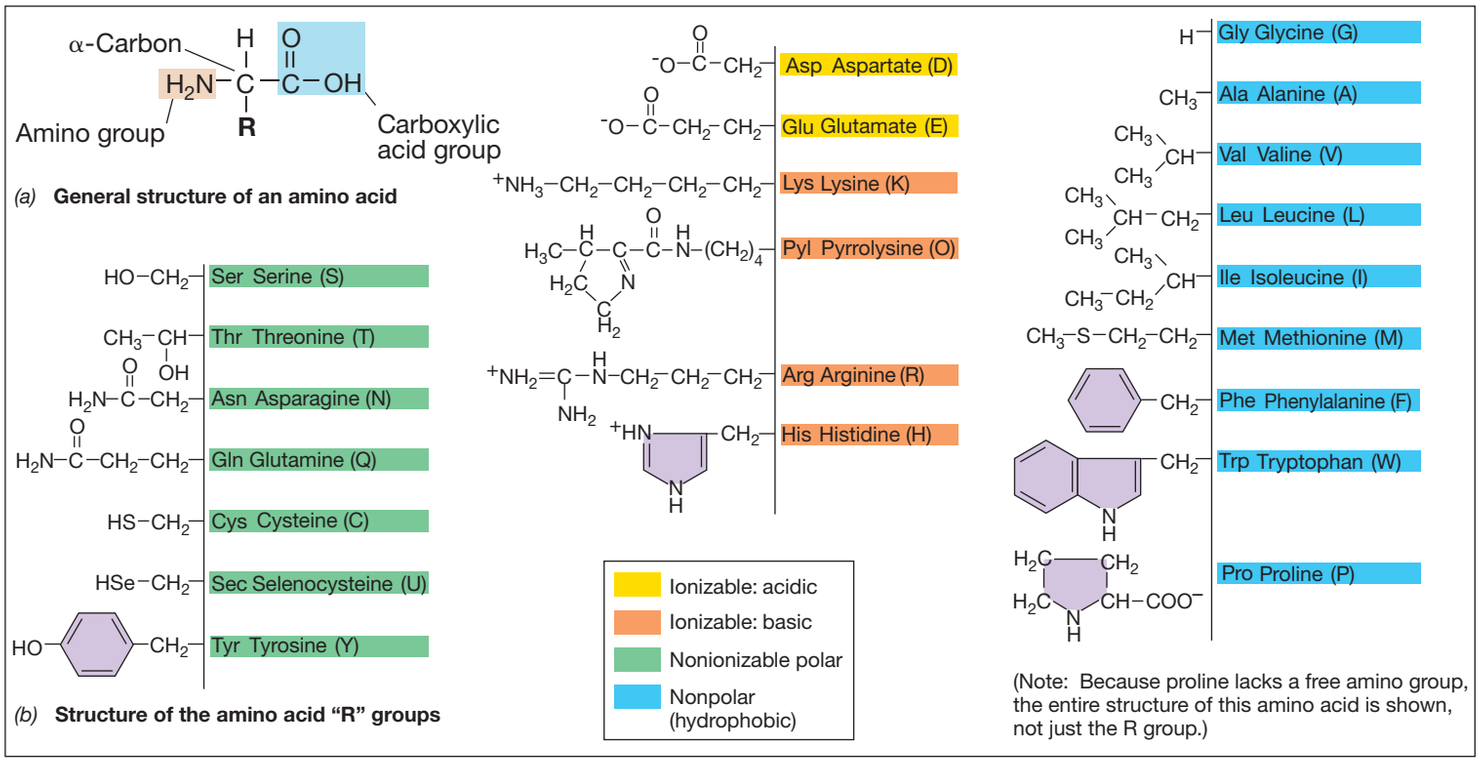


Figure 6.27 Structure of the 22 genetically encoded amino acids. *(a)* General structure. *(b)* R group structure. The three-letter codes for the amino acids are to the left of the names, and the one-letter codes are in parentheses to the right of the names. Pyrrolysine has thus far been found only in certain methanogenic *Archaea*.

the carboxyl carbon of one amino acid and the amino nitrogen of a second (formed through the elimination of water) are known as **peptide bonds** (Figure 6.28). Two amino acids bonded by peptide linkage constitute a *dipeptide*; three amino acids, a *tripeptide*; and so on. When many amino acids are linked, they form a **polypeptide**. A protein consists of one or more polypeptides. The number of amino acids differs greatly from one protein to another, from as few as 15 to as many as 10,000 (see Figure 6.41).

Each amino acid has a unique side chain (abbreviated R) bonded to its α-carbon; it is this side chain that governs the chemical properties of the amino acid. Side chains vary considerably, from as simple as a hydrogen atom in the amino acid glycine to aromatic rings in phenylalanine, tyrosine, and tryptophan (Figure 6.27*b*). Amino acids with chemically related side chains often show similar chemical properties and are thus grouped into related amino acid "families" (Figure 6.27*b*). For example, the side chain may contain a carboxylic acid group, as in aspartic acid or glutamic acid, rendering the amino acid *acidic*. Others contain additional amino groups, making them positively charged and *basic*. Several amino acids contain hydrophobic side chains and are grouped together as *nonpolar* amino acids. Cysteine contains a sulfhydryl group (−SH). Using their sulfhydryl groups, two cysteines can form a disulfide linkage (R–S–S–R) that connects two polypeptide chains.

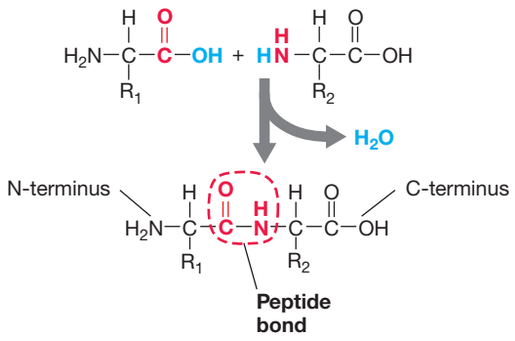


Figure 6.28 Peptide bond formation. R_1 and R_2 refer to the side chains of the amino acids. Note that following peptide bond formation, a free OH group is present at the C-terminus for formation of the next peptide bond.

Protein Diversity and Structures

The diversity of chemically distinct amino acids makes possible an enormous number of structurally unique proteins that can have widely different biochemical properties. If one assumes that an average polypeptide contains 300 amino acids, then 22^{300} different polypeptide sequences are theoretically possible. No cell has anywhere near this many different proteins. A cell of *Escherichia coli* contains around 2000 different kinds of proteins; the exact number of different kinds produced is highly dependent on the resources (nutrients) and growth conditions employed.

The linear sequence of amino acids in a polypeptide is its *primary structure*. This ultimately determines the folding pattern of the polypeptide, which in turn determines its biological activity. Even as

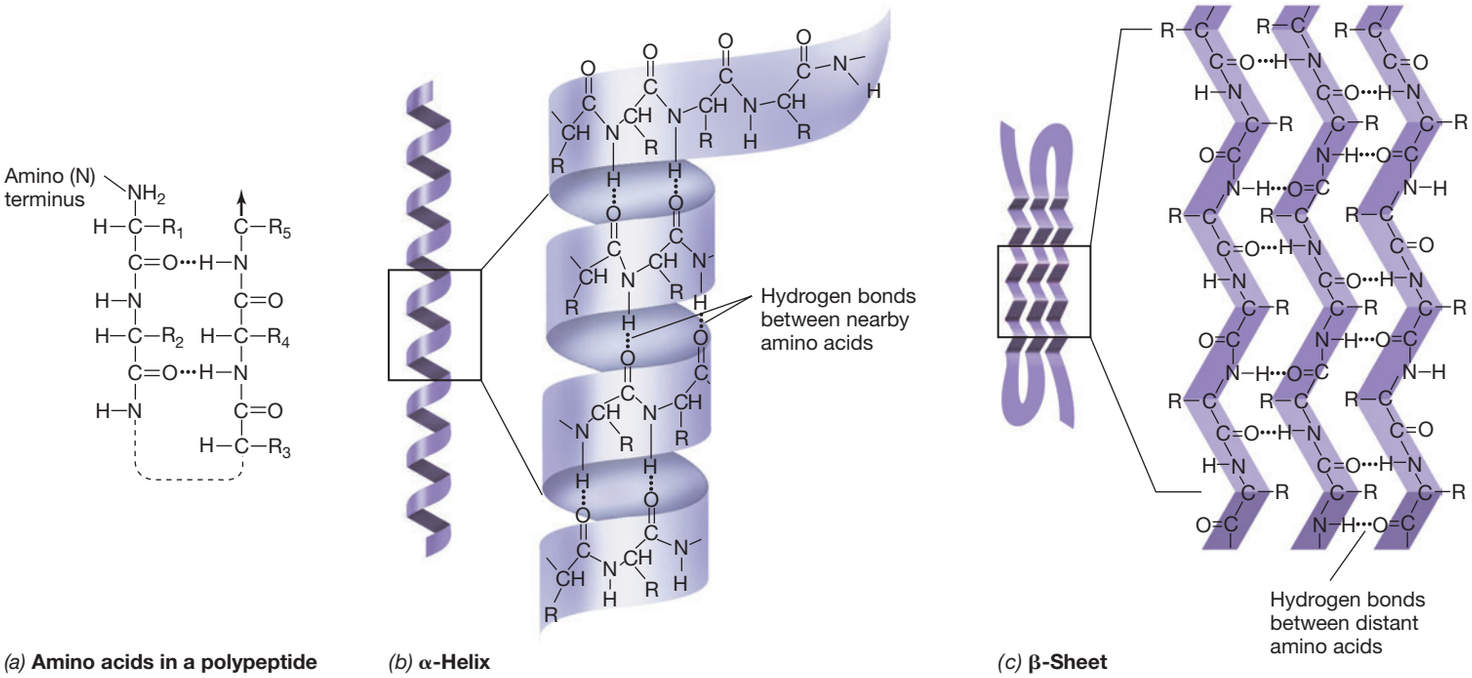


Figure 6.29 **Secondary structure of polypeptides.** *(a)* Hydrogen bonding in protein secondary structure. R represents the side chain of the amino acid. *(b)* α-Helix secondary structure. *(c)* β-Sheet secondary structure. Note that the hydrogen bonding is between atoms in the peptide bonds and does not involve the R groups.

little as a single amino acid change in the primary structure of a protein can affect its folding and thus its activity. Once formed, a polypeptide proceeds to fold to form a more stable structure. Hydrogen bonding between the oxygen and nitrogen atoms of two peptide bonds generates the *secondary structure*, either as an *α-helix* (imagine a polypeptide wound around a cylinder) or as a *β-sheet* (a repeated "back and forth" type of folding) (**Figure 6.29**). A single polypeptide can contain regions, called *domains*, of α-helix and regions of β-sheet secondary structure. The type of folding and its location in the molecule are determined by the primary structure and the available opportunities for hydrogen bonding.

Interactions between the R groups of the amino acids in a polypeptide generate higher-order structures. A protein's **tertiary structure** depends largely on hydrophobic interactions, with lesser contributions from hydrogen bonds, ionic bonds, and disulfide bonds, and generates the overall three-dimensional form of the polypeptide (**Figure 6.30**). Many proteins consist of two or more polypeptides and thus show **quaternary structure**. In such proteins, the quaternary structure describes the number and secondary structure of polypeptides (referred to as *subunits*) in the molecule. Quaternary structures may be stabilized by various interactions and also by disulfide bonds; if cysteines located in two different polypeptides are joined, the disulfide bond links the two subunits.

When proteins are exposed to extremes of heat or pH or to certain chemicals that affect their folding, they may undergo **denaturation**. This results in the loss of a protein's secondary, tertiary, and quaternary structure along with its biological properties. However, because peptide bonds are usually not broken, the denatured polypeptide retains its primary structure. Depending on the severity of the denaturing conditions, the polypeptide may properly refold after the denaturant is removed. However, if refolding is not correct, the protein is permanently inactive and is degraded by proteases to release its amino acids for new protein synthesis.

Check Your Understanding

- Draw the structure of a dipeptide containing the amino acids alanine and tyrosine and outline the peptide bond.
- Differentiate between the different classes of protein structure.
- What is denaturation and why is the process harmful to a cell?

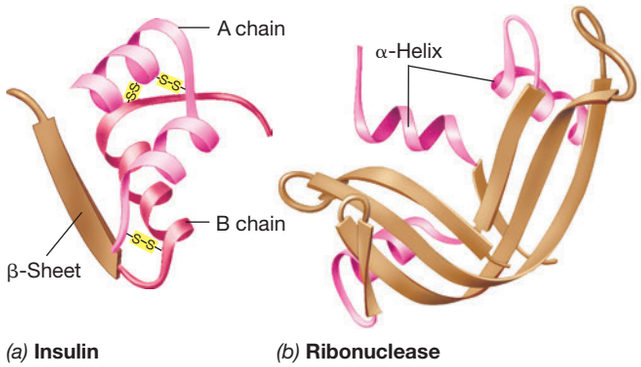


Figure 6.30 **Tertiary structure of polypeptides.** *(a)* Insulin, a protein containing two polypeptide chains; note how the B chain contains both α-helix and β-sheet secondary structure and how disulfide linkages (S—S) help in dictating folding patterns (tertiary structure). *(b)* Ribonuclease, a large protein with several regions of α-helix and β-sheet secondary structure.

6.8 Transfer RNA

With a primer on proteins behind us, we now consider protein synthesis. But to do so, we must first understand the role of transfer RNA (tRNA). Transfer RNAs function to carry amino acids to the translation machinery. To ensure that they carry the correct amino acid, each tRNA contains a specific three-nucleotide sequence called the **anticodon**, the group of three bases that recognizes a codon (a three-base code for an amino acid) on the mRNA (Section 6.10). The correct amino acid (called the *cognate* amino acid) is linked to a specific tRNA by an enzyme called an **aminoacyl-tRNA synthetase**. For each amino acid, a separate aminoacyl-tRNA synthetase exists that specifically binds both the cognate amino acid and the tRNA that contains the corresponding anticodon, thus ensuring that each tRNA receives its correct amino acid. If an amino acid is encoded by more than one codon, a single aminoacyl-tRNA synthetase will recognize the anticodon on any of the tRNAs for that amino acid.

General Structure of Transfer RNA

There are about 60 different tRNAs in prokaryotic cells and 100–110 in human cells. Transfer RNAs are short (73–93 nucleotides), single-stranded molecules that contain extensive secondary structure. Certain base sequences and secondary structures are invariant for tRNAs, whereas other parts are variable. Transfer RNAs also contain some purine and pyrimidine bases that are modified from the bases found in other classes of RNA, and these modifications occur after transcription. These unusual bases include pseudouridine (Ψ), inosine, dihydrouridine (D), ribothymidine, methyl guanosine, dimethyl guanosine, and methyl inosine. The mature and active tRNA also contains extensive double-stranded regions formed by internal base pairing when the single-stranded molecule folds back on itself (**Figure 6.31**).

A tRNA can be depicted in the shape of a cloverleaf (Figure 6.31*a*). Some regions of tRNA secondary structure are named after the modified bases found there (for example, the TΨC and D loops) or after their functions (for example, the anticodon loop and acceptor stem). The three-dimensional model of a tRNA shown in Figure 6.31*b* is a more realistic view of the molecule and shows how bases that appear widely separated in the cloverleaf model are actually much closer together when viewed in 3D. This close proximity allows some of the bases in one loop to pair with bases in another loop (Figure 6.31*b*).

At the 3′ end (the acceptor end) of all tRNAs are three unpaired nucleotides. The sequence of these three nucleotides is always *c*ytosine-*c*ytosine-*a*denine (CCA), and they are absolutely essential for function. However, in most organisms the 3′ CCA is not encoded in the tRNA gene on the chromosome; instead, each nucleotide is added sequentially by a protein called *CCA-adding enzyme*, using CTP and ATP as substrates. The cognate amino acid is then covalently attached to the terminal adenosine of the CCA end of its corresponding tRNA. From this location, the amino acid is incorporated into the growing polypeptide chain on the ribosome by a mechanism to be described in Section 6.10.

Recognition, Activation, and Charging of tRNAs

Recognition of the correct tRNA by an aminoacyl-tRNA synthetase is obviously crucial to the fidelity of translation and requires that specific contacts be made between regions of the tRNA and the synthetase (**Figure 6.32**). As might be expected because of its unique sequence, the anticodon of the tRNA is important in recognition by

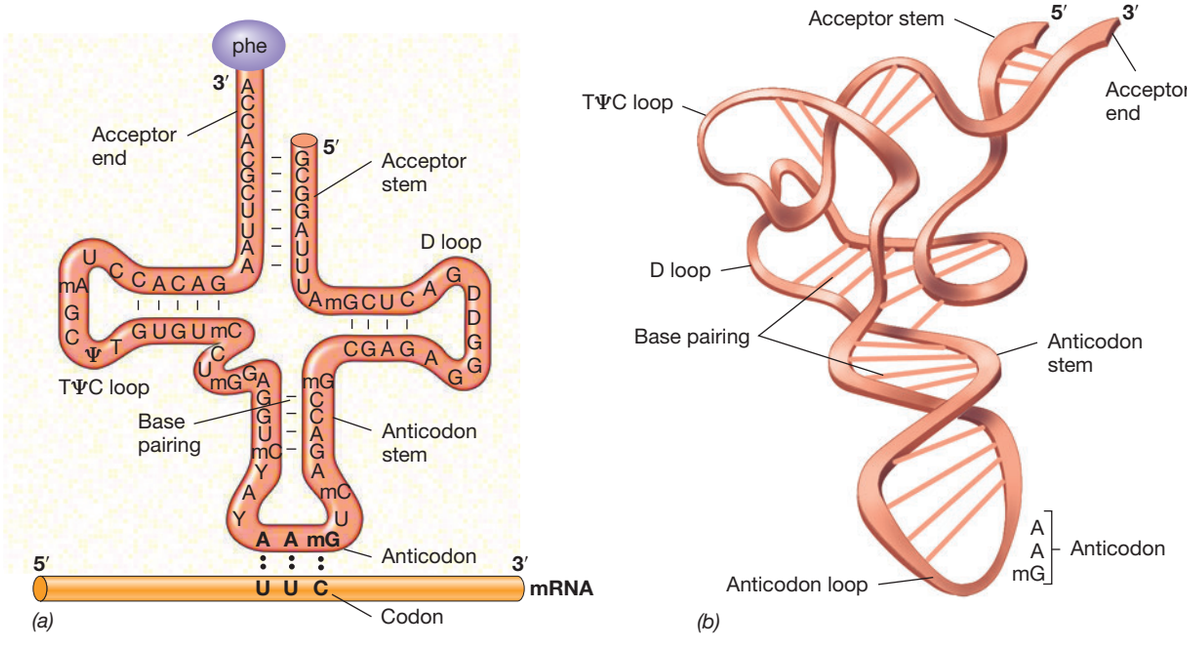


Figure 6.31 Structure of a transfer RNA. *(a)* The conventional cloverleaf structural drawing of yeast phenylalanine tRNA. The amino acid is attached to the ribose of the terminal A at the acceptor end. A, adenine; C, cytosine; U, uracil; G, guanine; T, thymine; Ψ, pseudouracil; D, dihydrouracil; m, methyl; Y, a modified purine. *(b)* The tRNA molecule folds so that the D loop and TΨC loops are close together and associate by hydrophobic interactions.

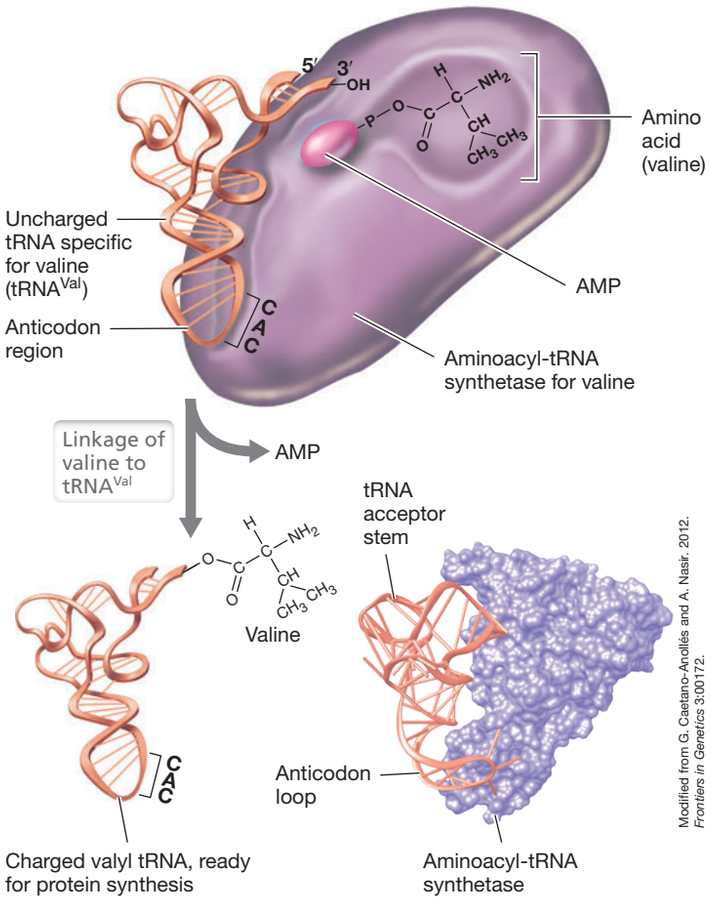


Figure 6.32 Aminoacyl-tRNA synthetase. Mode of activity of an aminoacyl-tRNA synthetase. Recognition of the correct tRNA by a particular synthetase involves contacts between specific nucleic acid sequences in the D loop and acceptor stem of the tRNA and specific amino acids of the synthetase. In this diagram, valyl-tRNA synthetase (specific for the amino acid valine) is shown catalyzing the final step of the reaction, where the valine in valyl-AMP is transferred to tRNA. The computer model on the bottom right shows the interaction of the prolyl-tRNA synthetase from *Thermus thermophilus* with its tRNA.

the synthetase. However, other contact sites between the tRNA and the synthetase are also important, including parts of the acceptor stem and D loop of the tRNA (Figure 6.31*a*).

The specific reaction between amino acid and tRNA catalyzed by the aminoacyl-tRNA synthetase begins with *activation* of the amino acid by reaction with ATP:

$$\text{Amino acid} + \text{ATP} \leftrightarrow \text{aminoacyl}—\text{AMP} + \text{P}—\text{P}$$

The aminoacyl-AMP intermediate formed remains bound to the tRNA synthetase until collision with the appropriate tRNA molecule. Then, as shown in Figure 6.32, the activated amino acid is bonded to the CCA stem of its tRNA to form a *charged tRNA*:

$$\text{Aminoacyl}—\text{AMP} + \text{tRNA} \leftrightarrow \text{aminoacyl}—\text{tRNA} + \text{AMP}$$

The pyrophosphate (PP_i) formed in the first reaction is split into two molecules of inorganic phosphate. Because ATP is used and AMP is formed in these reactions, a total of *two* energy-rich phosphate bonds are expended to charge a tRNA with its cognate amino acid. After activation and charging, the aminoacyl-tRNA leaves the synthetase. In the next step, it will be bound by a ribosome where actual polypeptide synthesis occurs.

Check Your Understanding

- What is the function of the anticodon of a tRNA?
- What is the function of the acceptor stem of a tRNA?
- What steps are required to form a charged tRNA?

UNIT 2

6.9 Translation and the Genetic Code

The heart of genetic information transfer is the correspondence between the nucleic acid template and the amino acid sequence of a polypeptide. This correspondence is rooted in the **genetic code**. An mRNA triplet of three bases, called a **codon**, encodes each specific amino acid (the codons themselves are encoded by the organism's genome). The 64 possible codons (four bases taken three at a time = 4^3) are shown in **Table 6.4**. Note that in addition to the codons for amino acids, there are also codons for starting and stopping translation. Here we focus on translation in *Bacteria*, with *Escherichia coli* as the model.

Properties of the Genetic Code

There are 22 naturally occurring amino acids and because there are 64 codons, several amino acids can be encoded by more than one codon. A code such as this that lacks one-to-one correspondence between "word" (that is, the amino acid) and code (codon) is called a *degenerate code*. A codon is recognized by specific base pairing with a complementary sequence on the *anticodon*, located on a tRNA (Section 6.8 and Figures 6.31 and 6.32). If this base pairing were always the standard pairing of A with U and G with C, then at least one specific tRNA would be needed to recognize each codon. In some cases, this is true. For instance, there are six different tRNAs in *Escherichia coli* for the amino acid leucine, one for each codon (Table 6.4). By contrast, some tRNAs can recognize more than one codon. For example, although there are *two* lysine codons in *E. coli*, there is only *one* lysyl tRNA, whose anticodon can base-pair with *either* AAA or AAG. In these cases, the anticodon forms standard base pairs at only the first two positions of the codon and tolerates irregular base pairing at the third position. This phenomenon is called **wobble** and is illustrated in **Figure 6.33**.

Mastering Microbiology
Microbiology Animation: The Genetic Code

If an amino acid is encoded by multiple codons, the codons are typically closely related in base sequence, usually differing at only their third position (Table 6.4) to allow for wobble (Figure 6.33). Interestingly, not all multiple codons for a given amino acid are used at the same frequency, leading to a **codon bias** that varies from organism to organism. Codon bias is correlated with a corresponding bias in the concentration of different tRNA molecules. That is, a tRNA whose anticodon corresponds to a rarely used codon is typically produced at low levels.

Start and Stop Codons and Reading Frames

Messenger RNA is translated in *Bacteria* beginning with its **start codon** (AUG, Table 6.4), which encodes a chemically modified methionine called *N-formylmethionine* (although AUG at the *beginning* of a coding region encodes *N*-formylmethionine, AUG *within*

TABLE 6.4 The genetic code as expressed by triplet base sequences of mRNA

Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
UUU	Phenylalanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine
UUC	Phenylalanine	UCC	Serine	UAC	Tyrosine	UGC	Cysteine
UUA	Leucine	UCA	Serine	UAA	None (stop signal)	UGA	None (stop signal)
UUG	Leucine	UCG	Serine	UAG	None (stop signal)	UGG	Tryptophan
CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine
CUC	Leucine	CCC	Proline	CAC	Histidine	CGC	Arginine
CUA	Leucine	CCA	Proline	CAA	Glutamine	CGA	Arginine
CUG	Leucine	CCG	Proline	CAG	Glutamine	CGG	Arginine
AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine
AUC	Isoleucine	ACC	Threonine	AAC	Asparagine	AGC	Serine
AUA	Isoleucine	ACA	Threonine	AAA	Lysine	AGA	Arginine
AUG (start)[a]	Methionine	ACG	Threonine	AAG	Lysine	AGG	Arginine
GUU	Valine	GCU	Alanine	GAU	Aspartic acid	GGU	Glycine
GUC	Valine	GCC	Alanine	GAC	Aspartic acid	GGC	Glycine
GUA	Valine	GCA	Alanine	GAA	Glutamic acid	GGA	Glycine
GUG	Valine	GCG	Alanine	GAG	Glutamic acid	GGG	Glycine

[a]AUG encodes *N*-formylmethionine at the beginning of polypeptide chains of *Bacteria*.

the coding region encodes methionine). By contrast, the translational machinery in *Archaea* and *Eukarya* insert an unmodified methionine as the first amino acid in a polypeptide.

With a triplet code it is critical for translation to begin at the correct nucleotide. If it does not, the whole reading frame of the mRNA will be shifted and thus an entirely different (and likely inactive) protein will be made. Alternatively, if the shift introduces a stop codon (Table 6.4) into the reading frame, the polypeptide will terminate prematurely. The reading frame that when translated yields the polypeptide encoded by the gene is called the *0* (zero) *frame*; the other possible reading frames (−1 and +1) do not encode the same amino acid sequence (**Figure 6.34**). Reading frame fidelity is governed by interactions between mRNA and rRNA within the ribosome. In *Bacteria*, ribosomal RNA recognizes a specific AUG on the mRNA as a start codon with the aid of an upstream sequence in the mRNA called the *ribosome-binding site* (RBS) (also called the *Shine–Dalgarno sequence* after its discoverers). This upstream alignment requirement explains why some mRNAs from *Bacteria* can use other start codons, such as GUG. However, because of the RBS, even these unusual start codons direct the incorporation of *N*-formylmethionine as the initiator amino acid (see Figure 6.36).

The codons UAA, UAG, and UGA (Table 6.4) are **stop codons**, and they signal the termination of translation of a protein-coding sequence on the mRNA. Stop codons are also called **nonsense codons**, because they interrupt the "sense" of the growing polypeptide when they terminate translation. In a few rare cases in *Bacteria* and *Archaea*, nonsense codons encode the unusual amino acids

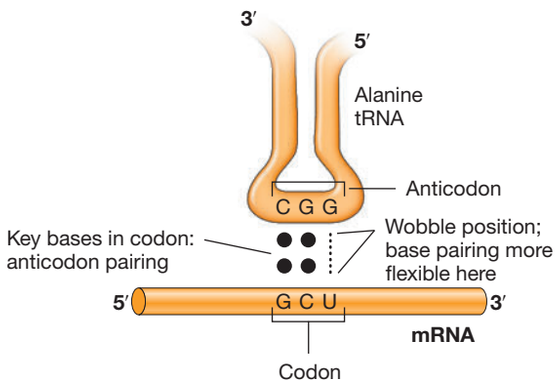


Figure 6.33 The wobble concept. Base pairing is more flexible for the third base of the codon than for the first two. Only a portion of the tRNA is shown here.

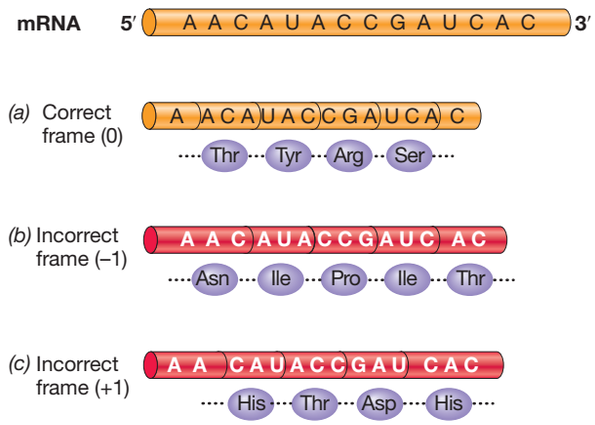


Figure 6.34 Possible reading frames in an mRNA. An interior sequence of an mRNA is shown. *(a)* The amino acids that would be encoded if the ribosome is in the correct reading frame (designated the "0" frame). *(b)* The amino acids that would be encoded by this region of the mRNA if the ribosome were in the −1 reading frame. *(c)* The amino acids that would be encoded if the ribosome were in the +1 reading frame.

selenocysteine and pyrrolysine (Figure 6.27). When this occurs, specific tRNAs are employed whose anticodons read these stop codons. What controls this unusual occurrence is a recognition sequence just downstream of the now-coding stop codon that signals the incorporation of tRNAs containing selenocysteine or pyrrolysine rather than stopping translation. A few other microbes use conventional stop codons to encode amino acids, but in these cases the organisms have simply dispensed with using these particular stop codons as translational stop sites.

If an mRNA can be translated, it is because it contains an **open reading frame (ORF)**: a start codon followed by a number of codons and then a stop codon in the same reading frame as the start codon. Using computational methods, a DNA base sequence can be scanned to search for open reading frames. In addition to looking for start and stop codons, computer analyses may include a search for promoters and ribosome-binding sequences as well in order to confirm the ORF as protein encoding.

The search for ORFs is central to the field of genomics (Chapter 10), for if an unknown piece of DNA has been sequenced, the presence of an ORF implies that it can encode protein. In this way, computational analyses of sequenced microbial genomes can reveal much about the biology of an organism.

Check Your Understanding

- What are start codons and stop codons? Why is it important for the ribosome to read "in frame"?
- What is codon bias?
- If you were given a nucleotide sequence, how would you find ORFs?

6.10 The Mechanism of Protein Synthesis

Protein synthesis is a dynamic process that can be broken down into three major steps: *initiation*, *elongation*, and *termination*. In addition to mRNA, tRNA, and ribosomes, translation requires a number of initiation, elongation, and termination proteins and the energy-rich compound guanosine triphosphate (GTP) to provide the energy to drive the process.

Ribosomes and the Initiation of Translation

Ribosomes are large complexes of proteins and RNAs where the cell's proteins are biosynthesized (**Figure 6.35**). A cell may have many thousands of ribosomes, the number increasing at higher growth rates. Each ribosome consists of two subunits. *Bacteria* and *Archaea* have 30S and 50S ribosomal subunits that yield intact 70S ribosomes. Each ribosomal subunit contains specific ribosomal RNAs (rRNAs) and ribosomal proteins. The 30S subunit contains 16S rRNA and 21 proteins, and the 50S subunit contains 5S and 23S rRNA and 31 proteins. Thus, in *Escherichia coli*, there are 52 distinct ribosomal proteins, most present at one copy per ribosome. The ribosome is a highly dynamic structure, and its subunits alternately associate and dissociate during the translational process (see Figures 6.36 and 6.37) while interacting with many other proteins. In addition, several cytoplasmic proteins called *translation factors* are essential for translation and interact with the ribosome at various stages of the translational process.

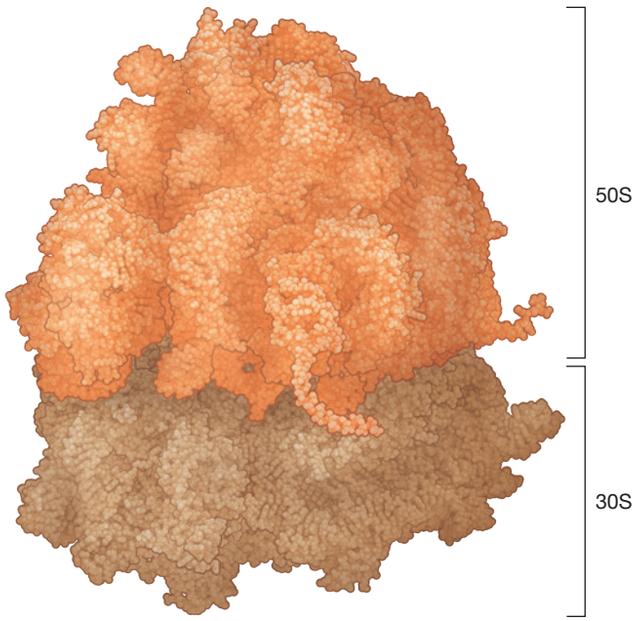


Figure 6.35 Ribosome structure. Crystal structure of the *Thermus thermophilus* 70S ribosome. 50S and 30S subunits are indicated. Each subunit is composed of several distinct proteins; the 50S subunit contains 23S and 5S rRNAs, and the small subunit contains 16S rRNA.

In *Bacteria*, initiation of protein synthesis begins with a free 30S ribosomal subunit (**Figure 6.36**). From this, an *initiation complex* forms consisting of the 30S subunit, mRNA, formylmethionine tRNA (the initiator tRNA in *Bacteria*; after polypeptide completion, the formyl group is removed), and the initiation factors IF1, IF2, and IF3. GTP is also required for this step. Next, a 50S ribosomal subunit is added to the initiation complex to form the active 70S ribosome (Figures 6.35 and 6.36). Just preceding the start codon on the mRNA is a sequence of three to nine nucleotides that compose the ribosome-binding site (Figure 6.36). This site is toward the 5′ end of the mRNA and is complementary to base sequences in the 3′ end of the 16S rRNA, which is part of the ribosome. Base pairing between these two RNAs holds the ribosome–mRNA complex securely together in the correct reading frame. Polycistronic mRNA (Section 6.5) contains several RBS sequences, one upstream of each coding sequence. This allows bacterial ribosomes to translate several genes on the same mRNA because the ribosome can locate each initiation site within a message by binding to its RBS.

Mastering Microbiology
Microbiology Animation: Translation: The Process

Elongation, Translocation, and Termination

During translation, the mRNA threads through the ribosome as its nucleotides bind to the 30S subunit. The ribosome contains other sites where the tRNAs interact. Two of these sites are located primarily on the 50S subunit and are termed the *A* (*acceptor*) *site* and the *P* (*peptide*) *site* (**Figure 6.37**). The A site is where the incoming charged tRNA first attaches, and the loading of a tRNA into the A site is assisted by the elongation factor EF-Tu. The P site is where the growing polypeptide chain is attached to the prior tRNA. During peptide bond formation, the growing polypeptide chain moves to the tRNA at the A site as a new peptide bond is formed. In addition to EF-Tu, elongation factor EF-Ts, as well as more GTP, is required (Figure 6.37).

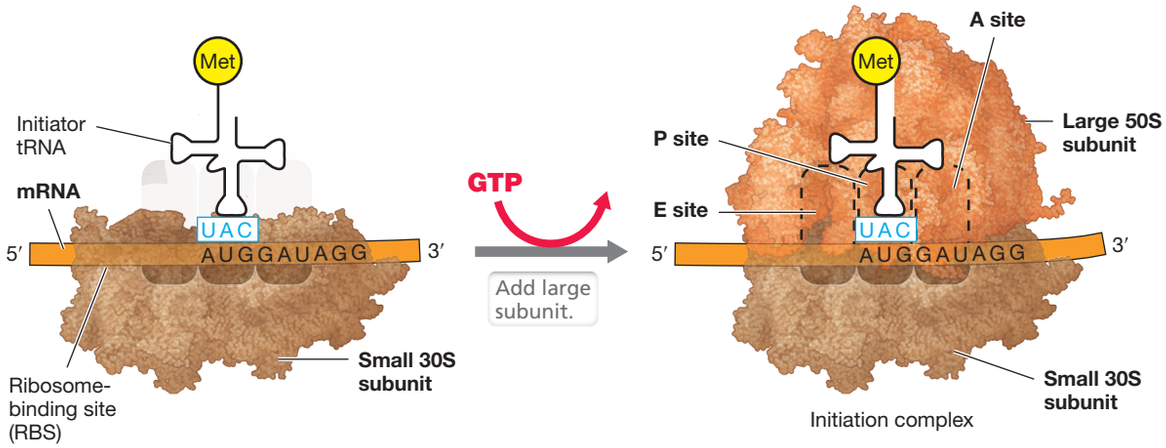


Figure 6.36 **The ribosome and initiation of protein synthesis.** The mRNA and initiator tRNA, carrying *N*-formylmethionine ("Met"), bind first to the small subunit of the ribosome. Initiation factors (not shown) use energy from GTP to promote the addition of the large ribosomal subunit. The initiator tRNA starts out in the P site (labeled in the structure on the right-hand side of the arrow).

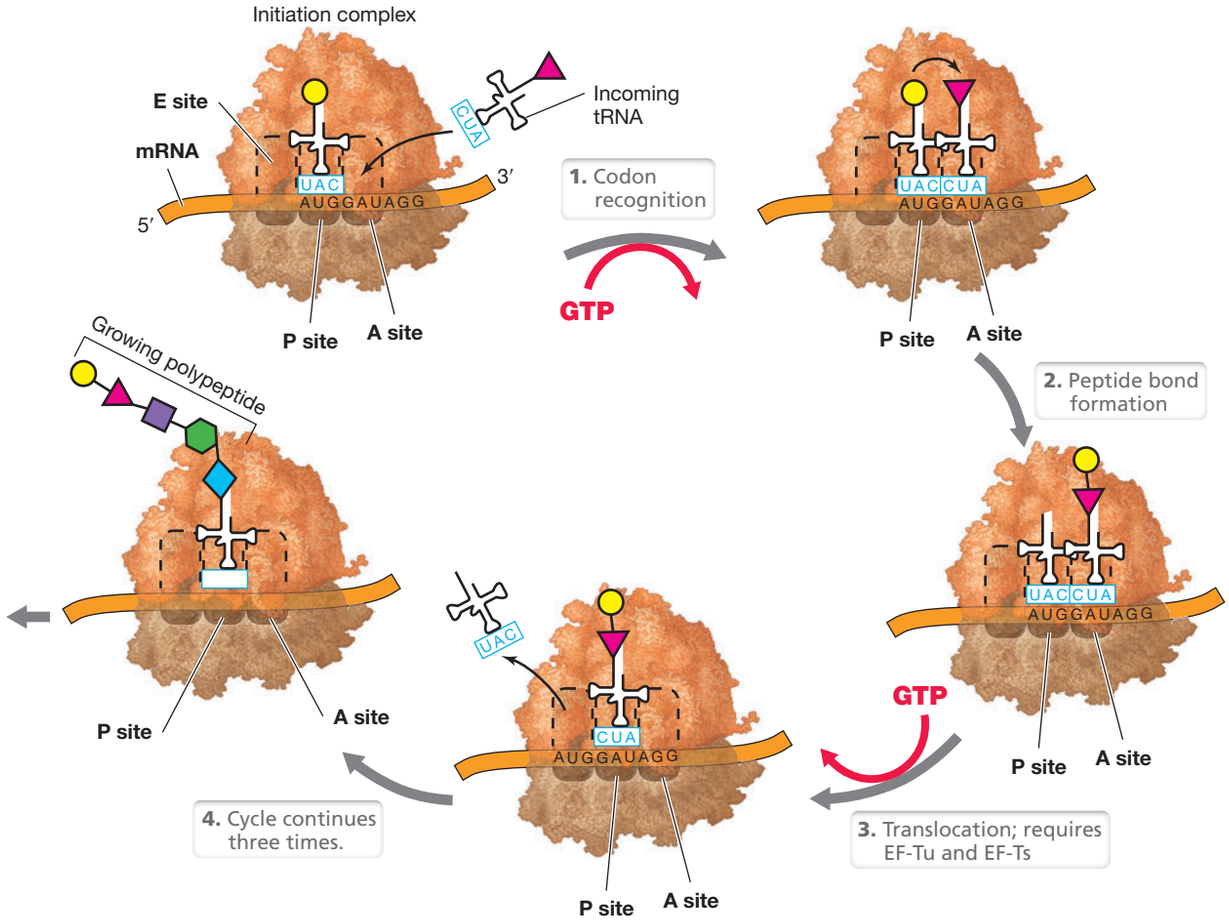


Figure 6.37 **Elongation cycle of translation.** 1. Elongation factors (not shown) use GTP to install the incoming tRNA into the A site. 2. Peptide bond formation is then catalyzed by the 23S rRNA. 3. Translocation of the ribosome along the mRNA from one codon to the next requires hydrolysis of another GTP and results in movement of the tRNA with the growing peptide to the P site. The outgoing tRNA is released from the E site. 4. The next charged tRNA binds to the A site and the cycle repeats. The genetic code, expressed as codons of mRNA, is shown in Table 6.4. Translation of a given polypeptide ends when the ribosome reaches any one of three stop codons (Table 6.4) because no tRNA has an anticodon sequence (Figure 6.33) complementary to the three-base sequence in a stop codon.

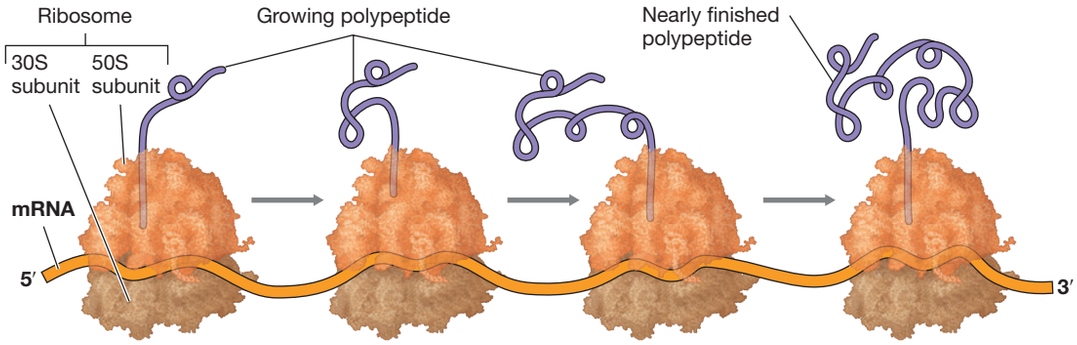


Figure 6.38 Polysomes. Translation by several ribosomes on a single messenger RNA forms the polysome. Note how the ribosomes nearest the 5′ end of the message are at an earlier stage in the translation process than ribosomes nearer the 3′ end, and thus only a relatively short portion of the final polypeptide has been made.

Following elongation, the tRNA holding the polypeptide is translocated from the A site to the P site, thus opening the A site for a new charged tRNA; this requires elongation factor EF-G and one molecule of GTP for each translocation event (Figure 6.37). In each translocation, the ribosome advances three nucleotides (one codon) along the mRNA, exposing a new codon at the A site. Translocation pushes the now amino acid–free tRNA to a third site, called the E (exit) site, and it is from here that the tRNA is released from the ribosome. The precision of the translocation step is critical to the accuracy of protein synthesis. That is, the ribosome must move *exactly one codon* at each step or the reading frame will change and the fidelity of translation will be compromised (Section 6.9).

Several ribosomes can translate a single mRNA molecule simultaneously, forming a complex called a *polysome* (**Figure 6.38**). Polysomes increase both the speed and efficiency of translation because each ribosome in the polysome makes a complete polypeptide. Note in Figure 6.38 how ribosomes in the polysome that are closest to the 5′ end (the beginning) of the mRNA molecule have short polypeptides attached to them because only a few codons have been read, while ribosomes closest to the 3′ end of the mRNA have nearly finished polypeptides.

Translation terminates when the ribosome reaches a stop codon (Table 6.4) because no tRNA binds to a stop codon. Instead, proteins called *release factors* (RFs) recognize the stop codon and cleave the attached polypeptide from the final tRNA, releasing the finished product. Following this, the ribosomal subunits dissociate, and the 30S and 50S subunits are then free to form new initiation complexes (Figure 6.36) and repeat the process.

Role of Ribosomal RNA in Protein Synthesis

Ribosomal RNA plays major roles in all stages of translation, from initiation to termination. By contrast, the primary role of the proteins in the ribosome is to form a scaffold to position key sequences in the ribosomal RNAs (Figure 6.35). In *Bacteria*, 16S rRNA facilitates initiation by base pairing with the ribosome-binding site on the mRNA, and, along with ribosomal proteins, it holds the mRNA in position on either side of the A and P sites. Ribosomal RNA also plays a role in ribosome subunit association, as well as in positioning tRNAs on the ribosome (Figures 6.36 and 6.37). Although charged tRNAs recognize the correct codon by codon–anticodon base pairing (Figure 6.33), they are also bound to the ribosome by interactions between the anticodon stem–loop of the tRNA and specific sequences in the 16S rRNA. Moreover, the acceptor end of the tRNA (Figures 6.36 and 6.37) base-pairs with sequences in 23S rRNA.

In addition to roles in mRNA alignment and translocation along the transcript, ribosomal RNA also catalyzes the actual formation of peptide bonds. The peptidyl transferase reaction (formation of peptide bonds, Figure 6.28) occurs on the 50S subunit of the ribosome and is catalyzed solely by 23S rRNA. This rRNA also plays a role in translocation and interacts with the elongation factors. Thus, in addition to its role as the structural backbone of the ribosome, ribosomal RNA plays a major catalytic role in the translation process.

Freeing Trapped Ribosomes and *Trans*-Translation

A defective mRNA that lacks a stop codon cannot be properly translated. Such a defect may arise, for example, from a mutation that removed the stop codon, from defective synthesis of the mRNA, or when partial degradation of an mRNA occurs before it is translated. If a ribosome reaches the end of an mRNA molecule and there is no stop codon, release factor cannot bind and the ribosome cannot be released from the mRNA; the ribosome is effectively "trapped."

To deal with this problem, bacterial cells initiate a process called *trans-translation*. This process produces a small RNA molecule called *tmRNA* that frees stalled ribosomes (**Figure 6.39**). The "tm" in its

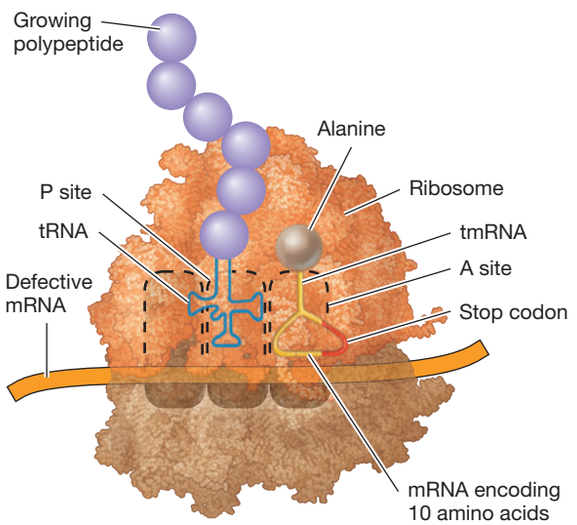


Figure 6.39 Freeing of a stalled ribosome by tmRNA. A defective mRNA lacking a stop codon stalls a ribosome that has a partly synthesized polypeptide attached to a tRNA (blue) in the P site. Binding of tmRNA (yellow) in the A site allows translation to continue up to the stop codon provided by the tmRNA.

name refers to the fact that tmRNA mimics both *t*RNA, in that it carries an amino acid (alanine), and *m*RNA, in that it contains a short stretch of RNA that can be translated. When tmRNA collides with a stalled ribosome, it binds alongside the defective mRNA. Protein synthesis can then proceed, first by adding the alanine on the tmRNA and then by translating the short tmRNA message. The tmRNA contains a stop codon that allows release factor to bind and disassemble the ribosome. The protein made as a result of this rescue operation is defective and is subsequently degraded. This is accomplished by a short sequence of amino acids encoded by tmRNA and added to the end of the defective protein; the sequence is a signal for a specific protease to degrade the protein. Thus, through the activity of *trans*-translation, stalled ribosomes are not inactivated but instead are freed up to participate in protein synthesis once again.

We complete this chapter by considering what happens to proteins *after* they are made but *before* they become functional. For some proteins the answer is "nothing," but for others, functionality depends on additional activities, and we survey these now.

Check Your Understanding

- What are the components of a ribosome? What functional roles does rRNA play in protein synthesis?
- How is a completed polypeptide chain released from the ribosome?
- How does tmRNA free stalled ribosomes?

V • Protein Processing, Secretion, and Targeting

Not all proteins that exit the ribosome are "ready to go," as some need additional folding or other processing and others need to be forwarded to specific cellular locations—such as the cytoplasmic membrane—if they are to carry out their functions properly.

While translation is the final step in genetic information flow (Figures 6.1*a* and 6.5), some proteins require subsequent *processing* or *targeting* before they become functional. These activities often require accessory proteins to assist with folding and transport as well as intrinsic signals within the translated protein itself.

6.11 Assisted Protein Folding and Chaperones

In Section 6.7 we saw how proteins show several levels of structure including primary, secondary, tertiary, and, in multi-subunit proteins, quaternary structure. Many proteins fold spontaneously into their active form, even while they are being synthesized (Figure 6.38). However, some do not, and these require assistance to achieve an active functional state.

Major Chaperones of *Bacteria*

Bacteria produce a series of proteins called **chaperones** that catalyze a variety of macromolecular folding events. These events include folding proteins that do not fold spontaneously, refolding partially denatured proteins, assembling multiprotein complexes, preventing the improper aggregation of proteins, untangling RNAs, and incorporating cofactors into enzymes. Chaperones are found in all domains of life, and their amino acid sequences are highly conserved among all organisms.

Four key chaperones in *Escherichia coli* are the proteins DnaK, DnaJ, GroEL, and GroES. DnaK and DnaJ are ATP-dependent enzymes that bind to newly formed polypeptides and prevent them from folding too quickly, which would increase the risk of improper folding (**Figure 6.40*a***). If the DnaKJ complex is unable to fold the protein properly, it may transfer the partially folded protein to the two multi-subunit proteins GroEL and GroES. The protein first enters GroEL, a large, barrel-shaped protein that uses the energy of ATP hydrolysis to fold the protein properly. GroES assists in this (Figure 6.40*a*). It is estimated that about 100 of the several thousand different proteins in a cell of *E. coli* need help from the GroEL–GroES complex to become properly folded, and of these, a dozen or so are proteins essential for the survival of the bacterium.

Other Functions of Chaperones

Chaperones can also refold proteins that have partially denatured in the cell. A protein may denature for many reasons, but often it is because the organism has temporarily experienced high temperatures. Chaperones are thus one type of *heat shock protein*, and their synthesis is greatly accelerated when a cell is stressed by excessive heat (▶ Section 7.11). The heat shock response is an attempt by the cell to refold its partially denatured proteins for reuse before proteases recognize them as improperly folded and destroy them. In contrast to heat shock proteins, *cold shock proteins* are produced when the cell experiences a sudden shift to very cold temperatures. Cold (but nonfreezing) conditions do not affect most proteins but can affect RNAs. CspA, a major cold shock protein of *E. coli*, is an RNA chaperone instead of a protein chaperone and prevents the spontaneous formation of secondary structures in mRNA that could compromise their ability to be translated. Many other cold shock proteins are known in *E. coli* including ones that refold cold-sensitive proteins rather than acting on mRNAs.

Some chaperones help assemble cofactor-containing enzymes such as those that catalyze redox or electron transport reactions (◀ Section 3.8). For example, several multi-subunit molybdoenyzmes such as the *E. coli* membrane-bound nitrate reductase (◀ Section 3.10 and Figure 3.23*b*) require that a molybdenum cofactor (Moco) be inserted during folding, and cofactor insertion is assisted by enzyme-specific chaperone proteins (Figure 6.40*b*). Nitrate reductase is a key enzyme in anaerobic respiration (◀ Section 3.10 and ▶ Section 14.11) and is composed of three subunits: NarG, NarH, and NarI. For the enzyme to be functional, the cytoplasmic chaperone protein NarJ must first insert the Moco

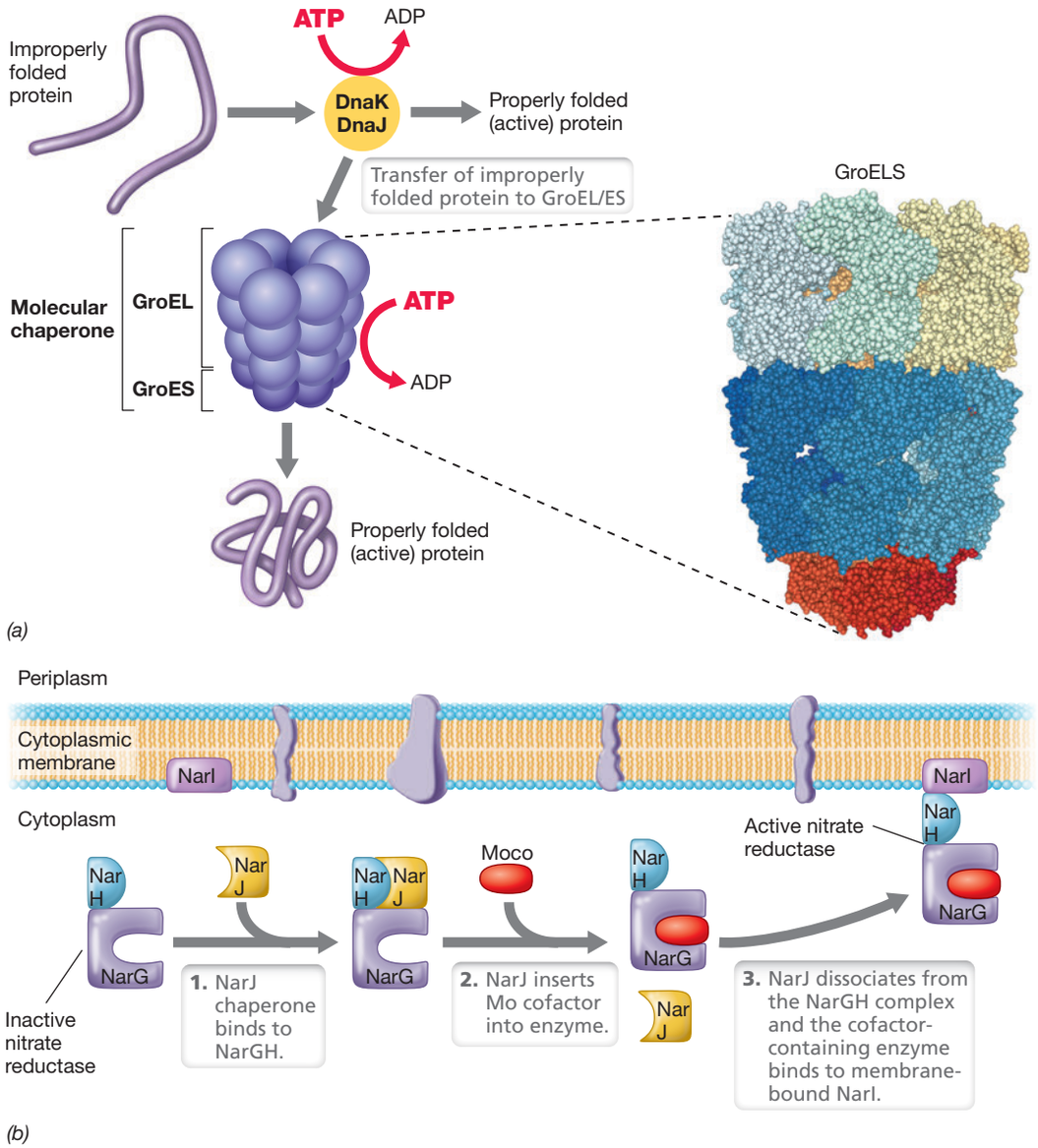


Figure 6.40 The activity of chaperone proteins. *(a)* An improperly folded protein can be refolded by either the DnaKJ complex or by the GroEL–GroES complex. In both cases, energy for refolding comes from ATP. Inset: Crystal structure of GroELS complex. *(b)* Formation of an active nitrate reductase enzyme (◀ Section 3.10 and Figure 3.23*b*) by chaperone-facilitated incorporation of a cofactor. The chaperone protein NarJ carries the molybdenum cofactor (Moco) to the inactive cytoplasmic NarGH complex. After incorporation of Moco, NarJ dissociates and the complex binds to NarI in the membrane, becoming an active enzyme.

group into NarG. Once this cofactor has been inserted, the NarG and NarH subunits associate with the membrane-bound NarI subunit to form the active nitrate reductase enzyme complex (Figure 6.40*b*).

Check Your Understanding

- What are molecular chaperones and why are they necessary?
- What macromolecules are protected by heat shock proteins?
- How do chaperones assist the *Escherichia coli* cell to make nitrate reductase?

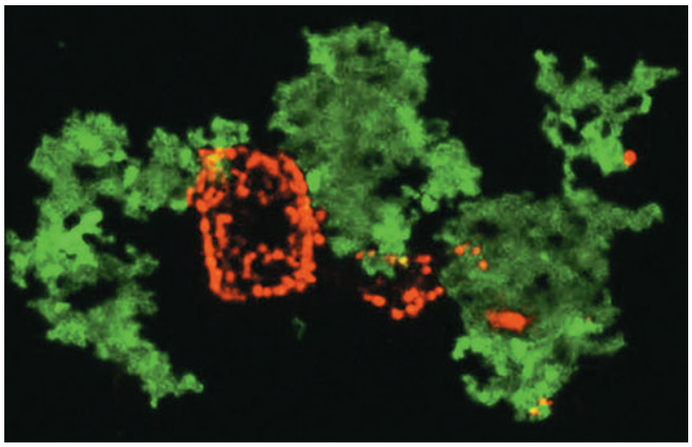
6.12 Protein Secretion: The Sec and Tat Systems

While many proteins exist in the cell's cytoplasm, some must be transported outside the cytoplasmic membrane into the periplasm (of gram-negative cells) or inserted into the cytoplasmic or outer membranes to facilitate nutrient transport or bioenergetic events. Other proteins such as toxins and extracellular enzymes (exoenzymes) must be secreted from the cell entirely to be active in the environment or to invade another cell. These secreted proteins must get from their site of synthesis on ribosomes into or through the cytoplasmic membrane in order to be functional, and the cell has evolved several systems that require either ATP (or GTP) or the proton motive force (Chapter 3) to get this job done.

One example of an unusually large protein that must be secreted to perform its function is halomucin. Halomucin is produced by the halophilic archaeon *Haloquadratum walsbyi* and shares a structural similarity with animal mucin, which protects eyes and bronchial epithelium from dehydration. Thus halomucin is believed to protect *H. walsbyi* from desiccation when growing in its highly saline brine pool habitat (◀ Section 4.15). At 9159 amino acids, halomucin is one of the largest proteins produced by any life form (the average cellular protein is <500 amino acids). For this large protein to confer protection to *H. walsbyi*, the cell must specifically recognize halomucin in the cytoplasm and orchestrate its secretion across the cytoplasmic membrane (**Figure 6.41**). While little is known about the mechanisms *H. walsbyi* uses to secrete proteins, however this is accomplished, a significant energy input would be needed to secrete a protein the extreme size of halomucin.

The Signal Sequence

Proteins called *translocases* transport specific proteins into or through the membranes of *Bacteria* and *Archaea*. For example, the Sec translocase system both exports unfolded proteins and inserts integral



Zenke, R. et al. 2015. Frontiers in Microbiology 6: 249

Figure 6.41 Secretion of halomucin. Fluorescent micrograph illustrating secreted halomucin (labeled with green fluorescent protein, ▶ Section 12.5) by the archaeon *Haloquadratum walsbyi* (red). Note that cells of *H. walsbyi* are square and are visualized here by using a red stain for storage polymers (poly-β-hydroxyalkanoates; ◀ Section 2.7).

membrane proteins into the cytoplasmic membrane. By contrast, the Tat translocase system transports previously folded proteins through the cytoplasmic membrane. Both the general secretion (Sec) and Tat systems are universal in *Bacteria* and *Archaea*.

Most proteins that must be transported into or through bacterial or archaeal cytoplasmic membranes are synthesized with an amino acid sequence of 15–20 residues—called the **signal sequence**—at the beginning (N-terminus, Figure 6.28) of the protein molecule. Signal sequences are variable, but they typically contain a few positively charged amino acids at the beginning, a central region of hydrophobic residues, and then a more polar region at their end. The signal sequence is so named because it "signals" to a translocase system that this particular protein is to be exported and also helps prevent the protein from completely folding, a process that could interfere with its secretion. Because the signal sequence is the first part of the protein to be synthesized, the early steps in export may actually begin before the protein is completely synthesized (**Figure 6.42**).

Sec and Tat Translocases

In the Sec system, unfolded proteins to be exported from the cytoplasm are recognized by either the *SecA protein* or the *signal recognition particle* (*SRP*) (Figure 6.42). Typically, SecA binds polypeptides that are to be exported into the periplasm and transports the unfolded polypeptide to a membrane-bound channel. The polypeptide is then translocated through the channel using ATP as the energy source. Conversely, the SRP binds polypeptides that are to be inserted into the membrane (but not released on the other side) through a process called *cotranslation*. The bacterial SRP, which consists of a single protein and a small, noncoding RNA molecule (4.5S RNA), coordinates to both recognize polypeptides that are destined for the membrane and to move the ribosome actively translating these proteins to the membrane surface. Thus during cotranslation, the polypeptide is actively translated into the membrane, driven by the hydrolysis of GTP. After the protein has been inserted into the membrane (SRP-mediated) or has crossed the membrane (SecA-mediated), the signal sequence is removed by a protease and the proteins fold to their active form.

Some proteins must be folded before they are translocated because they contain cofactors that must be inserted as the protein folds; for example, iron–sulfur proteins, cytochromes, and other respiratory enzymes (◀ Section 3.8). Such proteins are processed by the Tat translocase system (for example, nitrate reductase shown in Figure 6.40*b*). Tat stands for "*t*win-*a*rginine *t*ranslocase" because the transported proteins contain a short signal sequence that has a pair of arginine residues. This signal sequence is recognized by TatBC, which carries the folded protein to TatA—the membrane transporter. Following transport, which is fueled by the proton-motive force, the signal sequence is removed by a protease.

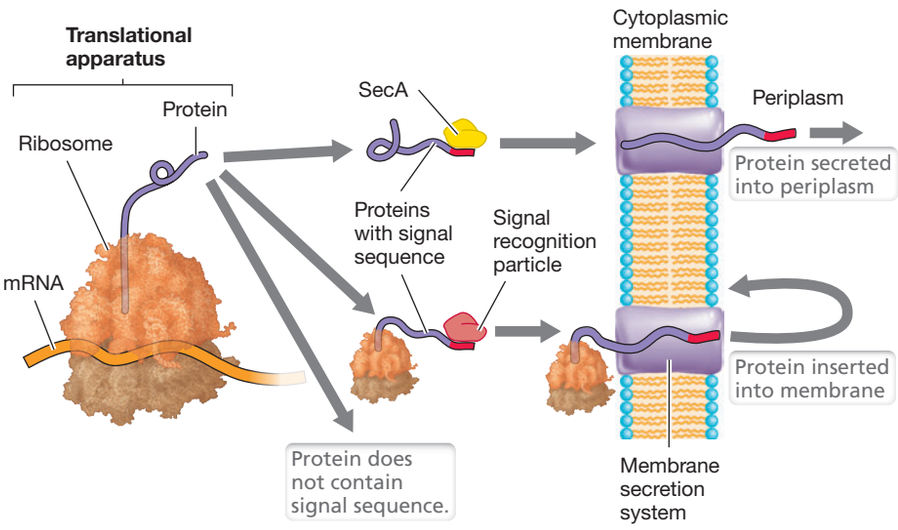


Figure 6.42 Export of proteins via the SecA secretory system. The signal sequence is recognized either by SecA or by the signal recognition particle, which carries the protein to the membrane secretion system. The signal recognition particle binds proteins that are inserted into the membrane, whereas SecA binds proteins that are secreted across the cytoplasmic membrane.

Check Your Understanding

- What is the signal sequence and what does it signal?
- What is the signal recognition particle composed of?
- How do the translocases Sec and Tat differ in the molecules they secrete?

6.13 Protein Secretion: Gram-Negative Systems

In order to insert proteins or other small molecules known as *effectors* into the outer membrane of gram-negative *Bacteria*, or to secrete them outside of the cell, special secretion systems known as types I through VI are used. Proteins secreted by these systems are in their final folded form once outside of

the cell. Gram-positive bacteria and *Archaea* possess secretion systems similar to some of these, but in these organisms, the machinery only has to transport the proteins or effector molecules across the cytoplasmic membrane.

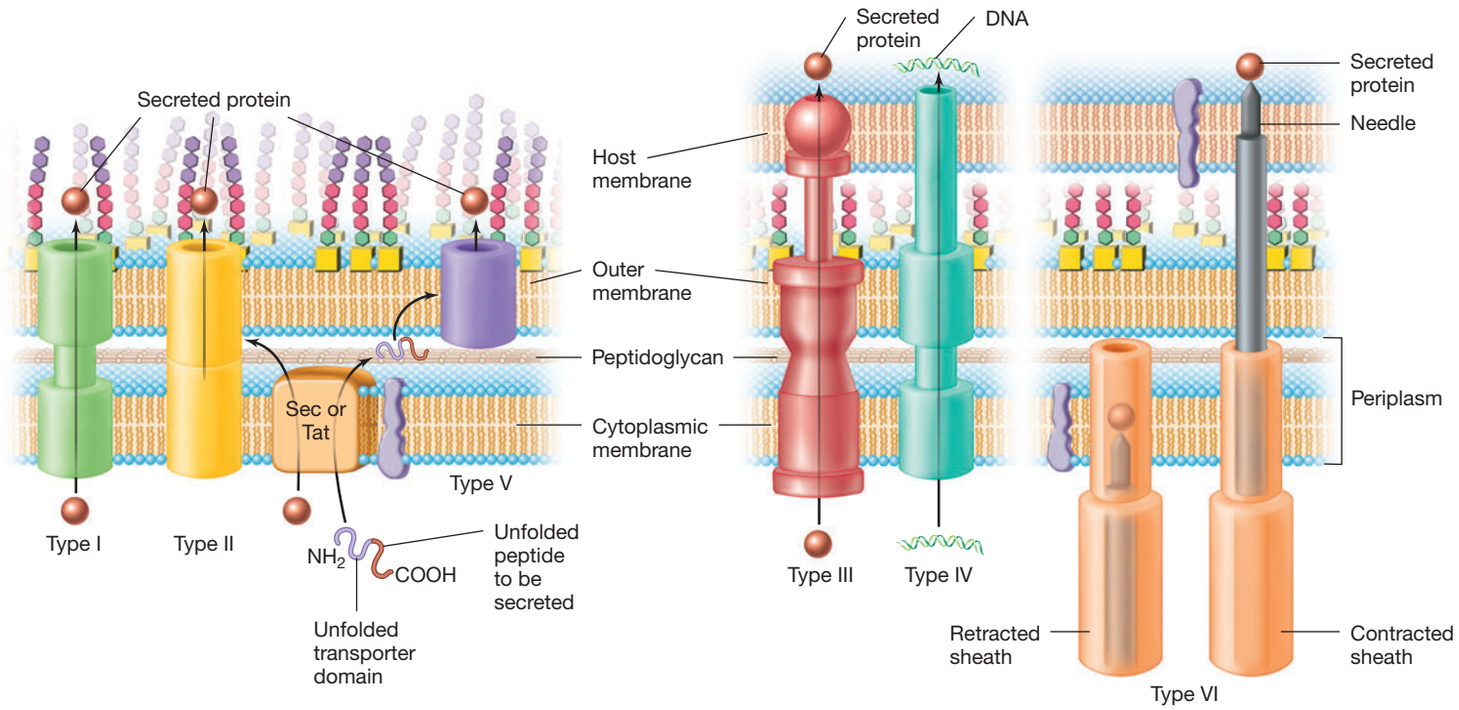
Types I–VI secretion systems facilitate several cellular activities including symbiotic interactions, biofilm formation, extracellular enzyme secretion, transfer of DNA, release of antibiotics, and delivery of proteins into host cells. Thus, molecules secreted by these systems allow bacteria to interact with the environment and other organisms. Each of these systems specifically recognizes its substrate based on amino acid residues and is composed of a large complex of proteins that forms a translocase channel spanning one or more membranes through which the secreted molecule travels (**Figure 6.43**).

Types II and V Secretion Systems

Secretion systems can be grouped into *one-step* and *two-step* classes. Type II and type V systems are two-step translocases because they depend on either the Sec or Tat system (Section 6.12) to transport the secreted protein or a portion of the translocase channel from the cytoplasm through the inner membrane (Figure 6.43*a*).

Type II systems are found in a wide variety of gram-negative pathogenic and nonpathogenic bacteria, and they transport proteins from the periplasm to the extracellular environment. The translocase machinery of type II systems includes a secretion pore in the outer membrane that is anchored to the cytoplasmic membrane by proteins that span the periplasm (Figure 6.43*a*). While the type II system does possess cytoplasmic membrane and periplasmic components, proteins to be secreted do not translocate through these and are instead delivered to the secretion pore in the outer membrane by either the Sec or Tat system. A key to the specificity of the type II system is that the secretion pore only opens to proteins specific to the type II system. Examples of proteins secreted by a type II system include the toxin produced by *Vibrio cholerae* (the causative agent of cholera) and a glucanase exoenzyme produced by *Klebsiella pneumoniae* to degrade large extracellular starch molecules.

Type V systems are the structurally simplest of the secretion systems (Figure 6.43*a*) and are also called *autotransporters* in that the protein to be secreted is fused to a transmembrane protein domain essential to the protein's transport across the outer membrane. After this unfolded multidomain protein is itself transported through the cytoplasmic membrane by the Sec system, a transmembrane domain called the transporter forms a secretion pore in the outer membrane that allows the remainder of the protein (the passenger domain) to pass through and ultimately be secreted outside of the cell (Figure 6.43*a*). Thus proteins to be moved outside of the cell by a type V system are initially transported in the unfolded state, and both the transporter and passenger domains require chaperone proteins (Section 6.11) for proper folding. The folding of the passenger domain (instead of ATP hydrolysis) drives type V secretion. Examples of proteins secreted by the type V system are adhesion proteins used by *Escherichia coli* and *Haemophilus influenzae* (a causative agent of pneumonia) to attach to host cells.



(a) **Types I, II, and V** *(b)* **Types III, IV, and VI**

Figure 6.43 Secretion systems in gram-negative bacteria. *(a)* Types I, II, and V secrete proteins outside of the bacterial cell. Type I systems secrete proteins in a single step. Types II and V first require the Sec or Tat system to transport the protein to be secreted across the inner membrane. Note that during type V secretion, the Sec system first transports the unfolded transporter domain linked to the unfolded secretion protein through the inner membrane. The translocase then folds in the outer membrane and delivers the folded secretion protein through the outer membrane. *(b)* Types III, IV, and VI secrete molecules outside of the bacterial cell and into a host cell. Type III systems have been termed the injectisome, (see page 201) while type IV systems are similar to a pilus (◀ Section 2.6) and also secrete DNA into a host cell. Type VI systems contain a sheath or needle in the cytoplasm that contracts to deliver a protein into a host cell.

Types I, III, IV, and VI Secretion Systems

The second class of translocases moves proteins through the outer membrane in a single step. Types I, III, IV, and VI are one-step systems because they form channels through both the cytoplasmic and outer membranes and do not require Sec or Tat. *Type I systems* are ABC transporters (◀ Section 2.2) that are characterized by three protein components: (1) a cytoplasmic membrane transporter coupled to (2) an outer membrane pore by (3) a membrane fusion protein. The cytoplasmic membrane transporter binds specifically to the protein to be secreted and requires ATP to initiate transport to the outside of the cell (Figure 6.43*a*). While the cytoplasmic membrane transporter is specific to its substrate, a wide range of polypeptide sizes can be secreted. Type I systems secrete small molecules such as a toxic protein (bacteriocin) produced by *E. coli* to kill competing bacterial cells and can also secrete large proteins such as those necessary for biofilm formation on plant surfaces by the soil bacterium *Pseudomonas fluorescens*.

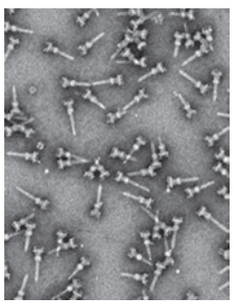
Type III systems are commonly used by pathogenic bacteria not only to secrete toxic proteins outside of the cell but to inject these molecules directly into eukaryotic host cells (Figure 6.43*b*). The entire type III structure is highly complex and composed of over 100 proteins that facilitate substrate recognition, coordinated assembly of the machinery for translocations, and the transport process itself. ATP hydrolysis provides the energy for the secretion and injection. The entire structure, 30–70 nm in length, has been termed the "injectisome" for its similarity to a syringe in both structure and function (**Figure 6.44*a*** and page 201). Proteins injected into host cells by the type III system often aid in pathogen infection and host invasion. Type III–injected proteins include certain effector molecules of *Chlamydia* (the cause of trachoma and a sexually transmitted disease) and *Salmonella* (a foodborne and waterborne pathogen). However, type III secretion systems are not limited to pathogens. Nitrogen-fixing bacteria deliver molecules critical for establishing a symbiotic relationship with plant roots (root nodules) through type III secretion machinery (▶ Section 23.4).

Type IV systems (Figure 6.43*b*) are the most ubiquitous and are present in many *Bacteria* and *Archaea*. This system is also used to deliver secreted proteins or other molecules *into* other cells. While this system can be used to transport toxins into host cells, its primary role is to transfer DNA to other cells though the process of conjugation (▶ Section 9.8), one mechanism that prokaryotic cells have to exchange genes. The type IV translocase is similar to a pilus that extends through the outer membrane into another cell during conjugation. Once the tip of the pilus makes contact with a receptor on the host cell, the DNA is recognized by an inner membrane protein of the donor and then transferred using ATP-facilitated transport (▶ Section 9.8). Not only can DNA be transferred from one bacterial cell to another using a type IV system, DNA from the plant pathogenic bacterium *Agrobacterium* can be transferred to host plant cells through the system encoded on the Ti plasmid (resulting in crown gall disease, ▶ Section 23.6).

Type VI systems are widely distributed in gram-negative bacteria, and like type IV systems they are capable of delivering a diversity of proteins directly into the cytoplasm of other cells using a one-step, ATP-requiring process similar to type III and IV systems. However, unlike the injectisome or pilus-like structure of the type III and IV translocase systems, the type VI translocase is cytoplasmic and forms a needle-like protein with a pore-forming protein that contracts all the way through the donor cell's two membranes and directly into a host cell once a substrate molecule is recognized (Figures 6.43*b* and 6.44*b*). The overall delivery process is similar to the mechanism that the tailed bacteriophage T4 uses to deliver DNA into an *E. coli* cell through tail contraction (◀ Section 5.5). Type VI systems are used by bacteria as weapons to compete with other bacterial cells or to attack eukaryotic cells. In *Pseudomonas aeruginosa*, a type VI system is used to inject a toxin into competing bacterial cells. Similarly, *Vibrio cholerae* uses its type VI system (Figure 6.44*b*) to inject enzymes into competing bacteria; the enzymes degrade the cell wall and membrane. *V. cholerae* also uses the system to deliver toxins to human intestinal cells during a cholera infection.

It should be obvious by now that protein secretion is a crucial aspect of the biology of *Bacteria* and *Archaea* and that these cells have evolved diverse structures and unique mechanisms to deal with protein secretion. In fact, three other systems (types VII–IX) have also been discovered, but their mechanisms have not been fully described. All of these elaborate systems are of interest for reasons beyond basic science. Since many of these systems are essential virulence factors (▶ Section 25.3) for the pathogenic bacteria that produce them, they are currently targets for vaccine development. The rationale here is quite simple: If the activity of these systems could be blocked by the immune system, the pathogen would be unable to establish an infection and cause disease.

We now move on from our focus on the central dogma of biology, protein processing, and protein secretion to tackle the concepts of Chapter 7, where we will examine how gene expression and protein activity are regulated. Without regulatory systems, cells would not be able to quickly respond to ever-changing conditions in their environments, and thus their very survival would be at stake.



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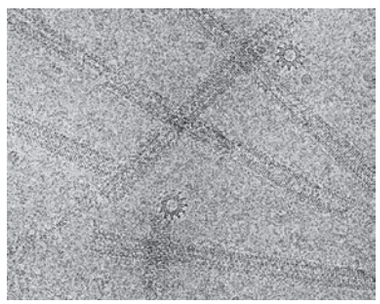

M. Kudryashev, H. Stahlberg, and M. Basler

(b)

Figure 6.44 Electron micrographs of secretion machinery. *(a)* Purified type III injectisomes from *Salmonella enterica* (*typhimurium*) (see also page 201). *(b)* Purified type VI contractile sheaths from *Vibrio cholerae*.

Check Your Understanding

- Compared with gram-positive bacteria, why is it important for gram-negative bacteria to have additional secretion pathways?
- How do the types I–VI secretion processes differ from Sec and Tat secretion?
- Why is the injectisome so named?

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CHAPTER REVIEW

I • Molecular Biology and Genetic Elements

6.1 The informational content of a nucleic acid is determined by the sequence of nitrogenous bases along the polynucleotide chain. Both RNA and DNA are informational macromolecules, as are the proteins they encode. DNA forms a double-stranded helix whose strands are complementary and antiparallel. DNA is supercoiled before it is packaged into cells. The three key processes of macromolecular synthesis are: (1) DNA replication; (2) transcription (RNA synthesis); and (3) translation (protein synthesis).

Q Describe the central dogma of molecular biology. With regards to DNA, what is supercoiling and what is meant by the terms antiparallel and complementary?

6.2 In addition to the chromosome, other genetic elements can exist in cells. Plasmids are DNA molecules that exist separately from the chromosome and may confer a selective growth advantage under certain conditions. Viruses contain an RNA or DNA genome, and transposable elements exist as a part of other genetic elements.

Q How are chromosomes and plasmids similar, and how do they differ? What are R plasmids and why are they of medical concern?

II • Copying the Genetic Blueprint: DNA Replication

6.3 Both strands of the DNA helix are templates for the synthesis of new strands (semiconservative replication). The new strands are elongated by addition of deoxyribonucleotides to the 3′ end. DNA polymerases require a primer made of RNA by the enzyme primase, and synthesis begins at an origin of replication. The double helix is unwound by helicase and is stabilized by single-strand binding proteins. Extension of the DNA occurs continuously on the leading strand but discontinuously on the lagging strand, resulting in fragments that must be joined together.

Q What is meant by the term semiconservative replication? What are the functions of DNA Pol I and III, helicase, and primase? How does a leading strand differ from a lagging strand?

6.4 Starting from a single origin on a circular chromosome, two replication forks synthesize DNA simultaneously in both directions until the forks meet at the terminus region. The proteins at the replication fork form a large complex known as the replisome. Most errors in base pairing that occur during replication are corrected by the proofreading functions of DNA polymerases.

Q What is the replisome and what does it contain? Why can replication occur faster on circular DNA than on linear DNA? What is proofreading and why is it important?

III • RNA Synthesis: Transcription

6.5 In *Bacteria*, promoters are recognized by the sigma subunit of RNA polymerase. Alternative sigma factors allow joint regulation of large families of genes in response to growth conditions. Transcription by RNA polymerase continues until specific sites called transcription terminators are reached. These terminators function at the level of RNA. In *Bacteria* and *Archaea* a single mRNA molecule may encode more than one polypeptide. A cluster of genes that are transcribed together from a single promoter is called an operon.

Q What do promoters do in *Bacteria*? How is a transcript in *Bacteria* terminated?

6.6 The transcription apparatus and the promoter architecture of *Archaea* and *Eukarya* have many features in common, although the components are usually relatively more simple in *Archaea*. In contrast, the processing of eukaryotic primary transcripts is unique and has three distinct steps: splicing, capping, and adding a poly(A) tail.

Q How does the archaeal RNA polymerase differ from that in *Bacteria*? How does the initiation of transcription in the two domains differ? Why do eukaryotic mRNAs have to be "processed" whereas most prokaryotic RNAs do not?

IV • Protein Synthesis: Translation

6.7 Polypeptide chains contain many amino acids linked via peptide bonds. Twenty-two different amino acids are genetically encoded. The primary structure of a protein is its amino acid sequence, but the higher-order structure (folding) of the polypeptide determines its cellular function.

Q Describe the two types of secondary structure a polypeptide can attain. Which proteins can achieve quaternary structure? Which protein structure(s) are altered by denaturation?

6.8 One or more tRNAs exist for each amino acid incorporated into polypeptides by the ribosome. Enzymes called aminoacyl-tRNA synthetases bond amino acids to their cognate tRNAs.

Q Why are tRNAs important in translation? Do genes for tRNAs have promoters, and are tRNAs translated? What are aminoacyl-tRNA synthetases, what are their substrates, and what do they do?

6.9 The genetic code is expressed as RNA, and a single amino acid may be encoded by several different but related codons. In addition to the stop (nonsense) codons, there is also a specific start codon that signals the initiation of translation.

Q Why is the genetic code a degenerate code? What is wobble and how does it accommodate fidelity in the genetic code?

6.10 Translation occurs on the ribosome and requires mRNA and aminoacyl-tRNAs. The ribosome has three sites: acceptor, peptide, and exit (A, P, and E). During each step of translation, the ribosome advances one codon along the mRNA, and the tRNA in the acceptor site moves to the peptide site. Protein synthesis terminates when a stop codon, which does not have a corresponding tRNA, is reached.

Q Where on the ribosome do tRNAs bind, and what is the energy source that supports translocation?

V • Protein Processing, Secretion, and Targeting

6.11 Polypeptides do not remain linear in structure following translation but require proper folding and additional processing for functional activity. Proteins called chaperones help fold some proteins that are unable to fold spontaneously and also assist in the incorporation of cofactors and refolding partially denatured proteins.

Q What is the primary function of chaperones? How do cells sometimes reuse partially denatured proteins?

6.12 Many proteins need to be transported into or through the cytoplasmic membrane. These proteins contain a signal sequence that is recognized by the cellular translocase systems of Sec and Tat.

Q What are the functions of the Sec and Tat systems and why are they necessary? How do these systems know which proteins to act upon?

6.13 A large diversity of secretion systems are employed by gram-negative bacteria to secrete proteins into or through their outer membrane and even into other cells, and types I–VI secretion systems function in this regard. Types II and V are two-step translocases and rely on the activity of Sec or Tat, whereas types I, III, IV, and VI are one-step translocases. In most cases, energy is required to drive secretion of proteins outside the cell.

Q What are the major differences between one-step and two-step translocases? Which are used to secrete proteins into other cells?

APPLICATION QUESTIONS

1. The genome of the bacterium *Neisseria gonorrhoeae* consists of one double-stranded DNA molecule that contains 2220 kilobase pairs. If 85% of this DNA molecule is made up of the open reading frames of genes encoding proteins, and the average protein is 300 amino acids long, how many protein-encoding genes does *Neisseria* have? What kind of genetic information is present in the other 15% of the DNA?
2. Compare and contrast the activity of DNA and RNA polymerases. What is the function of each? What are the substrates of each? What is the main difference in the behavior of the two polymerases?
3. What would be the result (in terms of protein synthesis) if RNA polymerase initiated transcription one base upstream of its normal starting point? Why? By inspecting Table 6.4, discuss how the genetic code has evolved to help minimize the impact of mutations.

CHAPTER GLOSSARY

Amino acid one of the 22 different monomers that are attached to one another by peptide bonds to form proteins; chemically, a two-carbon carboxylic acid containing an amino group and a characteristic substituent on the alpha carbon

Aminoacyl-tRNA synthetase an enzyme that catalyzes attachment of an amino acid to its cognate tRNA

Anticodon a sequence of three bases in a tRNA molecule that base-pairs with a codon during protein synthesis

Antiparallel in reference to double-stranded DNA, the two strands run in opposite directions (one runs 5′ → 3′ and the complementary strand 3′ → 5′)

Bacteriocin a toxic protein secreted by bacteria that inhibits or kills other, related bacteria

Chaperone a protein that helps other proteins fold or refold from a partly denatured state

Chromosome a genetic element, usually circular in prokaryotic cells, carrying genes essential to cellular function

Codon a sequence of three bases in mRNA that encodes an amino acid

Codon bias the nonrandom usage of multiple codons encoding the same amino acid; the relative proportions of different codons encoding the same amino acid vary in different organisms. Same as codon usage

Complementary nucleic acid sequences that can base-pair with each other

Denaturation loss of the correct folding of a protein, leading (usually) to protein aggregation and loss of biological activity

DNA (deoxyribonucleic acid) a polymer of deoxyribonucleotides linked by phosphodiester bonds; the deoxyribonucleotide sequence encodes genetic information

DNA gyrase an enzyme found in *Bacteria* and most *Archaea* that introduces negative supercoils in DNA

DNA helicase an enzyme that uses ATP to unwind the double helix of DNA

DNA ligase an enzyme that seals nicks in the backbone of DNA

DNA polymerase an enzyme that synthesizes a new strand of DNA in the 5′ → 3′ direction using an antiparallel DNA strand as a template

Exons the coding DNA sequences in a split gene (contrast with intron)

Gene a segment of DNA specifying a protein (via mRNA), a tRNA, an rRNA, or any other noncoding RNA

Genetic code the correspondence between nucleic acid sequence and amino acid sequence of proteins

Genetic element a structure that carries genetic information, such as a chromosome, a plasmid, or a virus genome

Genome the total complement of genes contained in a cell or virus

Informational macromolecule any large polymeric molecule that carries genetic information, including DNA, RNA, and protein

Introns the intervening noncoding DNA sequences in a split gene (contrast with exons)

Lagging strand the new strand of DNA that is synthesized in short pieces and then joined together later

Leading strand the new strand of DNA that is synthesized continuously during DNA replication

Messenger RNA (mRNA) an RNA molecule that contains the genetic information to encode one or more polypeptides

Nonsense codon another name for a stop codon

Nucleoside a nitrogenous base (adenine, guanine, cytosine, thymine, or uracil) plus a sugar (either ribose or deoxyribose) but lacking phosphate

Nucleotide a monomer of a nucleic acid containing a nitrogenous base (adenine, guanine, cytosine, thymine, or uracil), one or more molecules of phosphate, and a sugar, either ribose (in RNA) or deoxyribose (in DNA)

Open reading frame (ORF) a sequence of DNA or RNA that could be translated to give a polypeptide

Operon two or more genes transcribed into a single RNA and under the control of a single regulatory site

Peptide bond a type of covalent bond linking amino acids in a polypeptide

Phosphodiester bond a type of covalent bond linking nucleotides together in a polynucleotide

Plasmid an extrachromosomal genetic element that is usually not essential to the cell

Polynucleotide a polymer of nucleotides bonded to one another by covalent bonds called phosphodiester bonds

Polypeptide a polymer of amino acids bonded to one another by peptide bonds

Primary structure the precise sequence of monomers in a macromolecule such as a polypeptide or a nucleic acid

Primary transcript an unprocessed RNA molecule that is the direct product of transcription

Primase the enzyme that synthesizes the RNA primer used in DNA replication

Primer an oligonucleotide to which DNA polymerase attaches the first deoxyribonucleotide during DNA synthesis

Promoter a site on DNA to which RNA polymerase binds to commence transcription

Protein a polypeptide or group of polypeptides forming a molecule of specific biological function

Purine one of the nitrogenous bases of nucleic acids that contain two fused rings; adenine and guanine

Pyrimidine one of the nitrogenous bases of nucleic acids that contain a single ring; cytosine, thymine, and uracil

Quaternary structure in proteins, the number and types of individual polypeptides in the final protein molecule

Replication in cells, synthesis of DNA using DNA as a template

Replication fork the site on the chromosome where DNA replication occurs and where the enzymes replicating the DNA are bound to untwisted, single-stranded DNA

Replisome a DNA replication complex that consists of two copies of DNA polymerase III, DNA gyrase, helicase, primase, and copies of single-strand binding protein

Ribosomal RNA (rRNA) the types of RNA found in the ribosome; some participate actively in protein synthesis

Ribosome a cytoplasmic particle composed of ribosomal RNA and protein, whose function is to synthesize proteins

RNA (ribonucleic acid) a polymer of ribonucleotides linked by phosphodiester bonds that plays many roles in cells, in particular, during protein synthesis

RNA polymerase an enzyme that synthesizes RNA in the 5′ → 3′ direction using a complementary and antiparallel DNA strand as a template

RNA processing the conversion of a primary transcript RNA to its mature form

Secondary structure the initial pattern of folding of a polypeptide or a polynucleotide, usually dictated by opportunities for hydrogen bonding

Semiconservative replication DNA synthesis yielding two new double helices, each consisting of one parental and one progeny strand

Signal sequence a special N-terminal sequence of approximately 20 amino acids that signals that a protein should be incorporated into or exported across the cytoplasmic membrane

Spliceosome a complex of ribonucleoproteins that catalyze the removal of introns from primary RNA transcripts

Start codon a special codon, usually AUG, that signals the start of a protein

Stop codon a codon that signals the end of a protein

Termination stopping the elongation of an RNA molecule at a specific site

Tertiary structure the final folded structure of a polypeptide that has previously attained secondary structure

Transcription the synthesis of an RNA molecule complementary to one of the two strands of a double-stranded DNA molecule

Transfer RNA (tRNA) a small RNA molecule used in translation that possesses an anticodon at one end and has the corresponding amino acid attached to the other end

Translation the synthesis of protein by a ribosome using the genetic information in a messenger RNA as a template

Transposable element a genetic element able to move (transpose) from one site to another on host DNA molecules

Wobble a less rigid form of base pairing allowed only in codon–anticodon pairing

7 Microbial Regulatory Systems

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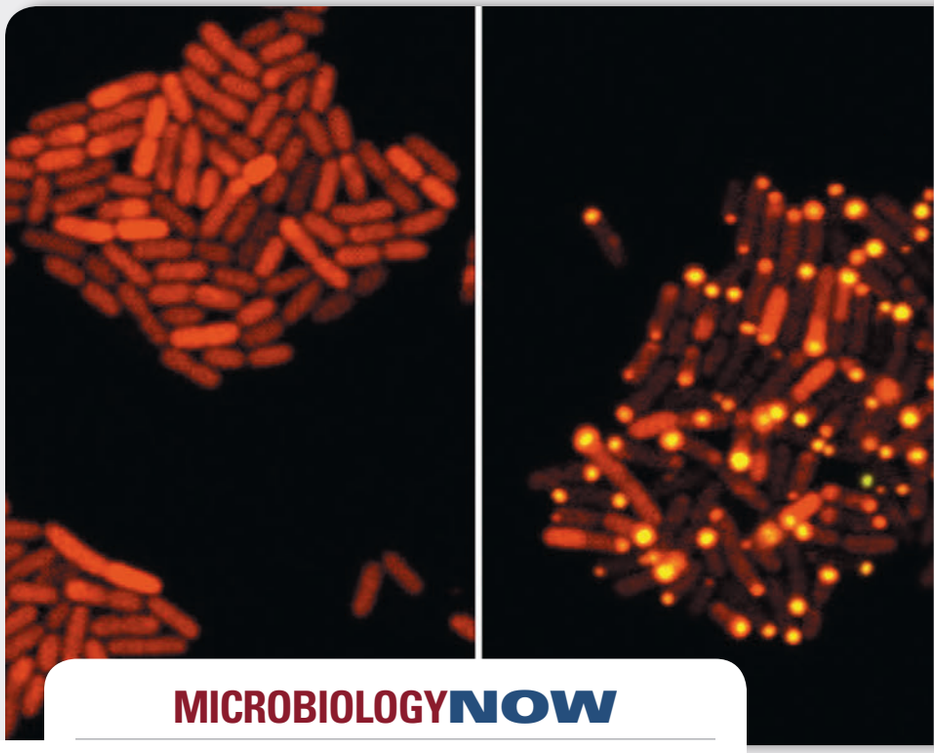


MICROBIOLOGYNOW

As Bacterial Cells Chatter, Viruses Eavesdrop

Despite their primitive nature, bacterial and archaeal cells participate in a form of communication called quorum sensing. Quorum sensing coordinates behaviors that require a certain cell density to be successful, such as virulence and biofilm formation. This communication is dependent on the microbial secretion of autoinducer molecules. As the population of autoinducer-producing cells increases in a given environment, so does the local concentration of autoinducer. Once the autoinducer reaches a certain threshold, regulatory proteins respond by activating genes whose products are necessary for group behavior.

As extensive research on quorum sensing continued, microbiologists began to ponder if viruses could respond to quorum sensing. Temperate bacteriophages have two possible "lifestyles"—their DNA can reside in dormancy within the bacterial host cell chromosome or they can aggressively multiply and lyse their host cell. Using elegant experiments, researchers discovered that certain dormant bacteriophages are actually "listening in" on their hosts' conversation. This eavesdropping occurs through a phage-encoded receptor protein that can sense host autoinducer concentrations. In response to autoinducer concentrations, this phage regulatory protein initiates a cascade of events that awakens the phage from dormancy.

The image to the left shows *Escherichia coli* cells harboring a fluorescently labeled phage repressor protein called cI that is distributed uniformly throughout the host cell and is essential for phage dormancy. The panel on the right shows the same cells with the addition of the newly discovered phage-encoded protein called Qtip, which is only produced in response to high autoinducer levels. Qtip functions by inactivating the cI protein by aggregating it (bright foci in right panel). Without free cI, the dormant phage produces thousands of progeny and lyses its host. This fascinating phenomenon illustrates how a nonliving microbe—the virus—both senses and responds to host cell communication to ensure its own successful spread!

Source: Silpe, J.E., and B.L. Bassler. 2019. A host-produced quorum-sensing autoinducer controls a phage lysis-lysogeny decision. *Cell 176*: 268.

To efficiently orchestrate the numerous reactions that occur in a cell and to maximally use available resources, cells must *regulate* the types, amounts, and activities of proteins and other macromolecules they make. Some proteins and RNA molecules are needed in the cell at about the same level under all growth conditions, and the production of these molecules is said to be *constitutive*. However, many proteins and RNAs are needed under some conditions but not others. For instance, enzymes required to catabolize the sugar lactose are useful only if lactose is available to the cell. Regulatory systems can thus be viewed as a means to improve the overall fitness of an organism and maximize its ability to produce offspring from the resources available.

Microbes regulate protein function in two distinct ways. One mechanism controls the *amount* of an enzyme or other protein, whereas the second controls the *activity* of a preformed enzyme or other protein (**Figure 7.1**). The amount of protein synthesized can be regulated at either the level of *transcription*—by varying the amount of mRNA made—or the level of *translation*—by translating or not translating the mRNA. Collectively, these regulatory processes are called **gene expression**. After a protein has been synthesized, *post-translational* regulatory processes can further regulate the activity of some proteins (Figure 7.1). In this chapter we focus on how the cell efficiently controls its metabolism by regulating both enzyme synthesis and enzyme activity.

I • DNA-Binding Proteins and Transcriptional Regulation

Transcriptional controls in *Bacteria* and *Archaea* typically function by binding a specific regulatory protein to a specific region of DNA, an event that either represses or activates transcription.

Although several microbial regulatory mechanisms are possible, our discussion begins with control at the level of transcription because this is the major means of regulation in prokaryotic cells.

7.1 DNA-Binding Proteins

Before delving into the themes of transcriptional regulation, we must first recall our discussion of how bacterial and archaeal gene

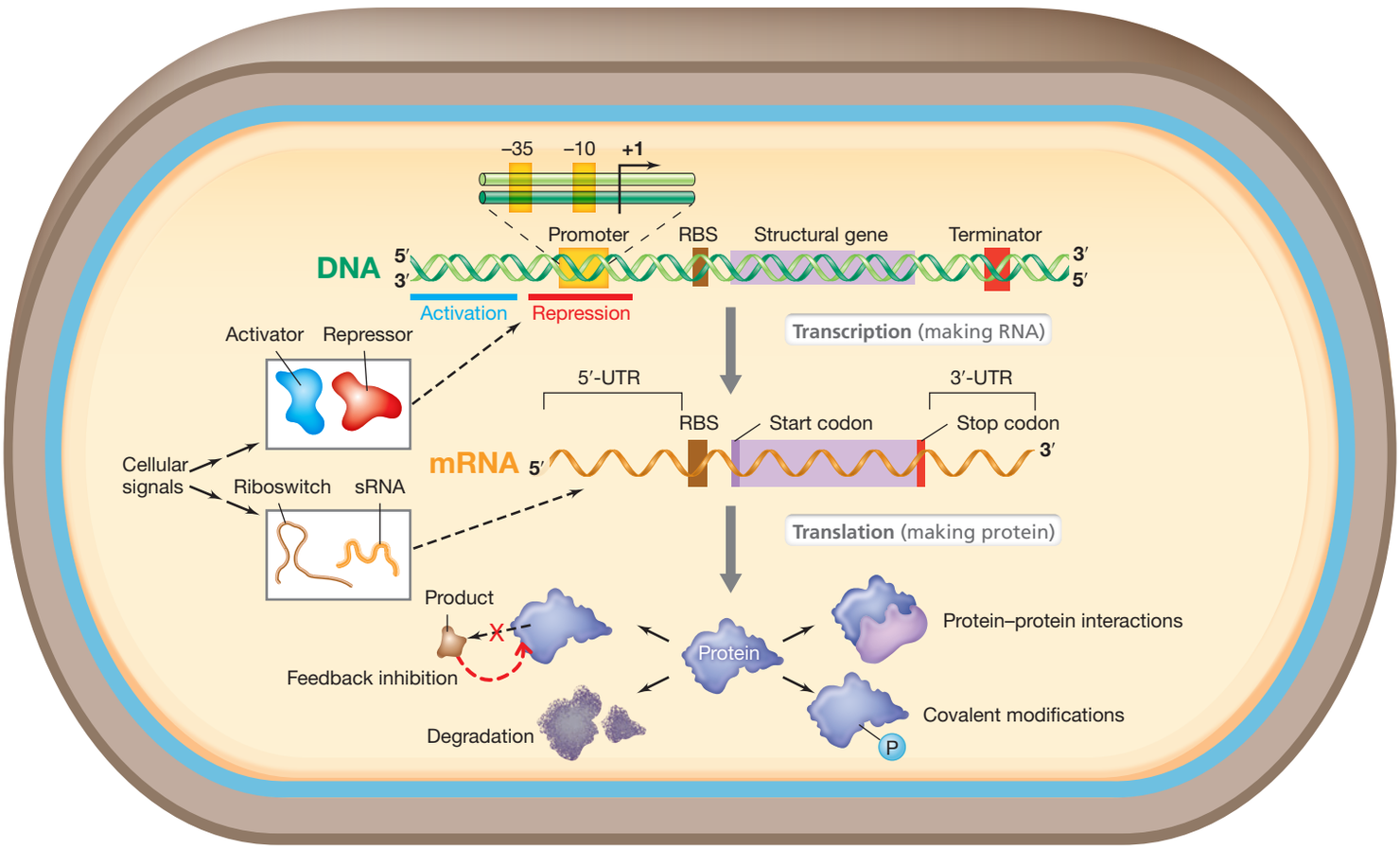


Figure 7.1 Gene expression and regulation of protein activity. For DNA, the promoter and terminator as well as regions related to transcriptional activation and repression are indicated. Cellular signals control the activity and DNA-binding ability of activator and repressor proteins. For mRNA, the 5′ untranslated region (5′-UTR) is a short region between the start of transcription and the start of translation, including the ribosome-binding site (RBS). The 3′ untranslated region (3′-UTR) is a short region between the stop codon and the transcription terminator. These are the regions where translational regulation often occurs, with riboswitches and small RNAs (sRNAs) often targeting the 5′-UTR in response to cellular signals. Mechanisms for regulating protein activity after translation are shown near the bottom.

arrangement differs from that of eukaryotes (Chapter 6). Besides lacking traditional introns, bacterial and archaeal genes can be arranged in **operons**—two or more genes transcribed under the control of a single regulatory site (◀ Section 6.5). This regulatory site, or *promoter region*, is located upstream of where RNA polymerase initiates transcription (Figure 7.1). Bacterial and archaeal promoter regions are characterized by distinct nucleotide sequences that must be recognized and bound by *DNA-binding proteins* for RNA polymerase to bind and allow transcription to occur (◀ Sections 6.5 and 6.6).

Interaction of Proteins with Nucleic Acids

Interactions between proteins and nucleic acids are central to replication, transcription, and translation, and also to the regulation of these processes. Protein–nucleic acid interactions may be specific or nonspecific, depending on whether the protein attaches anywhere along the nucleic acid or binds to a specific site. Most DNA-binding proteins interact with DNA in a sequence-specific manner. Specificity is provided by interactions between specific amino acid side chains of the proteins and specific chemical groups on the nitrogenous bases and the sugar–phosphate backbone of the DNA. Because of its size, the *major groove* of DNA (◀ Figure 6.3) is the main site of protein binding, and Figure 6.1*c* identified atoms of the bases in the major groove that are known to interact with proteins. To achieve high specificity, the binding protein must interact simultaneously with several nucleotides.

In DNA, an *inverted repeat* is a nucleotide sequence followed downstream by its inverted complement (◀ Section 6.5). Such inverted repeats are frequently the locations at which proteins bind specifically to DNA (**Figure 7.2**). Note that this interaction does not require the formation of stem–loop structures in the DNA as was described for the signal for termination of transcription (◀ Figure 6.23). DNA-binding proteins are often homodimeric, meaning they are composed of two identical polypeptide subunits, each subdivided into **domains**—regions of the protein with a specific structure and function. Each subunit has a domain that interacts specifically with a region of DNA in the major groove. When protein dimers interact with inverted repeats on DNA, each subunit binds to one of the inverted repeats. The dimer as a whole thus binds to both DNA strands (Figure 7.2).

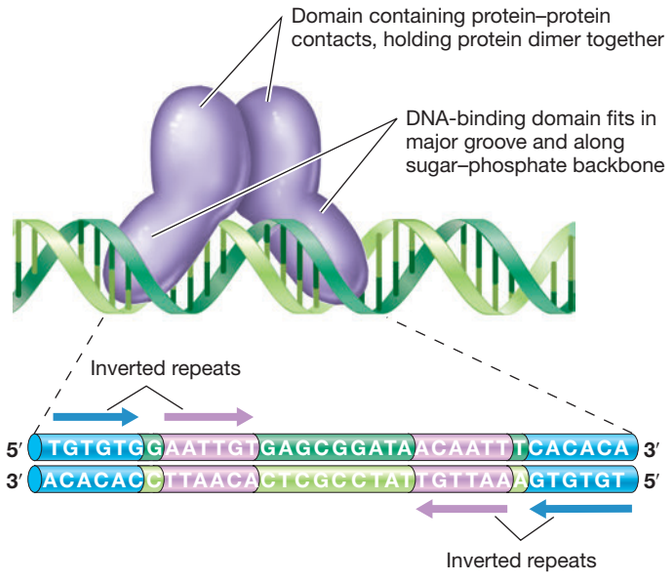


Figure 7.2 DNA-binding proteins. Many DNA-binding proteins are dimers that combine specifically with two sites on the DNA. The specific DNA sequences that interact with the protein are inverted repeats. The nucleotide sequence of the operator gene of the lactose operon (see Figure 7.6) is shown, and the inverted repeats, which are sites at which the *lac* repressor (LacI) makes contact with the DNA, are shown in purple and blue boxes.

Structure of DNA-Binding Proteins

DNA-binding proteins in both *Bacteria* and *Archaea* as well as eukaryotes possess several classes of protein domains that are critical for proper binding to DNA. One of the most common is the *helix-turn-helix* structure (**Figure 7.3*a***). This consists of two segments of polypeptide chain that have an α-helix secondary structure connected by a short sequence forming the "turn." The first helix is the *recognition helix*, which interacts specifically with DNA. The second helix, the *stabilizing helix*, stabilizes the first helix by interacting with it by way of hydrophobic interactions. The turn linking the two helices consists of three amino acid residues, the first of which is typically a glycine. Sequences are recognized by noncovalent interactions, including hydrogen bonds and van der Waals contacts, between the recognition helix of the protein and specific chemical groups in the sequence of base pairs on the DNA.

Many different DNA-binding proteins from *Bacteria* contain the helix-turn-helix structure. These include many repressor proteins, such as the *lac* and *trp* repressors of *Escherichia coli* (Section 7.3 and Figure 7.3 inset), and some proteins of bacterial viruses, such as the bacteriophage lambda repressor (Figure 7.3*b*). Indeed, over 250 different proteins with this motif bind to specific locations on DNA to regulate transcription in *E. coli*. Two other types of protein domains are commonly found in DNA-binding proteins. One of these, the *zinc finger*, is frequently found in regulatory proteins in eukaryotes and, as its name implies, binds a zinc ion. The other protein domain commonly found in DNA-binding proteins is the *leucine zipper*, which contains regularly spaced leucine residues that function to hold two recognition helices in the correct orientation to bind DNA.

Once a protein binds at a specific site on the DNA, several outcomes are possible. Some DNA-binding proteins are enzymes that catalyze a specific reaction on the DNA, such as transcription. In other cases, however, the binding event either blocks transcription or activates it, and we consider these now.

Check Your Understanding

- What is a protein domain?
- Why are most DNA-binding proteins specific to certain chemical groups within the DNA?

7.2 Transcription Factors and Effectors

Transcription is the first step in biological information flow; because of this, it is simple and efficient to control gene expression at this point. If one gene is transcribed more frequently than another, there will be more of its mRNA available for translation and therefore a

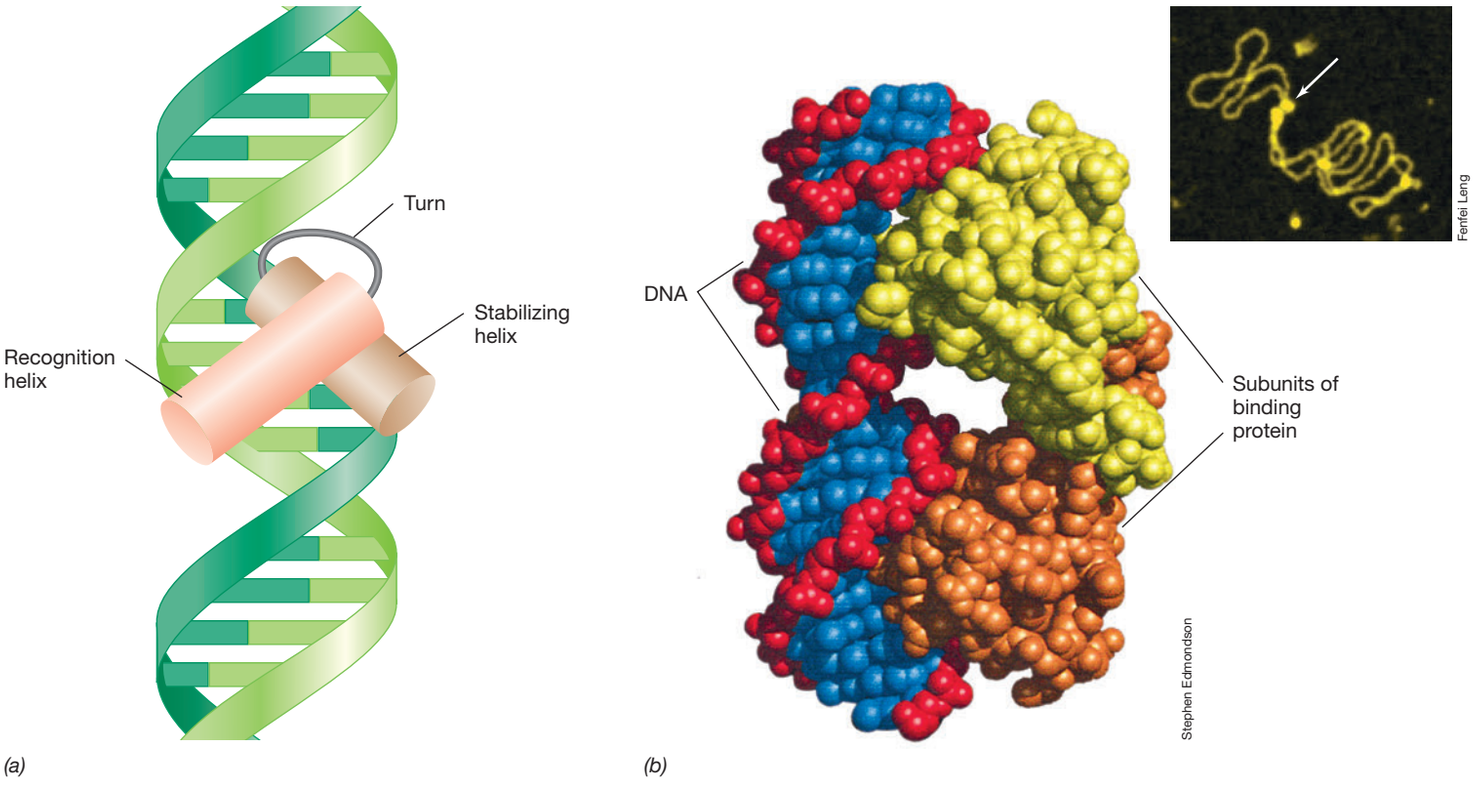


Figure 7.3 **The helix-turn-helix structure of some DNA-binding proteins.** *(a)* A simple model of the helix-turn-helix structure within a single protein subunit. *(b)* A computer model of both subunits of the bacteriophage lambda repressor bound to its operator. The complementary DNA strands are red and blue. One subunit of the dimeric repressor protein is shown in orange and the other in yellow. Each subunit contains a helix-turn-helix structure. Inset: Atomic force microscopy showing copies of the LacI repressor protein (arrow) bound to multiple operator sites on a DNA molecule.

greater amount of its protein product in the cell. This control is mediated by *transcription factors* and *effector molecules*.

Mechanisms of Transcription Factors

Proteins that control the rate of gene transcription by binding to specific DNA sequences are called **transcription factors**. Depending on the type of transcription factor, this DNA binding can lead to increased or decreased transcription of the associated gene. If binding to the DNA results in turning transcription *on*, the transcription factor is called an **activator protein** (Figure 7.4*a*). Conversely, binding of a **repressor protein** to DNA results in turning gene expression *off* (Figure 7.4*b*).

Activator proteins turn on transcription by binding to DNA and helping to recruit RNA polymerase, or a sigma (σ) factor in the case of bacterial transcription (◀ Section 6.5), to a specific promoter region (Figure 7.4*a*). Repressor proteins turn off transcription by binding to a specific DNA sequence of a gene called an *operator* region (Figure 7.4*b*). This region gets its name from the *operon*, a cluster of consecutive genes whose expression is under the control of a single operator (◀ Section 6.2). All of the genes in an operon are transcribed as a single unit yielding a single mRNA (◀ Section 6.5). The operator is located downstream of the promoter where synthesis of mRNA is initiated (Figure 7.4). The activity of activator and repressor proteins is described in more detail in the next section.

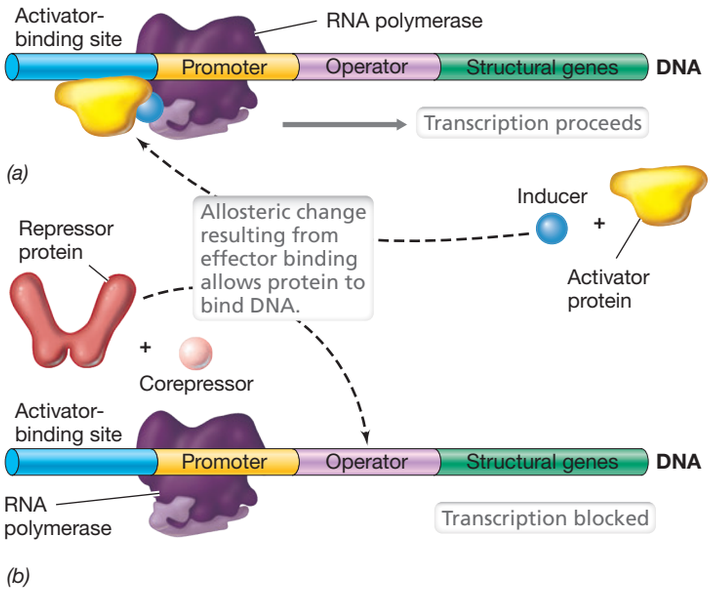


Figure 7.4 **Transcription factors, effectors, and allosterism.** Binding of effector molecules to activator and repressor proteins results in an allosteric change that affects the DNA-binding ability of the transcription factors. *(a)* Binding of an activator to the DNA results in recruiting RNA polymerase and turning transcription on. *(b)* Binding of a repressor protein to the operator region of the DNA results in blocking RNA polymerase and turning transcription off.

Effector Molecules

The ability of activator and repressor proteins to bind DNA is controlled by small molecules called *effectors*. Transcription factors are **allosteric proteins**; that is, their conformation is altered when the effector molecule binds to them (Figure 7.4 and Section 7.15). This conformational change determines if the transcription factor can bind DNA. If the binding of an effector to a transcription factor results in turning transcription *on*, the molecule is considered an *inducer* (Figure 7.4*a*). Conversely, *corepressor* molecules are those whose binding to a transcription factor results in turning transcription *off* (Figure 7.4*b*).

Effector molecules are typically cell metabolites, such as substrates or end products of the pathways that they regulate. As we will discuss with the *lac* operon—the operon encoding genes of lactose catabolism—structural analogs of effector molecules can also control transcription factor binding even though they are not themselves substrates for the enzymes that the operon encodes (Section 7.8). Thus, small molecules can indirectly regulate gene expression by binding to transcription factors, as many examples in this chapter will demonstrate.

Check Your Understanding

- What is a transcription factor and what are the two major types?
- What role do effectors have on transcription factors?

7.3 Repression and Activation

Mastering Microbiology
Activities: Microbiology Animation: Operons: Repression

The activities of activator and repressor proteins are simple forms of regulation that govern gene expression at the level of transcription, and the binding of these transcription factors to DNA plays the initial role in controlling the kinds and activities of enzymes in a cell. We begin our discussion of the activities of some common transcription factors by considering cellular conditions in which the expression of particular sets of enzymes are controlled.

Enzyme Repression and Induction

Often the enzymes that catalyze the synthesis of a specific product are not made if the product is already present in the growth medium in sufficient amounts. For example, in *Escherichia coli* and many other *Bacteria*, the enzymes needed to synthesize the amino acid arginine (◀ Figure 6.27) are made only when arginine is *absent* from the culture medium; an excess of arginine decreases the synthesis of these enzymes. This is called **enzyme repression**.

As can be seen in **Figure 7.5*a***, if arginine is added to a culture growing exponentially in a medium devoid of arginine, growth continues at the previous rate, but production of the enzymes for arginine synthesis stops. Note that this is a *specific* effect, as the synthesis of all other enzymes in the cell continues at the previous rate. This is because the enzymes affected by a particular repression event make up only a tiny fraction of the entire complement of proteins in the cell. Enzyme repression is widespread in *Bacteria* as a means of controlling the synthesis of enzymes required for the production of amino acids and the nucleotide precursor purines and pyrimidines (◀ Section 3.14). In most cases, the final product of a particular biosynthetic pathway is the effector molecule (corepressor) for that pathway and must be present in excess of biosynthetic needs to repress the enzymes of the pathway. Such enzyme repression ensures that the organism does not waste energy and nutrients synthesizing unneeded enzymes.

Enzyme induction is conceptually the opposite of enzyme repression. In enzyme induction, an enzyme is made only when its substrate is *present*. Enzyme repression typically affects biosynthetic (anabolic) enzymes. By contrast, enzyme induction usually affects the synthesis of specific degradative (catabolic) enzymes. To illustrate induction, consider the utilization of the sugar lactose as a carbon and energy source by *E. coli*. The enzymes for using lactose are

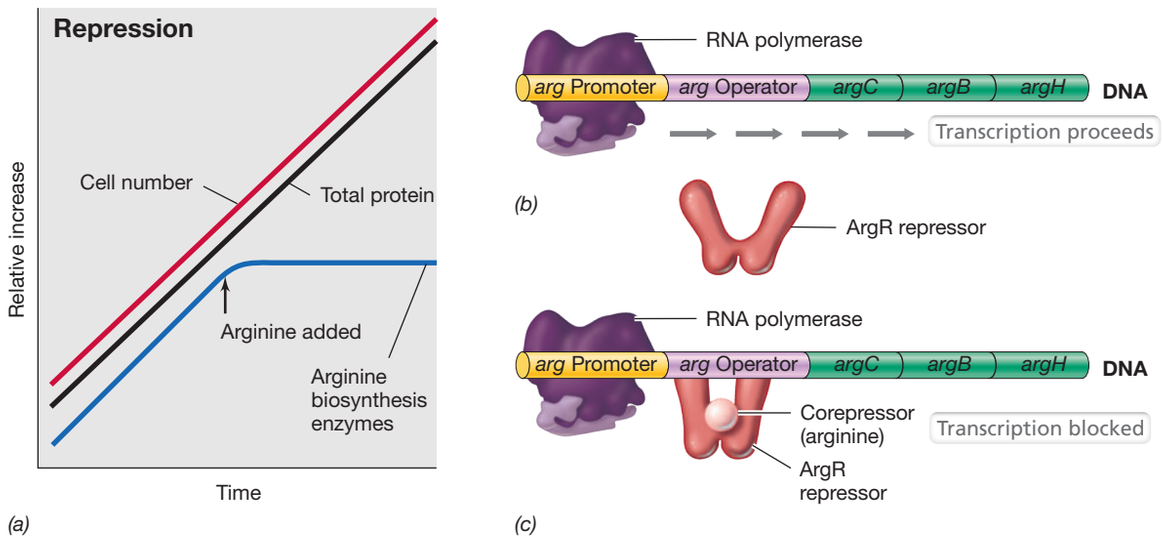


Figure 7.5 Enzyme repression and expression of the arginine operon. *(a)* In a growing bacterial culture, the addition of arginine to the medium specifically represses production of enzymes needed to make arginine. Net protein synthesis is unaffected. *(b)* The operon is transcribed because the repressor ArgR is unable to bind to the operator. *(c)* After the corepressor for the *argCBH* operon, the amino acid arginine, binds to the repressor, the repressor binds to the operator and blocks transcription; mRNA and the proteins it encodes are not made.

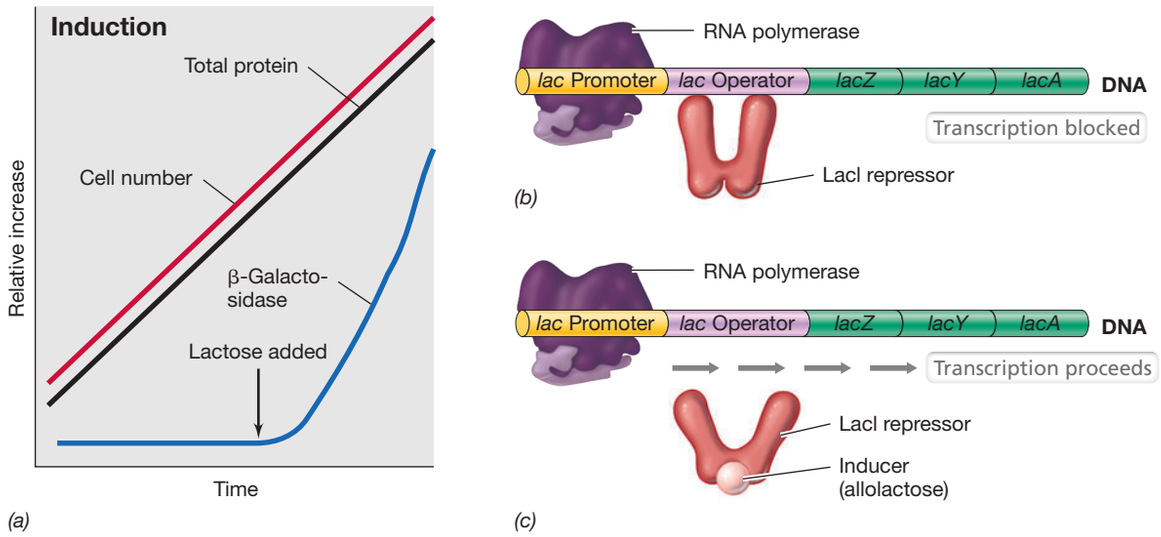


Figure 7.6 Enzyme induction and expression of the lactose operon. *(a)* In a growing bacterial culture, the addition of lactose to the medium specifically induces synthesis of the enzyme β-galactosidase and other proteins of lactose catabolism. Net protein synthesis is unaffected. *(b)* Binding of the LacI repressor protein to the operator blocks the activity of RNA polymerase. *(c)* Binding of the inducer molecule to the LacI repressor inactivates LacI so that it no longer can bind to the operator. This type of inducer binding results in derepression because RNA polymerase can now transcribe an mRNA for that operon. For the *lac* operon, the sugar allolactose rather than lactose is the actual inducer that binds to the LacI repressor.

encoded by the *lac* operon (**Figure 7.6**). Figure 7.6*a* shows the induction of β-galactosidase, the enzyme that cleaves lactose into glucose and galactose. This enzyme is required for *E. coli* to grow on lactose. If lactose is absent, the enzyme is not made, but synthesis begins almost immediately after lactose is added. The three genes in the *lac* operon encode three proteins, including β-galactosidase, that are induced simultaneously upon adding lactose. Such enzyme induction ensures that specific enzymes are synthesized only when needed.

Mechanisms of Repression and Derepression

As discussed in Section 7.2, repressor proteins that bind to DNA turn off transcription, and if a repressor binds DNA only in the presence of its effector, the effector is considered a corepressor. Figure 7.5*b* shows transcription of the arginine genes, which proceeds when the cell needs arginine. However, when arginine is plentiful, it becomes a corepressor. As Figure 7.5*c* shows, arginine binds to a specific repressor protein, the *arginine repressor* (ArgR), present in the cell. Binding of the corepressor to ArgR results in an allosteric change in this protein that allows it to bind to the operator. In this way, transcription is physically blocked because RNA polymerase can neither bind nor proceed, and in the absence of *arg* mRNA, the corresponding polypeptides encoded by the *arg* operon cannot be synthesized. If the mRNA is polycistronic (◀ Section 6.5), as the *arg* operon is, all the polypeptides encoded by this mRNA will be repressed (Figure 7.5*c*).

In contrast to the control of the *arg* operon, some repressor proteins actively bind to operator regions on the DNA in the *absence* of their effector molecule. Figure 7.6*b* shows the repression of the *lac* operon—an example of an inducible operon—by the binding of the lactose repressor (LacI) to the operator region. This binding of LacI completely blocks transcription. If the LacI effector molecule is present, it combines with the repressor protein, causing an allosteric change that prevents LacI from binding DNA; when this occurs, inhibition is overcome and transcription can proceed (Figure 7.6*c*). Because control of inducible operons is the opposite of repression, it is also called *derepression*. The corresponding effector molecule (corepressor) in this case is called an *inducer* as its presence leads to inactivation of the repressor and ultimately enzyme synthesis.

Mastering Microbiology
Activities: Microbiology Animation: Operons: Induction

For the *lac* operon example in Figure 7.6*c*, allolactose, an isomer made from lactose, is the actual inducer molecule that leads to the expression of the *lac* operon and ultimately synthesis of the enzyme β-galactosidase (encoded by *lacZ*). However, isopropylthiogalactoside (IPTG), a nonmetabolizable analog of allolactose that also induces the *lac* operon, is often used in the laboratory for detailed studies of lactose utilization and β-galactosidase activity in *E. coli*.

All regulatory systems employing repressors have the same underlying mechanism: prevention of mRNA synthesis by the activity of specific repressor proteins that are themselves under the control of specific small effector molecules. And, as previously noted, because the repressor's role is to stop transcription, regulation by repressors is called *negative control*. Another point to note is that genes are not turned on and off completely like light switches. DNA-binding proteins vary in concentration and affinity and thus control is quantitative. Even when a gene is "fully repressed" there is often a very low level of basal transcription.

Mechanisms of Activation

In contrast to repressor proteins binding and preventing transcription of DNA, some operons are transcribed only if a specific activator protein is first bound to the DNA (**Figure 7.7**). Activator protein binding is necessary in these cases because the promoter sequences in these operons are poor matches to consensus promoter sequences (◀ Section 6.5) and hence bind RNA polymerase only weakly. Thus, even with the correct sigma (σ) factor, the RNA polymerase has difficulty recognizing these promoters. And, in contrast to the case where operons are under repressor control (Figures 7.5 and 7.6), genes controlled

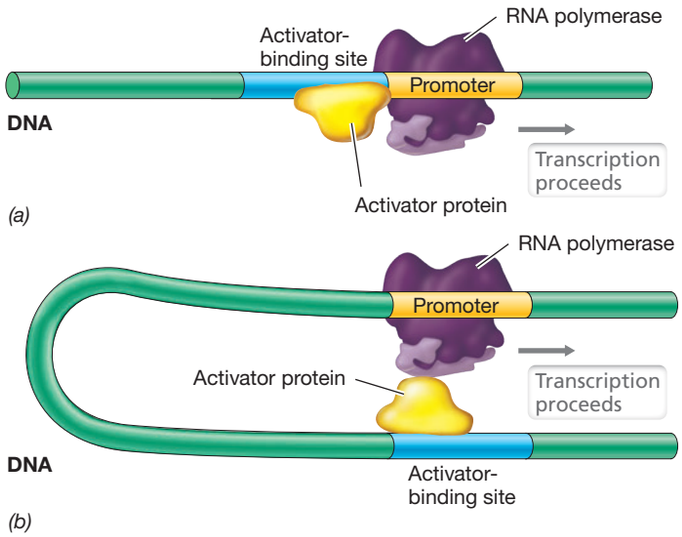


Figure 7.7 Activator protein interactions with RNA polymerase. *(a)* The activator-binding site is near the promoter. *(b)* The activator-binding site is several hundred base pairs from the promoter. In this case, the DNA must be looped to allow the activator and the RNA polymerase to interact.

by activator proteins are under *positive control* because the role of activator proteins is to facilitate rather than block transcription.

The function of activator proteins is to help the RNA polymerase recognize the promoter and begin transcription. For example, the activator protein may modify the structure of the DNA by bending it (**Figure 7.8**), allowing the RNA polymerase to make necessary contacts with nucleotides in the promoter region to begin transcription. Alternatively, the activator protein may interact directly with the RNA polymerase. This can happen either when the activator-binding site is close to the promoter (Figure 7.7*a*) or when it is several hundred base pairs away from the promoter, a situation in which DNA looping is required to make the necessary contacts between protein and nucleic acid (Figure 7.7*b*).

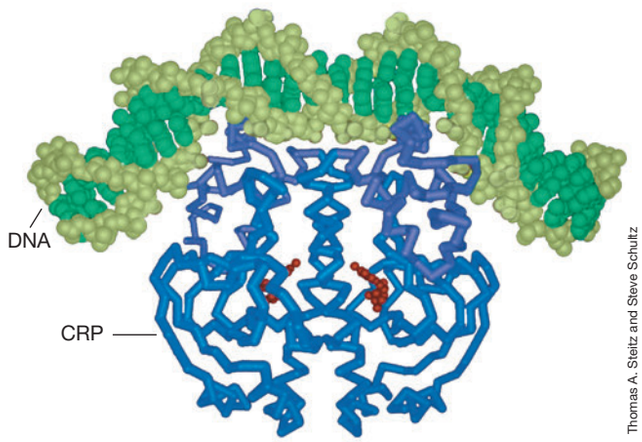


Figure 7.8 Computer model of a positive regulatory protein interacting with DNA. This model shows the cyclic AMP receptor protein (CRP), a regulatory protein that controls several operons. The α-carbon backbone of this protein is shown in blue and purple, while residues that bind cyclic AMP are shown in red. The protein is binding to a DNA double helix (shades of green). Note that binding of the CRP protein to DNA has bent the DNA.

An example of activation is the regulation of the maltose operon in *Escherichia coli*, which encodes the enzymes necessary for the catabolism of the disaccharide sugar maltose. This operon possesses a maltose activator protein called MalT that cannot bind to the DNA unless it first binds to its effector molecule, maltose (**Figure 7.9**). Thus, maltose is also considered an inducer molecule because the enzymes for maltose catabolism in *E. coli* are synthesized only after the addition of maltose to the medium. This follows the pattern shown for β-galactosidase in Figure 7.6*c* except that maltose rather than lactose is required to induce gene expression. When the maltose activator protein binds to DNA, it allows RNA polymerase to begin transcription (Figure 7.9*b*). Like repressor proteins, activator proteins bind specifically to particular sites within the DNA. However, the region on the DNA that is the binding site of the activator is not called an operator (Figures 7.5 and 7.6) but instead an *activator-binding site* (Figures 7.7 and 7.9). Nevertheless, the genes controlled by this activator-binding site are still called an operon.

Operons versus Regulons

In *E. coli*, the genes required for maltose utilization are spread out over the chromosome in several operons, each of which has an activator-binding site to which a copy of the maltose activator protein can bind (**Figure 7.10**). Therefore, the maltose activator protein actually controls the transcription of more than one operon. When more than one operon is under the control of a single regulatory protein, these operons are collectively called a **regulon**. Thus, the enzymes for maltose utilization are encoded by the maltose regulon. A set of operons whose promoters are recognized only by a specific alternative sigma (σ) factor (◀Table 6.3) is another example of a positively regulated regulon.

Regulons are known for operons under negative control or repression as well. For example, the arginine biosynthetic enzymes are

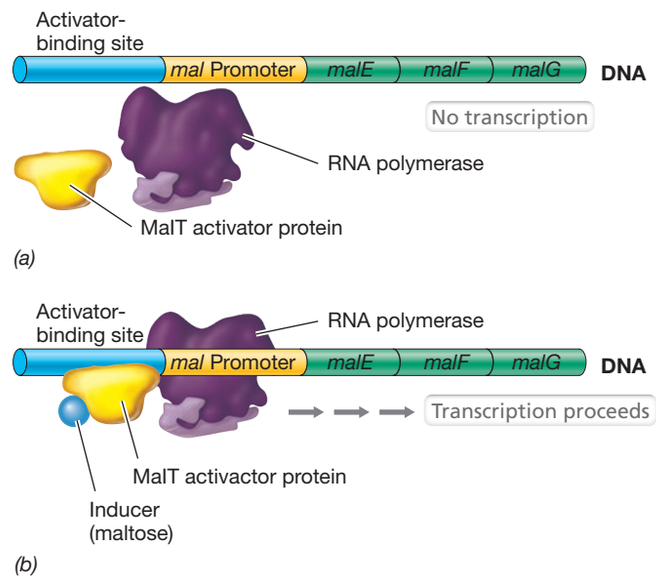


Figure 7.9 Positive control of enzyme induction in the maltose operon. *(a)* In the absence of an inducer, neither the MalT activator protein nor the RNA polymerase can bind to the DNA. *(b)* When the sugar maltose (the inducer of the *malEFG* operon) is present, it binds to the MalT activator protein, which in turn binds to the activator-binding site. This recruits RNA polymerase to bind to the *mal* promoter and begin transcription.

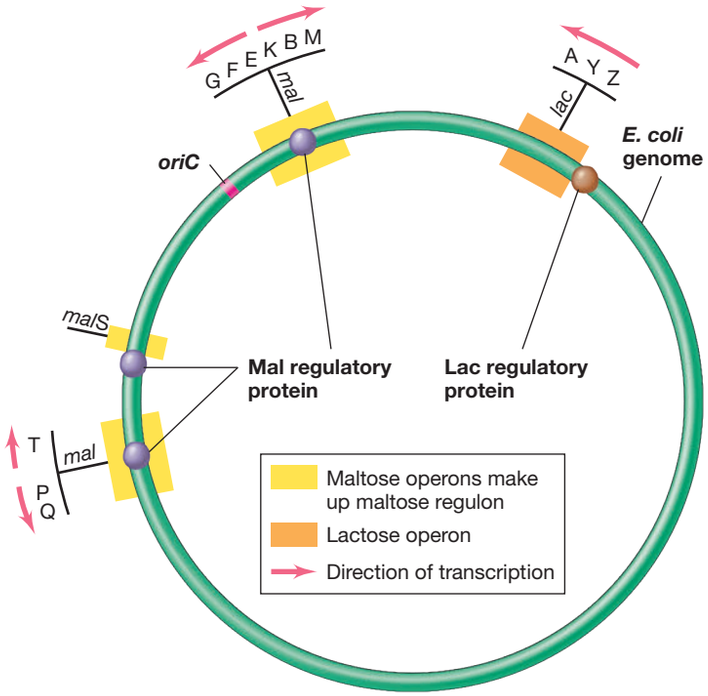


Figure 7.10 Maltose regulon of *Escherichia coli*. The genes and operons required for maltose utilization (*mal*) are dispersed throughout the *E. coli* genome and regulated by the same maltose regulatory protein (MalT). Note that the LacI repressor protein binds only to the *lac* operon, which is only located at one position on the chromosome, while the Mal repressor protein binds to multiple operons (the *mal* regulon).

encoded by the arginine regulon, whose operons are all under the control of the arginine repressor protein (only one of the arginine operons was shown in Figure 7.5). In regulon control, a specific DNA-binding protein binds only at those operons it controls regardless of whether it is functioning as an activator or repressor; other operons are not affected.

Many genes and regulons in *E. coli* have promoters under positive control and many have promoters under negative control. In addition, many operons have promoters with multiple types of control and some have more than one promoter, each with its own control system! Thus, the simple picture outlined above does not hold for all operons. Multiple control features are common in the operons and regulons of virtually all *Bacteria* and *Archaea*, and thus their overall regulation may require a network of interactions.

We now consider transcriptional control in *Archaea*, which has elements that both resemble and differ from those in *Bacteria*.

Check Your Understanding

- Compare and contrast the activities of an activator protein and a repressor protein.
- Distinguish between an operon and a regulon.

7.4 Transcription Controls in *Archaea*

As we have seen, there are two alternative approaches to regulating the activity of RNA polymerase. One strategy, common in *Bacteria*, is to use DNA-binding proteins that either block RNA polymerase activity (repressor proteins) or stimulate RNA polymerase activity (activator proteins). An alternative strategy, common in eukaryotes, is to coordinate the binding of several proteins on the DNA to interact with RNA polymerase. Because these proteins affect the rate at which a gene is transcribed, they are also considered *transcription factors* (Section 7.2). Given the greater overall similarity between the mechanism of transcription in *Archaea* and *Eukarya* (◀ Section 6.6), it is perhaps surprising that the regulation of transcription in *Archaea* more closely resembles that of *Bacteria*.

Few repressor or activator proteins from *Archaea* have been characterized in the detail of the *arg*, *lac*, or *mal* operons of *Escherichia coli* (Figures 7.5, 7.6, 7.9, and 7.10), but it is clear that *Archaea* have both positive and negative control systems. Archaeal repressor proteins either block the binding of RNA polymerase itself or block the binding of TBP (TATA-binding protein) and TFB (transcription factor B), proteins that are required for RNA polymerase to bind to the promoter in *Archaea* (◀ Section 6.6). At least some archaeal activator proteins function in just the opposite way, by recruiting TBP to the promoter, thereby facilitating transcription.

Control of Nitrogen Assimilation in *Archaea*

A well-studied example of an archaeal repressor is the NrpR protein from the methanogen *Methanococcus maripaludis* (▶ Section 17.2). NrpR represses genes encoding nitrogen assimilation functions (**Figure 7.11**), such as those for nitrogen fixation (◀ Section 3.12) and

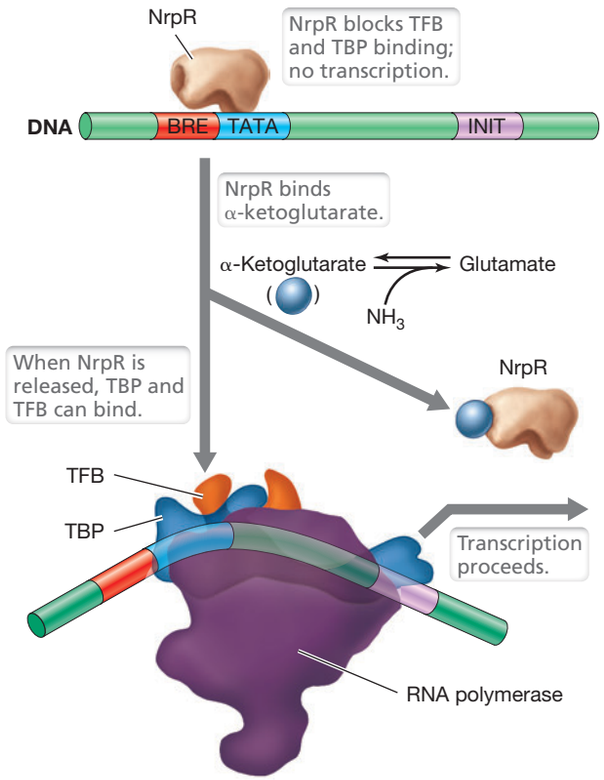


Figure 7.11 Repression of genes for nitrogen metabolism in *Archaea*. The NrpR protein of *Methanococcus maripaludis* functions as a repressor. It blocks the binding of the TFB and TBP proteins, which are required for promoter recognition, to the BRE (B recognition element) site and TATA box, respectively. If there is a shortage of ammonia, α-ketoglutarate is not converted to glutamate. The α-ketoglutarate accumulates and binds to NrpR, releasing it from the DNA and allowing TBP and TFB to bind. The latter promotes RNA polymerase binding and transcription of the operon.

synthesis of the amino acid glutamine. When organic nitrogen is plentiful in the *M. maripaludis* cell, NrpR represses nitrogen assimilation genes. However, if the level of organic nitrogen becomes limiting, the compound α-ketoglutarate accumulates to high levels. This occurs because α-ketoglutarate, a citric acid cycle intermediate, is also a major acceptor of ammonia during nitrogen assimilation (◀ Section 3.14).

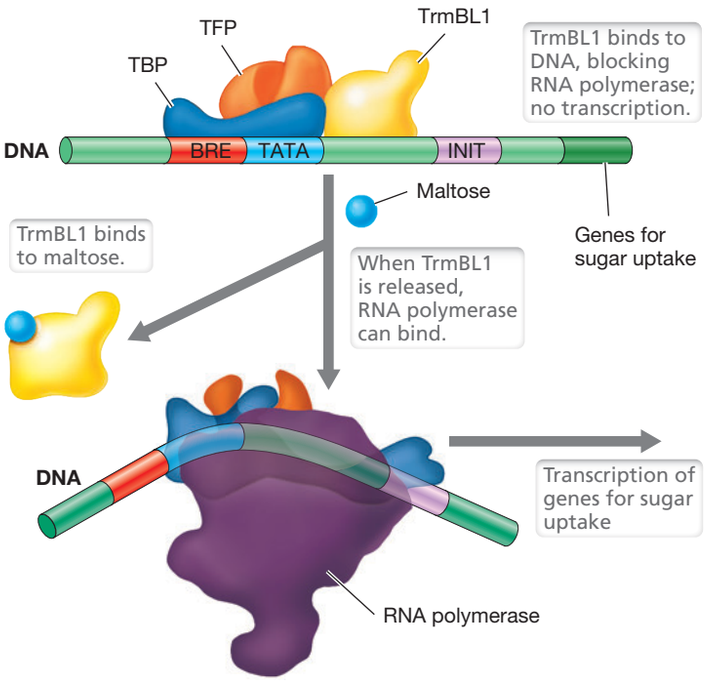
When levels of α-ketoglutarate rise, this signals the cell that ammonia is limiting and that additional pathways need to be activated for obtaining ammonia, such as nitrogen fixation or the activity of glutamine synthetase, the high-affinity nitrogen assimilation enzyme. Elevated levels of α-ketoglutarate function as an inducer by binding to the NrpR protein. In this state, NrpR loses its affinity for the promoter regions of its target genes and no longer blocks transcription from promoters. In this respect, the NrpR protein resembles the LacI repressor and similar-functioning proteins of *Bacteria* (Section 7.3 and Figure 7.6).

Dual-Acting Transcriptional Regulators in *Archaea*

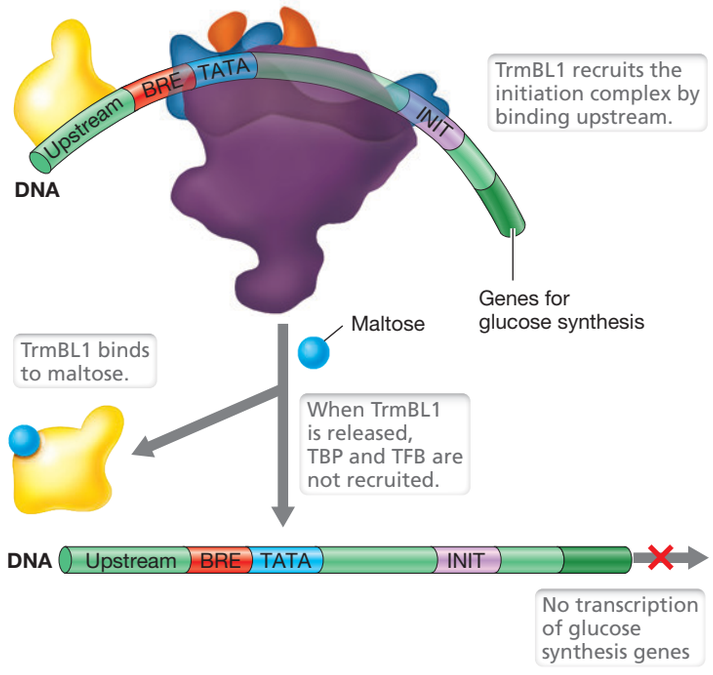
Some archaeal regulators possess *dual functionality* and can function as both a repressor and an activator. For example, the TrmB family of transcriptional regulators that primarily regulate sugar metabolism is widespread in *Archaea*, with more than 250 proteins identified. The actual activity of the allosteric regulator depends on its DNA binding site. In *Pyrococcus furiosus*, a hyperthermophile that does not possess a glucose transporter but can perform glycolysis, TrmBL1 simultaneously functions as a repressor of genes for other sugar transport systems present in this organism and as an activator of genes for glucose synthesis (gluconeogenesis, ◀ Section 3.13).

As a repressor, TrmBL1 binds to a specific DNA sequence *downstream* of the BRE (*B* recognition *e*lement)/TATA binding sites of maltodextrin and maltose/trehalose uptake genes (**Figure 7.12*a***). This binding blocks the binding of RNA polymerase to these sites and thus prevents gene expression. If cellular conditions shift and the inducer sugars maltose, maltotriose, or fructose are present, they bind to TrmBL1, resulting in an allosteric change in the protein such that it can no longer bind DNA. Without TrmBL1 bound to the DNA, RNA polymerase is able to bind to the promoter region and derepression of sugar transporter genes occurs (Figure 7.12*a*).

In other metabolic situations, TrmBL1 can function as an activator protein by binding to a separate and distinct DNA sequence associated with genes for glucose biosynthesis. This regulatory region differs from the one that TrmBL1 binds to as a repressor as it is located *upstream* of the BRE/TATA binding sites (Figure 7.12*b*). Binding of TrmBL1 to this DNA sequence helps recruit the archaeal transcription initiation complex (TBP, TFB, and RNA polymerase, ◀ Section 6.6), thus activating transcription. However, in the same manner as its role as a repressor, TrmBL1 as an allosteric activator will not bind to DNA if the effector molecules maltose, maltotriose, or fructose are present. Without TrmBL1 binding upstream of the BRE/TATA



(a)



(b)

Figure 7.12 Dual functionality of the *Pyrococcus furiosus* TrmBL1 regulator. *Pyrococcus furiosus* is a hyperthermophilic species of the *Euryarchaeota* with a growth temperature optimum of 100°C (▶ Section 17.4). *(a)* The TrmBL1 protein functions as a repressor of genes for sugar uptake. It binds to DNA downstream of the TATA box, blocking the binding of RNA polymerase. Binding of maltose (or another inducer) to TrmBL1 results in release of the regulator from the DNA. Now RNA polymerase can bind and transcribe sugar uptake genes. *(b)* The TrmBL1 protein functions as an activator of genes for glucose synthesis. It binds to a sequence upstream of the promoter region and recruits the transcription initiation complex. This in turn results in transcription of glucose synthesis genes. The presence of maltose results in release of TrmBL1 from the DNA. Without TrmBL1 bound to the DNA, TBP and TFB are not recruited and glucose synthesis genes are not transcribed. The DNA polymerase from *P. furious* is a valuable tool in biotechnology (▶ Section 12.1).

sequences and activating transcription, RNA polymerase and the other needed initiation factors cannot be recruited for transcription of genes necessary for glucose synthesis. This dual-functioning control conserves energy by preventing expression of the gluconeogenesis pathway when other sugars that can be converted into glucose are present in the cell.

The SurR protein of *P. furiosus* is another example of a regulatory protein that functions as both an activator and a repressor, depending on the location of its binding site within the promoter region. SurR controls a catabolic shift of *P. furiosus* from fermentation (which produces H_2) to anaerobic respiration with sulfur (S^0) as electron acceptor, forming H_2S (◀ Section 3.10 and ▶ Section 14.12). When S^0 is *absent*, SurR binds to regulatory regions that activate transcription of genes that encode the enzyme hydrogenase such that *P. furiosus* can grow by fermentation. At the same time, SurR functions as a repressor to prevent transcription of genes encoding proteins that participate in sulfur metabolism. However, when S^0 is *present*, SurR no longer binds to DNA. Interestingly, this inability of SurR to bind is not the result of S^0 binding to SurR as an effector molecule but to the oxidation of cysteine residues within the DNA-binding domain of this regulatory protein that triggers its release from the DNA. This event both activates expression of genes that participate in S^0 metabolism and represses the synthesis of hydrogenase, needed for fermentative metabolism.

Many regulatory mechanisms are triggered by environmental stimuli, and we move on to explore some of the most common ones in Part II of the chapter.

Check Your Understanding

- What is the major difference between transcriptional regulation in *Archaea* and eukaryotes?
- How do transcriptional activators in *Archaea* often differ in mechanism from those in *Bacteria*?
- Explain how the *Pyrococcus furiosus* TrmBL1 transcription regulator is able to act as both an activator and a repressor.

II • Sensing and Signal Transduction

Microbial genomes encode an assortment of multilevel regulatory mechanisms that monitor the environment for changes signaling better (or worse) growth conditions and then trigger a response that best favors survival of the cell.

Prokaryotic cells regulate cell metabolism in response to many different environmental fluctuations, including changes in temperature, pH, oxygen or nutrient availability, and even to changes in the number of other cells present. To do this, cells receive signals from the environment and transmit them to the specific target to be regulated. Some of these signals are small molecules that enter the cell and function as effectors. However, in many cases the external signal is not transmitted directly to the regulatory protein but instead is detected by a cell surface sensing system that transmits the signal to the rest of the regulatory machinery, a process called **signal transduction**.

7.5 Two-Component Regulatory Systems

Because most signal transduction systems contain two parts, they are called **two-component regulatory systems**. Characteristically, such systems consist of a specific **sensor kinase protein**, usually located in the cytoplasmic membrane, and a **response regulator protein**, present in the cytoplasm.

A kinase is an enzyme that phosphorylates compounds, typically using phosphate from ATP. Sensor kinases detect a signal from the environment and phosphorylate themselves (a process called *autophosphorylation*) at a specific histidine residue on the protein (**Figure 7.13**). Sensor kinases thus belong to the class of enzymes called *histidine kinases*. The phosphate is then transferred from the sensor to another protein inside the cell, the response regulator. The latter is typically a DNA-binding protein that regulates transcription in either a positive or a negative fashion (Section 7.3). In the example shown in Figure 7.13, regulation is negative; the phosphorylated response regulator functions as a repressor that binds DNA, thereby blocking transcription. Once dephosphorylated, the response regulator is released and transcription is permitted.

For a balanced regulatory system to work properly, it must have a *feedback loop*, that is, a way to complete the regulatory circuit and terminate the response. This resets the system for another cycle. This feedback loop employs a *phosphatase*, an enzyme that removes the phosphate from the response regulator at a constant rate. The response regulator itself often catalyzes this reaction, although in

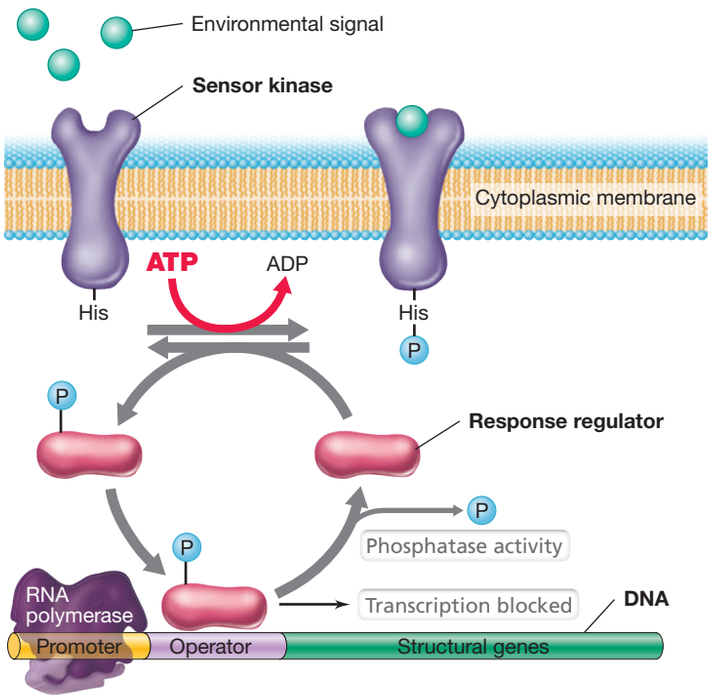


Figure 7.13 The control of gene expression by a two-component regulatory system. One component is a sensor kinase in the cytoplasmic membrane that phosphorylates itself in response to an environmental signal. The phosphoryl group is then transferred to the second component, a response regulator. The phosphorylated form of the response regulator then binds to DNA. In the system shown here, the phosphorylated response regulator is a repressor protein. The phosphatase activity of the response regulator slowly releases the phosphate from the response regulator and resets the system.

TABLE 7.1 Examples of two-component systems that regulate transcription in *Escherichia coli*

System	Environmental signal	Sensor kinase	Response regulator	Primary activity of response regulator[a]
Arc system	Oxygen	ArcB	ArcA	Repressor/activator
Nitrate and nitrite respiration (Nar)	Nitrate and nitrite	NarX NarQ	NarL NarP	Activator/repressor Activator/repressor
Nitrogen utilization (Ntr)	Shortage of organic nitrogen	NRII (=GlnL)	NRI (=GlnG)	Activator of promoters requiring RpoN/σ^{54}
Pho regulon	Inorganic phosphate	PhoR	PhoB	Activator/repressor
Porin regulation	Osmotic pressure	EnvZ	OmpR	Activator/repressor

[a]Note that many response regulator proteins act as both activators and repressors depending on the genes being regulated. Although ArcA can function as either an activator or a repressor, it functions as a repressor on most operons that it regulates.

some cases separate proteins are needed (Figure 7.13). Phosphatase activity is typically slower than kinase (phosphorylation) activity. However, if phosphorylation ceases due to reduced sensor kinase activity, phosphatase activity eventually returns the response regulator to the fully nonphosphorylated state, and the system is reset.

Examples of Two-Component Regulatory Systems

Two-component systems regulate a large number of genes in many different *Bacteria*, and some key examples include those that respond to phosphate limitation, nitrogen limitation, and osmotic pressure; we focus on the latter two systems here.

In *Escherichia coli* almost 50 different two-component systems are present, and several are listed in **Table 7.1**. In one example, the osmolarity (dissolved solute concentration) of the environment controls the relative levels of the proteins OmpC and OmpF in the *E. coli* outer membrane. OmpC and OmpF are *porins*, proteins that allow metabolites to cross the outer membrane of gram-negative bacteria (◀ Section 2.4). If osmotic pressure is *low*, the synthesis of OmpF, a porin with a larger pore, increases; if osmotic pressure is *high*, OmpC, a porin with a smaller pore, is made in larger amounts.

EnvZ, a sensor histidine kinase in the *E. coli* cytoplasmic membrane, detects changes in osmotic pressure. When a shift occurs, EnvZ is autophosphorylated and transfers its phosphate group to OmpR, the response regulator of this system (**Figure 7.14*a***). Under conditions of *low* osmotic pressure, phosphorylated OmpR (OmpR-P) *activates* transcription of the *ompF* gene. Conversely, when osmotic pressure is *high*, OmpR-P *represses* transcription of *ompF* and simultaneously activates transcription of *ompC* (Figure 7.14*a*). The expression of *ompF* is also regulated by an additional control mechanism—regulatory RNA—and we consider this additional level of regulation in Section 7.12.

Some signal transduction systems have a secondary regulatory element that is *not* RNA-based, and their activities can quickly become quite complex. Such is the case with the regulation of nitrogen assimilation in *Bacteria*. For instance, in the Ntr regulatory system (Figure 7.14*b*), the response regulator is an activator protein called *nitrogen regulator I* (*NRI*). In the phosphorylated state, NRI-P oligomerizes and binds to an activator-binding site over 100 base pairs upstream of the promoter region for nitrogen assimilation genes (Figure 7.14*b*). This binding causes the DNA to loop around such that NRI-P can make contact with RNA polymerase and the alternative sigma factor σ^{54} (RpoN) (◀ Section 6.5); this activates transcription of genes that encode key nitrogen assimilation enzymes (Figure 7.7*b*). Phosphorylation of NRI is controlled by the sensor kinase of the Ntr system called *nitrogen regulator II* (*NRII*), which senses ammonia levels in the cell. However, NRII can switch from *kinase* activity to *phosphatase* activity depending on the nitrogen status of the cell (Figure 7.14*b*).

The dual enzymatic activity of NRII is controlled by the state of another protein called *PII*, a signal-transducing protein we will visit later when we consider regulation of an important ammonia assimilation protein, glutamine synthetase (Section 7.16). Under nitrogen starvation conditions, a nucleotide called uridine monophosphate (UMP) is added to PII, forming PII–UMP (Section 7.16). If PII–UMP is present in the cell, it stimulates the kinase activity of NRII. Thus the presence of PII-UMP leads to NRI-P and activation of genes encoding enzymes of nitrogen assimilation (Figure 7.14*b*). Conversely, if nitrogen is present in excess in the cell, UMP is removed from PII. PII in its unmodified form promotes the phosphatase activity of NRII, which results in unphosphorylated NRI. Without phosphorylation of NRI, transcriptional activation of nitrogen assimilation genes does not occur (Figure 7.14*b*). This type of regulation in which a hierarchy of control systems are deployed in a cascading fashion is common for systems of central importance to cellular metabolism.

Although rare in *Archaea*, two-component systems closely related to those in *Bacteria* are also present in microbial eukaryotes, such as the yeast *Saccharomyces cerevisiae*, and even in plants. However, most eukaryotic signal transduction pathways are distinct systems that phosphorylate serine, threonine, or tyrosine residues of proteins and are unrelated to those of the bacterial two-component systems that phosphorylate histidine residues (Figures 7.13 and 7.14*a*).

Check Your Understanding

- What are kinases and what is their role in two-component regulatory systems?
- What are phosphatases and what is their role in two-component regulatory systems?

7.6 Regulation of Chemotaxis

We have previously seen that some motile cells of *Bacteria* and *Archaea* respond to challenges such as nutrient limitation and toxin accumulation by moving toward attractants and away from

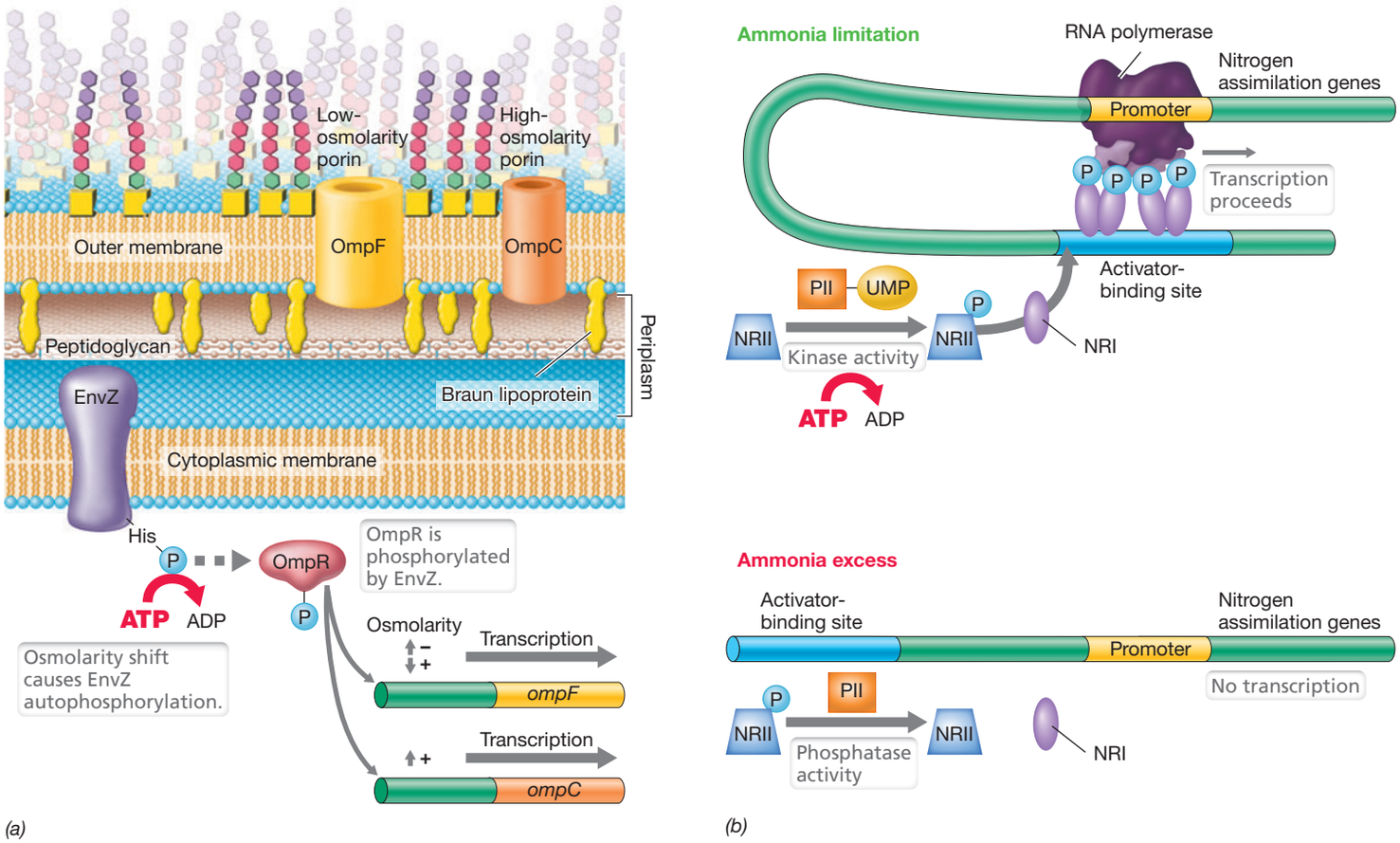


Figure 7.14 **Two examples of well-studied two-component systems in *Escherichia coli*.** *(a)* Regulation of synthesis of outer membrane proteins. The cytoplasmic membrane histidine kinase EnvZ autophosphorylates itself under osmotic pressure changes and then activates the transcriptional regulator OmpR by phosphorylation. OmpR-P binds upstream of the *ompF* gene and activates transcription under low osmotic pressure; conversely, it represses transcription of *ompF* under high osmotic pressure. OmpR-P only activates transcription of the *ompC* gene under conditions of high osmolarity. *(b)* Regulation of ammonia assimilation by the Ntr system. NRII is the sensor kinase and NRI is the response regulator. Genes encoding nitrogen assimilation proteins are only transcribed when cellular levels of ammonia are low.

repellents, a behavior called *chemotaxis* (◀ Section 2.11). We noted there that prokaryotic cells are too small to sense spatial gradients of a chemical, but they can respond to temporal gradients. That is, they can sense the *change* in concentration of a chemical over time rather than the absolute concentration of the chemical stimulus. Chemotactic activity of the opportunistic pathogen *Pseudomonas aeruginosa* scavenging nutrients from damaged epithelial cells is shown visually in **Figure 7.15**.

Chemotaxis has been well studied in *Bacteria*, which use a modified two-component regulatory system (Section 7.5) to sense temporal changes in attractants or repellents and process this information to regulate the direction of flagellar rotation. This differs from what was considered in the previous section in that the two-component system is used to regulate the activity of *preexisting proteins* (the flagellum protein complex) rather than to control the transcription of genes encoding flagellar proteins.

Response to Signal

The mechanism of chemotaxis depends upon a signal cascade of multiple proteins. Sensory proteins sense attractants or repellents and interact with cytoplasmic sensor kinases. These sensory proteins are called *methyl-accepting chemotaxis proteins* (*MCPs*). MCPs allow the cell to monitor the concentration of various substances over time. Thousands of MCPs cluster to form large hexagonal arrays of proteins known as chemoreceptors. Depending on the microbe, these chemoreceptors can be located in the cytoplasmic membrane and/or cytoplasm. *Vibrio* species, which are often associated with coastal marine waters, contain both transmembrane and cytoplasmic chemoreceptors for sensing (**Figure 7.16**), whereas *Escherichia coli* only contains transmembrane chemoreceptors.

E. coli possesses four transmembrane chemoreceptors, each composed of five different MCPs that are specific for certain compounds. For example, the Tar MCP of *E. coli* senses the attractants aspartate and maltose and the repellents cobalt and nickel. MCPs bind attractants or repellents directly or in some cases indirectly through interactions with periplasmic binding proteins (◀ Sections 2.2 and 2.4), which allows for a wider range of signals to be sensed.

MCP binding of an attractant or repellent triggers interactions with the cytoplasmic proteins CheA and CheW (**Figure 7.17**). CheA is the sensor kinase (Section 7.5) for chemotaxis. When MCPs bind to an attractant *or* release a repellent, CheW is inactive and phosphorylation of CheA is inhibited (Figure 7.17*a*). Thus, an increase

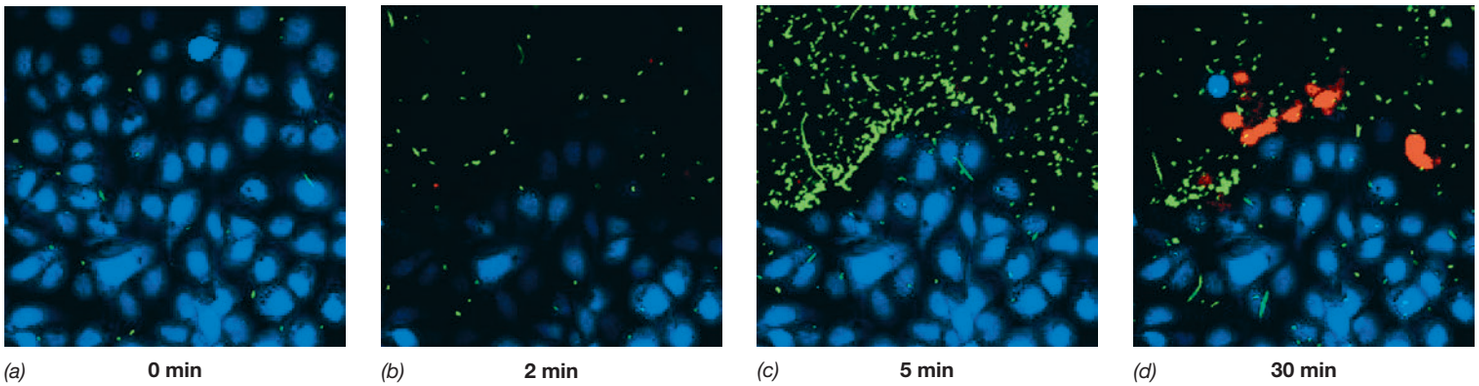


Figure 7.15 Chemotaxis of *Pseudomonas aeruginosa* cells toward nutrients from damaged epithelial cells. A time series of fluorescence micrographs illustrating epithelial cells (blue: alive; red: dead) and *P. aeruginosa* (green) pre– and post–epithelial cell wounding. *(a)* Before damage of the epithelial layer by scratching. *(b)* Two minutes after scratching the epithelial monolayer, very few bacterial cells are present. *(c)* Five minutes after wound induction, *P. aeruginosa* cells migrate to the damaged epithelial cells to devour nutrients released from the cells. *(d)* After 30 minutes, some of the epithelial cells die (red) and the bacterial hunters move on to scavenge more nutrients from living cells. Images adapted from Schwarzer, C., Fisher, H., and Machen, T.E. 2016. *PLoS ONE 11(3):* e0150109.

in attractant *decreases* the rate of CheA autophosphorylation and the response regulator CheY remains unphosphorylated. When an MCP binds a repellent *or* releases an attractant, it changes conformation and, with help from CheW, leads to the autophosphorylation of CheA to form CheA-P (Figure 7.17*b*). Thus, an increase in repellent concentration *increases* the rate of CheA autophosphorylation. CheA-P then transfers the phosphate to CheY (forming CheY-P); this is the response regulator (Section 7.5) that controls flagellar rotation. As described in the next subsection, CheY-P leads to clockwise flagellar rotation. CheA-P can also transfer the phosphate to a different protein, CheB, which plays a role in adaptation (Figure 7.17), to be detailed shortly.

Controlling Flagellar Rotation

CheY is a central protein in the regulatory system because it governs the direction of rotation of the flagellum. Recall that if rotation of the flagellum is counterclockwise, *Escherichia coli* will continue to move in a run (swim smoothly), whereas if the flagellum rotates clockwise, the cell will tumble (move randomly) (◀ Section 2.11 and Figure 2.33). When MCPs bind a repellent or release an attractant, CheY is phosphorylated. CheY-P interacts with the flagellar motor to induce clockwise flagellar rotation, which causes tumbling (Figure 7.17*b*). When MCPs bind an attractant or release a repellent, CheY is unphosphorylated and cannot bind to the flagellar motor. This results in the flagellum rotating counterclockwise, causing the cell to run (Figure 7.17*a*). Another protein, CheZ, dephosphorylates CheY, returning it to the form that allows runs instead of tumbles. Thus an increase in repellent concentration leads to an increase in the level of CheY-P and therefore tumbling. By contrast, if the cell is swimming toward attractants, the lower level of CheY-P suppresses tumbles and promotes runs.

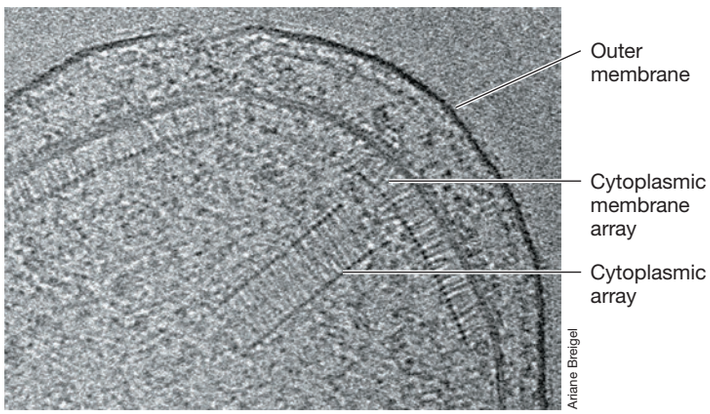


Figure 7.16 *Vibrio* chemoreceptor arrays. Electron tomograph of a cell of *Vibrio cholerae* showing membrane-bound and cytoplasmic chemoreceptor arrays.

Adaptation

Once an organism has successfully responded to a stimulus, it must stop responding and reset the sensory system to await further signals. This is known as *adaptation*. During adaptation of the chemotaxis system, a feedback loop resets the system. This relies on the response regulator CheB, mentioned earlier.

As their name implies, MCPs can be methylated. When MCPs are fully methylated they no longer respond to attractants but are sensitive to repellents. Conversely, when MCPs are unmethylated they respond strongly to attractants but are insensitive to repellents. Varying the methylation level thus allows adaptation to sensory signals. This is accomplished by methylation and demethylation of the MCPs. CheR is the chemotaxis protein that methylates MCPs, while phosphorylated CheB (CheB-P) is the protein that demethylates MCPs (Figure 7.17). Note that CheA-P donates a phosphate group not only to CheY as described above, but to CheB as well (Figure 7.17*b*).

If the level of an attractant remains high, the rate of CheA autophosphorylation is low. This leads to unphosphorylated CheY and CheB (Figure 7.17). Consequently, the cell swims smoothly in a run. Methylation of the MCPs increases during this period because CheB-P is not present to demethylate them. However, MCPs no longer respond to the attractant when they become fully methylated.

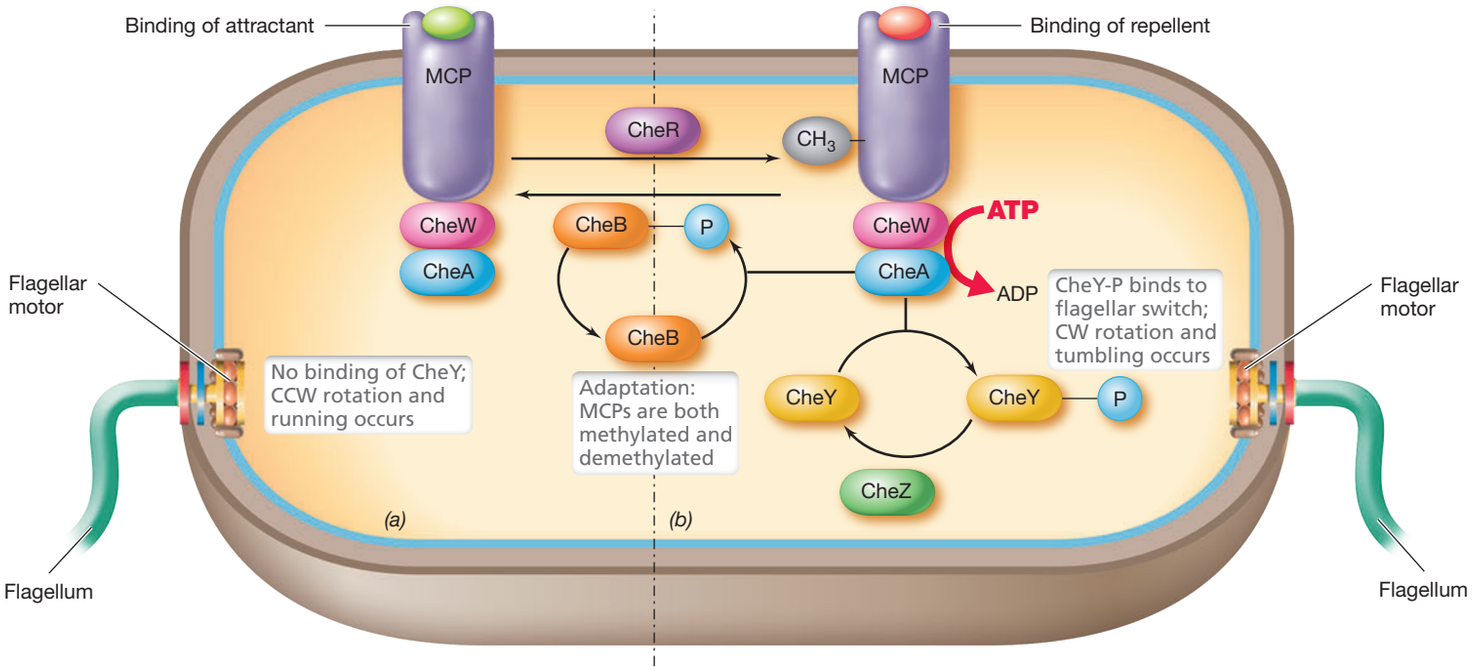


Figure 7.17 Mechanism of chemotaxis in *Escherichia coli*. Methyl-accepting chemotaxis proteins (MCPs) within chemoreceptor arrays form a complex with the sensor kinase CheA and the coupling protein CheW. *(a)* Binding of an attractant to MCP (or an empty MCP) inhibits CheA kinase activity. Without phosphorylation of CheY, the flagellar switch continues in a counterclockwise (CCW) rotation and cell running toward the attractant occurs. *(b)* Binding of a repellent to MCP stimulates CheA, which can then phosphorylate the response regulators CheB and CheY. Binding of phosphorylated CheY (CheY-P) to the flagellar motor switch results in clockwise (CW) rotation and cell tumbling. CheZ is responsible for dephosphorylating CheY-P so that the response can continue or cease depending on the activity of CheA. CheR continually adds methyl groups to the MCP, while CheB-P (but not CheB) removes them. The degree of methylation of the MCPs controls their ability to respond to attractants and repellents and leads to adaptation.

Therefore, if the level of attractant remains high but constant, the cell begins to tumble. Eventually, CheB becomes phosphorylated and CheB-P demethylates the MCPs. This resets the receptors, allowing them to once again respond to further increases or decreases in the level of attractants. Put another way, the cell stops swimming if the attractant concentration is constant. It only continues to swim if ever higher levels of attractant are encountered. The course of events is just the opposite for repellents. Fully methylated MCPs respond best to an increasing gradient of repellents and send a signal for cell tumbling to begin. The cell then moves off in a random direction while MCPs are slowly demethylated. With this mechanism for adaptation, chemotaxis successfully achieves the ability to monitor small changes in the concentrations of both attractants and repellents over time.

Other Taxes

In addition to chemotaxis, several other forms of taxis are known, for example, *phototaxis* (movement toward light) and *aerotaxis* (movement toward oxygen) (◀ Section 2.12). Interestingly, many of the cytoplasmic Che proteins that function to control flagellar activity in chemotaxis also play a role in these other taxes. For example, in phototaxis, a light sensor protein replaces the MCPs of chemotaxis, and in aerotaxis, a redox protein monitors levels of oxygen. These sensors then interact with cytoplasmic Che proteins to begin the cascade of events that direct runs or tumbles. Thus several different kinds of environmental signals converge on the same flagellar control system, and this allows the cell to economize on its regulatory systems.

Check Your Understanding

- What are the primary response regulator and the primary sensor kinase for regulating chemotaxis?
- Why is adaptation during chemotaxis important?
- How does the response of the chemotaxis system to an attractant differ from its response to a repellent?

7.7 Cell-to-Cell Signaling

Populations of *Bacteria* and *Archaea* are able to communicate with one another through the production of small extracellular molecules. Depending on the cell, these molecules can be either small peptides or nonpeptide organic molecules. Accumulation of these signaling molecules often leads to coordinated group behaviors such as biofilm formation (◀ Section 4.9 and ▶ Section 8.10), and because accumulation is essential, the density of cells producing specific signaling molecules in a local environment ultimately controls such pathways. This regulatory phenomenon is called **quorum sensing** (the word "quorum" in this sense meaning "sufficient numbers").

Mechanism of Quorum Sensing

Quorum sensing is a regulatory mechanism that assesses population density. Many bacteria use this approach to ensure that sufficient cell numbers of their own species are present before initiating activities that require a certain cell density to work effectively. For

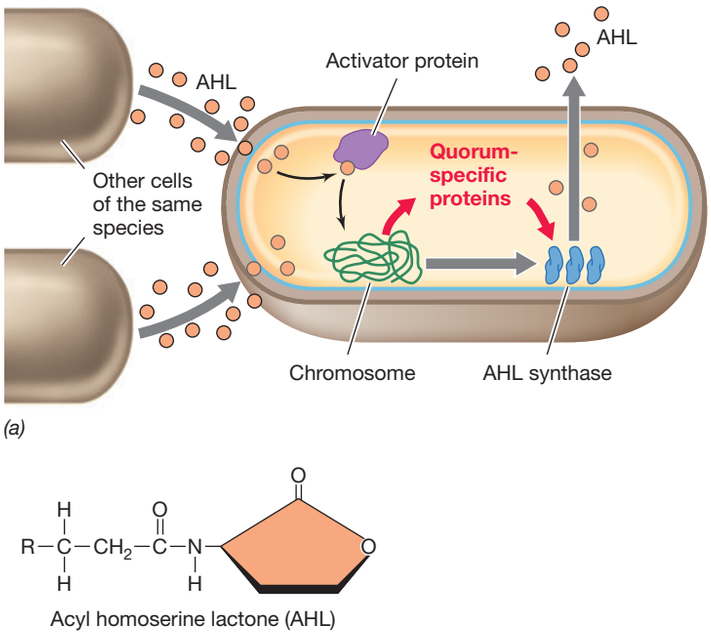


Figure 7.18 **Quorum sensing.** *(a)* A cell capable of quorum sensing expresses acyl homoserine lactone (AHL) synthase at basal levels. This enzyme makes the cell's specific AHL. When cells of the same species reach a certain density, the concentration of AHL rises sufficiently to bind to the activator protein, which activates transcription of quorum-specific genes. *(b)* General structure of an AHL. Different AHLs are variants of this parent structure. R = alkyl group (C_1–C_{17}); the carbon next to the R group is often modified to a keto group (C=O).

example, a pathogenic bacterium that secretes a toxin will have no effect as a single cell; production of toxin by one cell alone would merely waste the cell's resources. However, if a sufficiently large population of cells is present, the coordinated expression of the toxin may successfully cause disease and release resources from the host that can be used to support growth of the pathogen.

Mastering Microbiology
Art Activity: Figure 7.20 Quorum sensing

Quorum sensing is widespread among gram-negative *Bacteria* but is also found in many gram-positive species and in some *Archaea*. Each species that employs quorum sensing produces a specific signal molecule called an **autoinducer** (Figure 7.18). This molecule typically diffuses freely across the cell envelope in either direction. Because of this, the autoinducer reaches high concentrations inside the cell only if many cells are nearby, each making the same autoinducer. In the cytoplasm, the autoinducer binds to a specific transcriptional activator protein or a sensor kinase of a two-component system (Sections 7.3–7.5), ultimately triggering transcription of specific genes (Figure 7.18*a*).

While several different classes of autoinducers exist, the first to be identified were the *acyl homoserine lactones* (AHLs) (Figure 7.18*b*). Several different AHLs, with acyl groups (functional groups containing both carbonyl and alkyl groups) of different lengths, are found in different species of gram-negative bacteria. In addition, many gram-negative *Bacteria* make autoinducers such as AI-2 (a cyclic furan derivative) that can be used between many different species, thus allowing more widespread gram-negative interspecies communication. Gram-positive *Bacteria* and some *Archaea* generally use certain small peptides as autoinducers.

The phenomenon of quorum sensing was discovered as the mechanism by which light emission in bioluminescent bacteria is regulated (▶ Sections 16.4 and 23.10). Several bacterial species can emit light, including the marine bacterium *Aliivibrio fischeri*. **Figure 7.19** shows bioluminescent colonies of *A. fischeri*. The light is generated by an enzyme called *luciferase*. The *lux* operons encode the proteins needed for bioluminescence and are under control of the activator protein LuxR. The *lux* operons are induced when the concentration of the specific *A. fischeri* AHL, *N*-3-oxohexanoyl homoserine lactone, becomes high enough. When this occurs, LuxR binds to the *lux* operons and activates their transcription.

Quorum sensing has also been observed in microbial eukaryotes. For example, in the yeast *Saccharomyces cerevisiae*, specific aromatic alcohols are produced as autoinducers and control the transition between growth of *S. cerevisiae* as single cells and as elongated filaments. Similar transitions are seen in other dimorphic fungi (fungi capable of two different morphologies), some of which cause disease in humans. An example is *Candida*, whose quorum sensing is mediated by the long-chain alcohol farnesol. As the concentration of farnesol increases, the transition from budding yeast cells to elongated hyphae is prevented.

Virulence Factors

Various genes are controlled by quorum sensing, including some in pathogenic bacteria. For example, Shiga toxin–producing *Escherichia coli*, such as the notorious foodborne pathogen *E. coli* O157:H7 (▶ Section 33.11), produces an AHL called AI-3 that induces virulence genes. As the *E. coli* population increases in the intestine, bacterial cells produce AI-3 while host intestinal cells produce the stress hormones epinephrine and norepinephrine. All three of these signal molecules bind to two separate sensor kinases in the *E. coli* cytoplasmic membrane, resulting in the phosphorylation and activation of two transcriptional activator proteins (**Figure 7.20*a***). These proteins

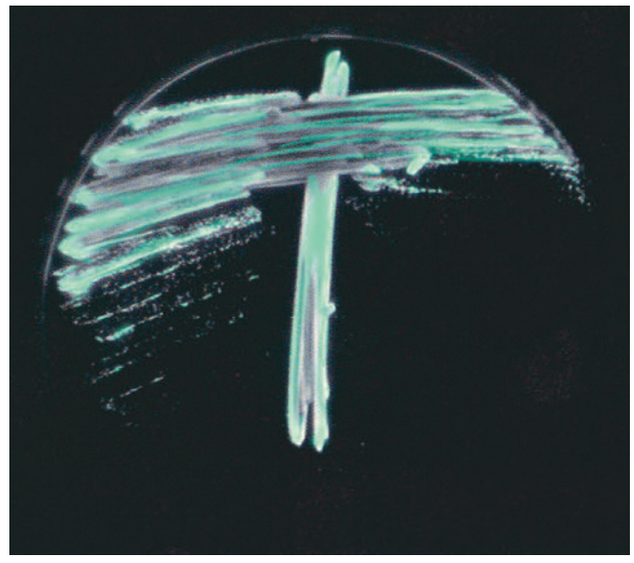
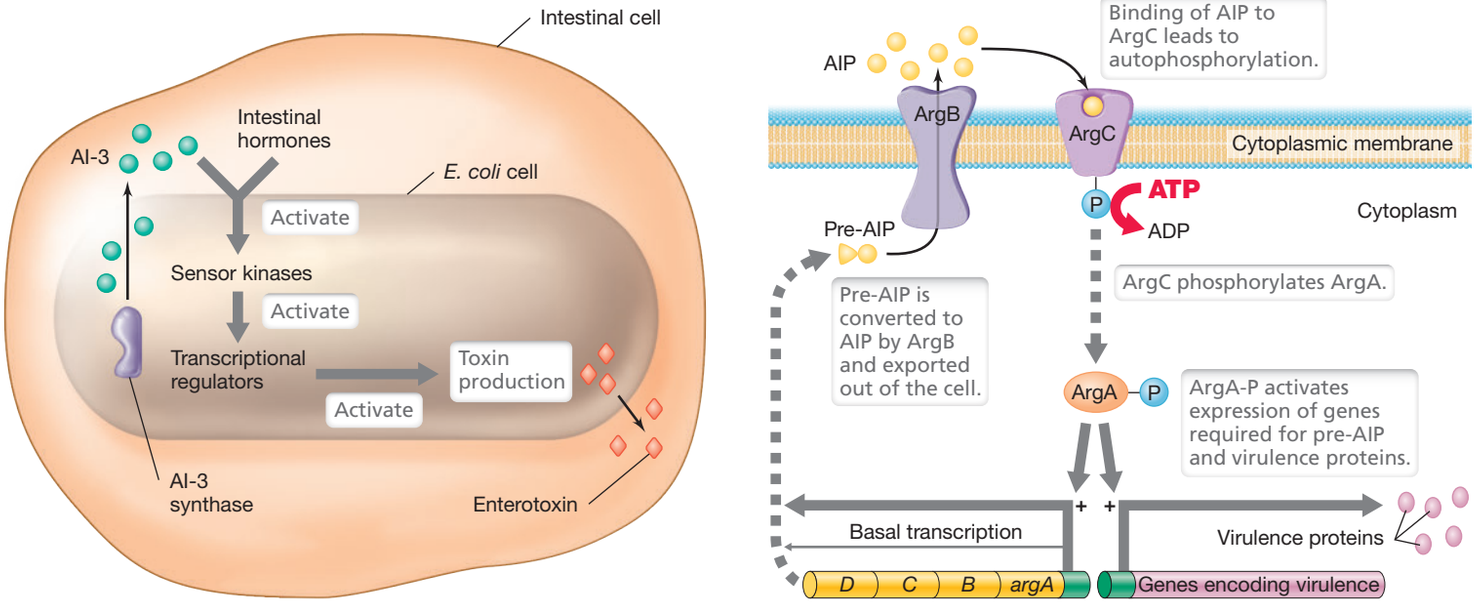


Figure 7.19 **Bioluminescent bacteria producing the enzyme luciferase.** Cells of the bacterium *Aliivibrio fischeri* were streaked on nutrient agar in a Petri dish and allowed to grow overnight. The photograph was taken in a darkened room using only the light generated by the bacteria.



(a) **Virulence factor production in Shiga toxin-producing *Escherichia coli***

(b) **Virulence factor production in *Staphylococcus aureus***

Figure 7.20 Quorum sensing regulation of virulence factors in two common bacterial pathogens. *Escherichia coli* and *Staphylococcus aureus* can be harmless saprophytes or potent pathogens, depending on the strain. As pathogens, the production of major virulence factors is controlled by quorum sensing. *(a)* As the bacterial population increases, AI-3 produced by *Escherichia coli* and epinephrine and norepinephrine produced by the intestinal cell accumulate and bind to sensor kinases, initiating a cascade of events necessary for production of virulence factors (for example, enterotoxin). *(b)* Basal level transcription of the *argABCD* operon in *Staphylococcus* leads to production of ArgD, the pre-*auto*inducing *p*eptide (AIP). ArgB trims ArgD into the functional AIP and exports it out of the cell. As the cell population increases, the AIP concentration increases and binds to ArgC, leading to autophosphorylation of ArgC. ArgC-P then activates the transcriptional activator ArgA by transfer of a phosphate group. ArgA-P increases transcription of the *argABCD* operon as well as activating the transcription of an RNA that leads to the production of virulence proteins.

activate transcription of genes encoding motility functions and secretion of the toxin as well as genes encoding proteins that form lesions on the host intestinal mucosa, thus releasing nutrients that can be used by cells of the pathogen. This is a rare example of a system that senses both bacterial and eukaryotic chemical signals to regulate gene expression.

The pathogenesis of the gram-positive bacterium *Staphylococcus aureus* (▶ Section 31.9) requires, among many other things, the production and secretion of small extracellular peptides that damage host cells or that interfere with the host's immune system. The genes encoding these virulence factors are under the control of a quorum-sensing system that uses a small peptide called the *autoinducing peptide* (AIP), encoded by the *argD* gene, as the autoinducer. After synthesis of ArgD (pre-AIP), the membrane-bound ArgB protein trims the peptide into its active AIP form and secretes the small peptide outside of the cell (Figure 7.20*b*). As the cell density of *S. aureus* increases, so does the concentration of AIP. ArgC is a membrane-bound sensor kinase (Section 7.5) that binds to AIP, resulting in autophosphorylation. ArgC-P then transfers its phosphate to the transcriptional activator ArgA. ArgA-P increases transcription of *argABCD* genes that encode the signal transduction system as well as an RNA molecule that controls production of a range of virulence proteins.

Some eukaryotes produce molecules that specifically interfere with bacterial quorum sensing. Most of these found so far have been furanone derivatives containing a halogen atom. These components mimic the AHLs or AI-2 and disrupt bacterial behavior that relies on quorum sensing. Because of this, quorum-sensing disruptors have been proposed as potential drugs to disperse bacterial biofilms (◀ Section 4.9 and ▶ Section 8.10) and prevent the expression of virulence genes.

In the next part we explore mechanisms of controlling large gene sets scattered across the chromosome, something the cell must do to coordinate the expression of genes not linked in a single operon.

Check Your Understanding

- What advantage do quorum-sensing systems confer on bacterial cells?
- What properties are required for a molecule to function as an autoinducer?
- How do the autoinducers used in quorum sensing by gram-negative bacteria differ from those used by gram-positive bacteria?

III • Global Control

Some microbial regulatory systems control the expression of several sets of genes scattered across the genome in order to activate or repress the synthesis of groups of functionally related proteins in a single control event.

An organism often needs to regulate many unrelated genes simultaneously in response to a change in its environment. Regulatory mechanisms that respond to environmental signals by

TABLE 7.2 Examples of global control systems known in *Escherichia coli*[a]

System	Signal	Primary activity of regulatory protein	Number of genes regulated
Aerobic respiration	Presence of O_2	Repressor (ArcA)	> 50
Anaerobic respiration	Lack of O_2	Activator (FNR)	> 70
Catabolite repression	Cyclic AMP level	Activator (CRP)	> 300
Heat shock	Temperature	Alternative sigma factors (RpoH and RpoE)	> 36
Nitrogen utilization	NH_3 limitation	Activator (NRI)/alternative sigma factor (RpoN)	> 12
Oxidative stress	Oxidizing agents	Activator (OxyR)	> 30
SOS response	Damaged DNA	Repressor (LexA)	> 20
General stress response	Stress conditions	Alternative sigma factor (RpoS)	> 400

[a]For many of the global control systems, regulation is complex. A single regulatory protein can play more than one role. For instance, the regulatory protein for aerobic respiration is a repressor for many promoters but an activator for others, whereas the regulatory protein for anaerobic respiration is an activator protein for many promoters but a repressor for others. Regulation can also be indirect or require more than one regulatory protein. Many genes are regulated by more than one global system.

regulating the transcription of many different genes comprising more than one regulon are called *global control systems*. There are several global control systems in *Escherichia coli* (and probably in all *Bacteria* and *Archaea*), and some key ones are listed in **Table 7.2**. Global control networks may include activators, repressors, signal molecules, two-component regulatory systems, regulatory RNA (Section 7.12), and alternative sigma (σ) factors (◀ Section 6.5) as components of the overall control system.

7.8 The *lac* Operon

Both the lactose operon and the maltose regulon respond to global controls in addition to their own controls discussed in Section 7.3. We begin our consideration of global regulation by revisiting the *lac* operon and considering how cells respond when more than one sugar is available.

Catabolite Repression

In its intestinal habitat, *Escherichia coli* likely encounters several different utilizable carbon sources, and for this reason, the organism is genetically equipped to catabolize many different sugars. When given several sugars, including glucose, do cells of *E. coli* use them simultaneously or one at a time? The answer is that *glucose is always used first*. It would be wasteful to induce enzymes for using other sugars when glucose is available because *E. coli* grows faster on glucose than on any other carbon source. **Catabolite repression** is a mechanism of global control that controls the use of carbon sources if more than one is present.

When cells of *E. coli* are grown in a medium that contains glucose, the synthesis of enzymes needed for the breakdown of other carbon sources (such as lactose or maltose) is repressed, even if those other carbon sources are present. Thus, the presence of a favored carbon source represses the induction of pathways that catabolize other carbon sources. Catabolite repression is sometimes called the "glucose effect" because glucose was the first substance shown to cause this response. But catabolite repression is not always linked to glucose; the key point is that the favored substrate is a better carbon and energy source than other available carbon and energy sources.

Why is catabolite repression called *global* control? In *E. coli* and other organisms for which glucose is the best energy source, catabolite repression prevents expression of most other catabolic operons as long as glucose is present. Dozens of catabolic operons are affected, including those for lactose (Figure 7.6), maltose (Figure 7.9), a host of other sugars, and most other commonly used carbon and energy sources for *E. coli*. In addition, genes for the synthesis of flagella are controlled by catabolite repression because if bacteria have a good carbon source available, there is no need to swim around in search of nutrients.

One consequence of catabolite repression is that it may lead to two exponential growth phases, a phenomenon called *diauxic growth*. If two usable energy sources are available, the cells first consume the better energy source. Growth stops when the better source is depleted, but following a lag period, it resumes on the other energy source. Diauxic growth is illustrated in **Figure 7.21** for a culture of *E. coli* grown on a mixture of glucose and lactose. The cells grow more rapidly on glucose than on lactose. Although glucose and lactose are both excellent energy sources for *E. coli*, glucose is superior and supports a higher growth rate.

The proteins of the *lac* operon, including the enzyme β-galactosidase, are required for using lactose and are induced in its presence (Figure 7.6). But the synthesis of these proteins is subject to catabolite

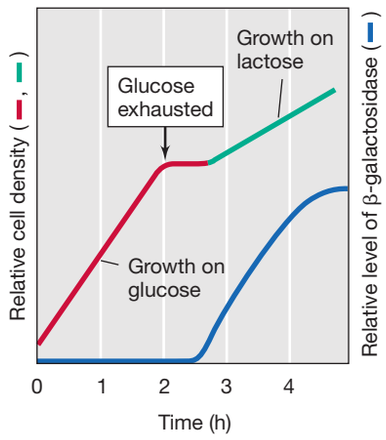


Figure 7.21 Diauxic growth of *Escherichia coli* on a mixture of glucose and lactose. The presence of glucose represses the synthesis of β-galactosidase, the enzyme that cleaves lactose into glucose and galactose. After glucose is depleted, there is a lag during which β-galactosidase is synthesized. Growth then resumes on lactose but at a slower rate, as indicated by the green line.

repression, and as long as glucose is present, the *lac* operon is not expressed and lactose is not used. However, when glucose is depleted, catabolite repression is abolished and the *lac* operon is expressed; shortly thereafter, cells begin to grow on lactose. How are all of these events controlled?

Cyclic AMP and Cyclic AMP Receptor Protein

Despite its name, catabolite repression relies on an activator protein and is actually a form of activation (Section 7.3). The activator protein is called the *cyclic AMP receptor protein* (*CRP*). A gene that encodes a catabolite-repressible enzyme is expressed only if CRP binds to DNA in the promoter region. This allows RNA polymerase to bind to the promoter. CRP is an allosteric protein and binds to DNA only if it has first bound a small molecule called *cyclic adenosine monophosphate* (*cyclic AMP* or *cAMP*), a derivative of the nucleotide adenosine present in RNA (**Figure 7.22**). Like many DNA-binding proteins (Section 7.1), CRP binds to DNA as a dimer.

Cyclic AMP is a key molecule in many metabolic control systems, both in prokaryotic cells and eukaryotes. Because it is derived from a nucleic acid precursor, it is a **regulatory nucleotide**. Other regulatory nucleotides include cyclic guanosine monophosphate (cyclic GMP; important mostly in eukaryotes), cyclic di-GMP (important in biofilm formation; ◀ Section 4.9 and ▶ Section 8.10), and guanosine tetraphosphate (ppGpp, important in the stringent response, Section 7.9). Cyclic AMP is synthesized from ATP by an enzyme called *adenylate cyclase* (Figure 7.22). However, glucose inhibits the synthesis of cyclic AMP and also stimulates cyclic AMP transport out of the cell. When glucose enters the cell, the cyclic AMP level is lowered, CRP cannot bind DNA, and RNA polymerase fails to bind to the promoters of operons subject to catabolite repression. Thus, catabolite repression is an *indirect* result of the presence of a better energy source (glucose); the *direct* cause of catabolite repression is a low level of cyclic AMP.

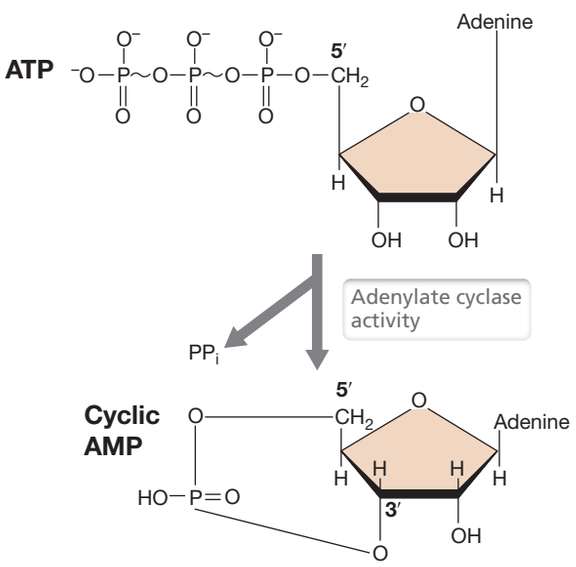


Figure 7.22 Cyclic AMP. Cyclic adenosine monophosphate (cyclic AMP) is made from ATP by the enzyme adenylate cyclase.

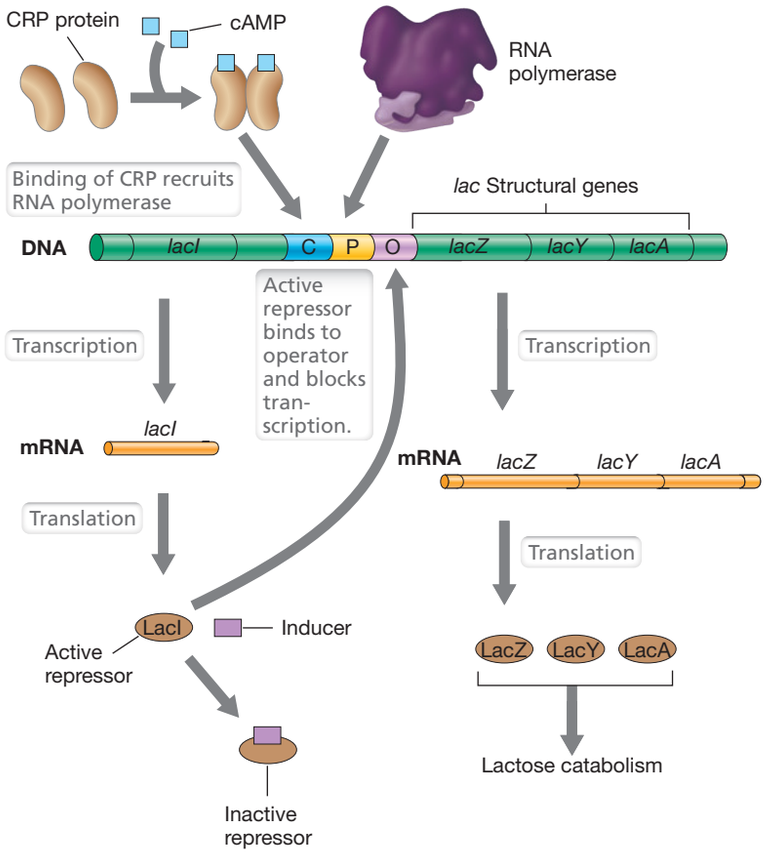


Figure 7.23 Overall regulation of the *lac* system. The *lac* operon consists of *lacZ*, encoding β-galactosidase, *lacY*, encoding lactose permease, and *lacA*, encoding lactose acetylase. The LacI repressor protein is encoded by a separate gene, *lacI*. LacI binds to the operator (O) unless the inducer is present. The cyclic AMP receptor protein (CRP) binds to the CRP activator site (C) when activated by cyclic AMP and recruits RNA polymerase to bind to the promoter (P). For the *lac* operon to be transcribed by RNA polymerase, the LacI repressor must be absent (that is, inducer must be present) and cyclic AMP levels must be high (due to the absence of glucose), allowing CRP to bind.

Let us return to the *lac* operon and include catabolite repression. The entire regulatory region of the *lac* operon is diagrammed in **Figure 7.23**. For *lac* genes to be transcribed, two requirements must be met: (1) The level of cyclic AMP must be high enough for the CRP protein to bind to the CRP-binding site (positive control), and (2) lactose or another suitable inducer must be present so that the lactose repressor (LacI protein) does not block transcription by binding to the operator (negative control). If these two conditions are met, the cell is signaled that glucose is absent and lactose is present; then and only then does transcription of the *lac* operon begin.

Mastering Microbiology
Art Activity: Figure 7.23 Overall regulation of the lac system

We now consider another form of global control distinct from catabolite repression but where regulatory nucleotides play a central role.

Check Your Understanding

- Explain how catabolite repression depends on an activator protein.
- What role does cyclic AMP play in glucose regulation?
- Explain how the *lac* operon is both positively and negatively controlled.

UNIT 2

7.9 Stringent and General Stress Responses

In nature microorganisms must often survive nutrient-limited conditions and exposure to environmental stressors such as extreme pH, oxidative stress, and antibiotic exposure. How do cells cope and adapt to such conditions? While some gram-positive bacteria undergo sporulation to withstand such harsh conditions (◀ Section 2.8), many *Bacteria* instead activate a variety of *stress survival pathways*—including both specific and general—that improve their chances of survival.

Mechanism of the Stringent Response

The **stringent response** is a widely distributed regulatory mechanism used by bacteria to survive nutrient deprivation, environmental stresses, and antibiotic exposure. Triggering of the stringent response ultimately leads to a shutdown of macromolecule synthesis and the activation of pathways to improve the cell's ability to survive and compete in nature.

Nutrient levels for microbes in nature can change significantly and rapidly. Such changing conditions can easily be simulated in the laboratory, and much work has been done on the regulation of gene expression following a "shift down" or "shift up" in nutrient status. These include, in particular, the regulatory events triggered by starvation for amino acids or energy, experiments that led to the discovery of the stringent response.

As a result of a shift down from amino acid excess to limitation, as occurs when a culture is transferred from a rich complex medium to a defined medium with a single carbon source (◀ Table 4.2), the synthesis of rRNA and tRNA ceases almost immediately, and no new ribosomes are produced (**Figure 7.24*a***). Protein and DNA synthesis are also curtailed, but the biosynthesis of new amino acids is activated. Following such a shift, new proteins must be made to synthesize the amino acids no longer available in the environment, and these proteins are made by existing ribosomes. After a while, rRNA synthesis (and hence, the production of new ribosomes) begins again but at a new rate commensurate with the cell's reduced growth rate (Figure 7.24*a*). This course of events is called the *stringent response* (or stringent control) and, like catabolite repression (Section 7.8), is another example of global control.

The stringent response is triggered by a mixture of two regulatory nucleotides, *guanosine tetraphosphate* (ppGpp, Figure 7.24*b*) and

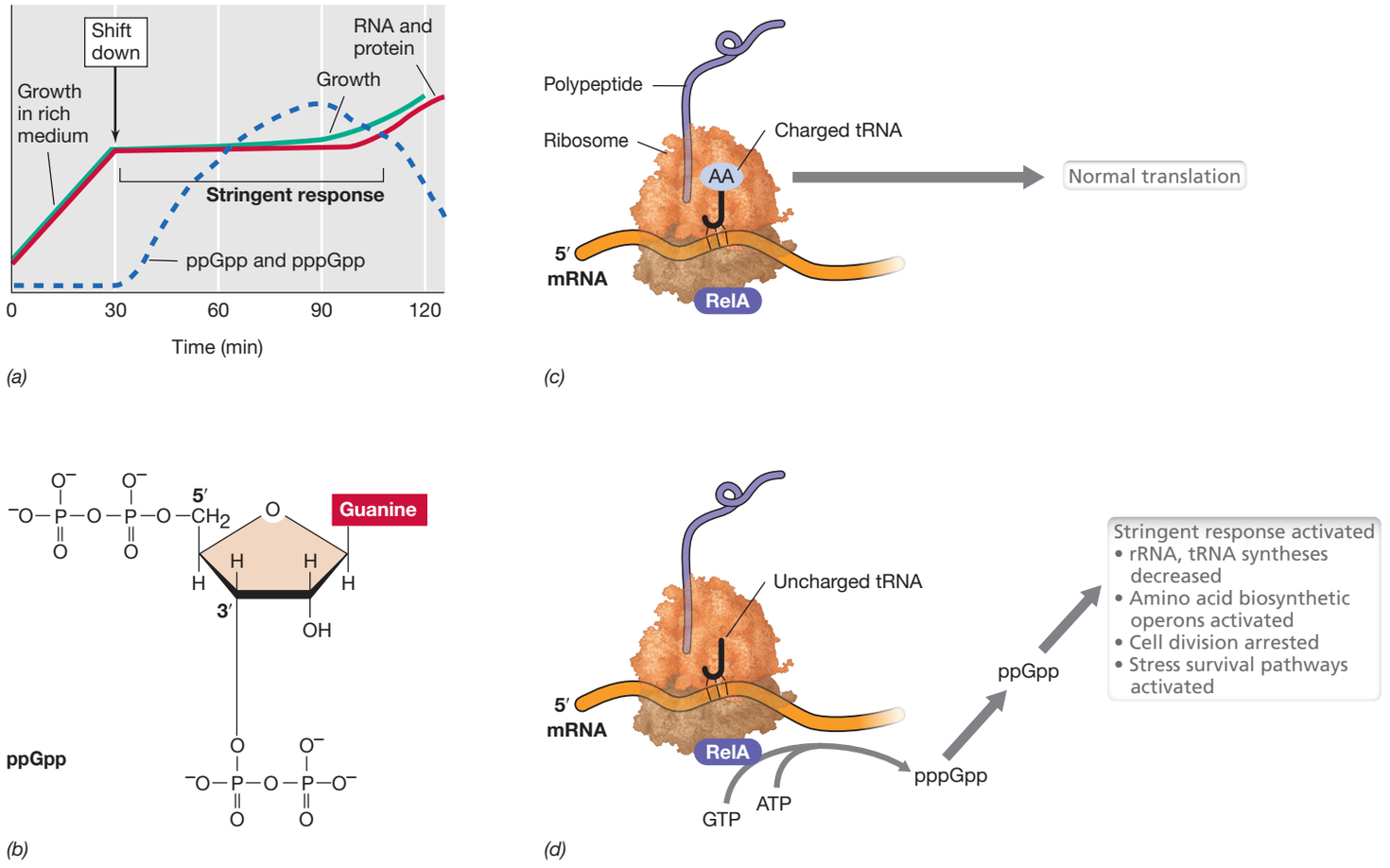


Figure 7.24 The stringent response in *Escherichia coli*. *(a)* Upon nutrient downshift, rRNA, tRNA, and protein syntheses temporarily cease. Sometime later, growth resumes at a decreased rate. *(b)* Structure of guanosine tetraphosphate (ppGpp), a trigger of the stringent response. *(c)* Normal translation, which requires charged tRNAs. *(d)* Synthesis of ppGpp. When cells are starved for amino acids, an uncharged tRNA can bind to the ribosome, which stops ribosome activity. This event triggers the RelA protein to synthesize a mixture of pppGpp and ppGpp.

guanosine pentaphosphate (pppGpp) [collectively, these are referred to as (p)ppGpp)]. In *Escherichia coli*, these nucleotides, which are also called *alarmones*, rapidly accumulate during stress or a shift down from amino acid excess to amino acid starvation. Alarmones are synthesized by a specific protein, called RelA, using ATP as a phosphate donor (Figure 7.24*c*, *d*). RelA adds two phosphate groups from ATP to GTP or GDP, thus producing pppGpp or ppGpp, respectively. RelA is associated with the 50S subunit of the ribosome and is activated by a signal from the ribosome during amino acid limitation. When the growth of the cell is limited by a shortage of amino acids, the pool of *uncharged* tRNAs increases relative to *charged* tRNAs (◀ Section 6.8). Eventually, an uncharged tRNA is inserted into the ribosome instead of a charged tRNA during protein synthesis. When this happens, the ribosome stalls, and this leads to (p)ppGpp synthesis by RelA (Figure 7.24*d*). The protein Gpp converts pppGpp to ppGpp so that ppGpp is the major overall product.

The alarmones ppGpp and pppGpp have global control effects. They strongly inhibit rRNA and tRNA synthesis by binding to RNA polymerase and preventing initiation of transcription of genes for these RNAs in gram-negative bacteria. In gram-positive bacteria, the same alarmones have been shown to interfere with initiating ribonucleotides for transcription. In both gram-negative and gram-positive *Bacteria*, the alarmones activate both the stress response pathways and the biosynthetic operons for certain amino acids. In *E. coli*, the stringent response also inhibits the initiation of new rounds of DNA synthesis and cell division and slows down the synthesis of cell envelope components, such as membrane lipids.

In addition to RelA, a second protein called SpoT helps trigger the stringent response. SpoT can either make (p)ppGpp or degrade it. Under most conditions, SpoT is responsible for degrading (p)ppGpp; however, SpoT synthesizes (p)ppGpp in response to certain stresses or when nutrient (and thus energy) deprivation is detected. Thus the stringent response results not only from the absence of precursors for protein synthesis, but also from the lack of energy for biosynthesis.

The Stringent Response and Microbial Ecology

Because the stringent response is a global mechanism that balances the metabolic state of the cell, the environment or habitat of the bacterium ultimately determines the response cascade. When cells of *E. coli* are voided in the feces and face a switch from the nutrient-rich intestine to an open water system, the reduction in nutrients triggers ppGpp synthesis to initiate the stringent response (**Figure 7.25*a***).

In contrast to *E. coli*, the aquatic bacterium *Caulobacter* naturally inhabits oligotrophic (nutrient-poor) freshwaters, and in this bacterium, the stringent response is triggered by carbon and ammonia starvation, not amino acid limitation. *Caulobacter* can form two types of cells, stalked cells and swarmer cells (Figure 7.25*b*). To increase its probability of survival, the production of ppGpp in *Caulobacter* leads to an increase in swarmer cell formation. Unlike stalked cells, swarmer cells are motile and can therefore swim and perhaps reach a niche containing more nutrients (▶ Section 8.8; Figure 7.25*b*).

In a disease example, the stringent response has also been implicated in the persistence of the aerobic bacterial pathogen

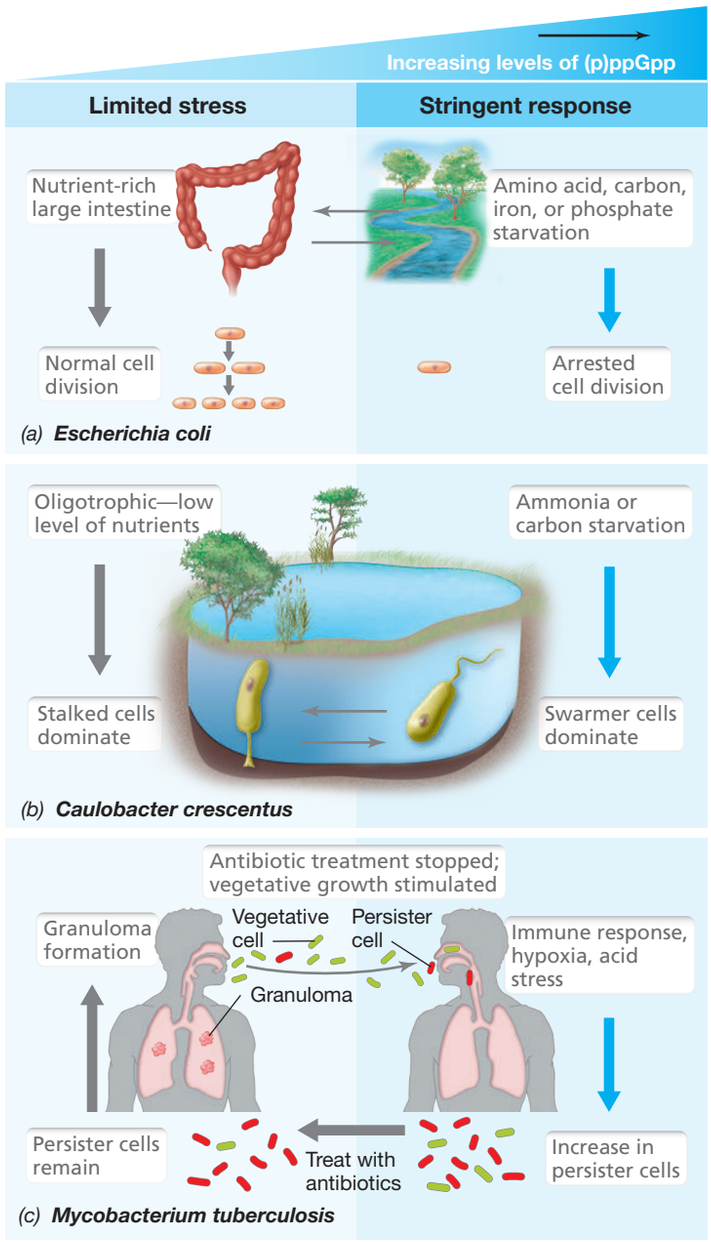


Figure 7.25 Environmental control of stringent response signaling in *Escherichia coli* and *Caulobacter crecentus* (both gram-negative bacteria), and *Mycobacterium tuberculosis* (a gram-positive bacterium). *(a) Escherichia coli.* When *E. coli* cells move from the nutrient-rich intestine to an open water source, they experience nutrient limitation. As ppGpp production increases, cell division is inhibited. *(b) Caulobacter crescentus. C. crescentus* naturally inhabits freshwater systems that are limited in nutrients. If cells encounter severe nutrient limitation, the stringent response is induced and ppGpp production increases. This increase leads to the cell morphology changing from stalked cells to swarmer cells that can swim to find more nutrients. *(c) Mycobacterium tuberculosis.* When *M. tuberculosis* cells enter a host, the bacterial cells are exposed to the host's immune response. This leads to production of ppGpp, which converts some cells to relatively dormant persister cells. These persister cells are resistant to antibiotics and can dominate more sensitive vegetative cells, forming granulomas. Once persister cells are aerosolized and relieved of stress, they revert back to infective vegetative cells. Unlike many other bacterial pathogens that acquire antibiotic resistance from mobile genetic elements, horizontal gene transfer is rare in *M. tuberculosis*. Antibiotic resistance in this pathogen is primarily due to chromosomal mutations affecting the target of the antibiotic.

Mycobacterium tuberculosis (tuberculosis, ▶ Section 31.4) in dormant granulomas. Within the lung of the human tuberculosis patient, the local environment is both hypoxic (low in O_2) and limited for phosphate, and these conditions trigger the stringent response in cells of *M. tuberculosis*. Activation of the stringent response also leads to a subpopulation of *M. tuberculosis* cells that convert to dormant *persister cells* (▶ Section 8.12); these cells are resistant to antibiotics and can survive in granulomas that form in the lung (Figure 7.25*c*). Once drug treatment of the infection has greatly reduced cell numbers and the environmental stresses have been relieved, the *M. tuberculosis* persister cells can then revert back to infective cells and trigger chronic tuberculosis, a not uncommon occurrence and one of the reasons drug therapy for tuberculosis is carried out over a long period.

The General Stress Response: The RpoS Regulon

Bacterial cells often respond to nutrient limitation and stressors by entering into the stationary phase of growth (◀ Section 4.6). This growth switch is controlled by activating a regulon recognized by the alternative sigma factor RpoS (σ^S or σ^{38}, ◀ Table 6.3), also called the *stationary phase sigma factor*. The RpoS regulon comprises over 400 genes including those associated with nutrient limitation, resistance to DNA damage, biofilm formation, and responses to osmotic, oxidative, and acid stresses. Thus, RpoS not only senses environmental changes but also relays signals to other regulators.

Examples of *E. coli* genes that are recognized by RpoS include *dinB*, which encodes DNA polymerase IV of the SOS DNA repair system (▶ Section 9.4 and Figure 9.10) and catalase genes necessary for combating reactive oxygen species (◀ Section 4.16). Because of the expansive nature of the RpoS regulon, this alternative sigma factor is itself controlled by several transcriptional, translational, and post-translational regulatory mechanisms. For example, transcription of the *rpoS* gene increases in response to the presence of the alarmone ppGpp, thus directly linking the RpoS regulon with the stringent response. Translation of *rpoS* mRNA is also positively regulated by the presence of small RNAs expressed during stress conditions (Section 7.12). Conversely, the RpoS protein is susceptible to degradation during nonstressful conditions. Overall, the general stress response highlights the global control nature of RpoS and the coordinated processes that a cell must undertake for adaptation and survival in nature.

We next consider a more specific global regulatory mechanism focused on phosphate, an essential nutrient for life (◀ Section 4.1).

Check Your Understanding

- Which *Escherichia coli* genes are activated and which are repressed during the stringent response, and why?
- How are the alarmones ppGpp and pppGpp synthesized?
- What are some other conditions that trigger the stringent response in other bacteria?

7.10 The Phosphate (Pho) Regulon

Phosphorus is not only essential for DNA, RNA, and membrane synthesis, but it is also critical for energy generation (◀ Section 4.1) and cell signaling. In nature, phosphorus is generally in the form of inorganic phosphate (PO_4^{3-}, abbreviated P_i) and is often the limiting nutrient in many environments. Because of this, P_i transformations are heavily regulated. The *phosphate* (*Pho*) regulon consists of a two-component regulatory system (Section 7.5) that responds to P_i limitation. This global control system includes not only genes encoding extracellular enzymes for extracting P_i from organic phosphates, but also genes encoding P_i transporters, enzymes for P_i storage, and other energy-requiring metabolic and biosynthesis pathways as well.

Streptomyces and Phosphate

Streptomyces is a genus of gram-positive *Bacteria* that contains many species that produce antibiotics (▶ Section 16.12) and whose phosphate metabolism has been well studied. While antibiotic production is an energy-intensive process, the process is limited by high phosphate concentrations. In the environment, *Streptomyces* produce antibiotics to kill competing cells. Thus, low phosphate levels signal *Streptomyces* that competition for resources is keen, and this results in antibiotic production.

In *Streptomyces*, the two-component Pho regulatory system that senses P_i limitation consists of a membrane-bound histidine kinase sensor protein PhoR and a cytoplasmic transcriptional regulator PhoP. While the mechanism for actually *sensing* extracellular P_i levels is unknown, low environmental P_i levels trigger PhoR kinase activity, yielding a phosphorylated PhoP (PhoP-P) (**Figure 7.26**). Once phosphorylated, PhoP-P binds to conserved promoter regions called "Pho boxes" scattered across the genome. This binding

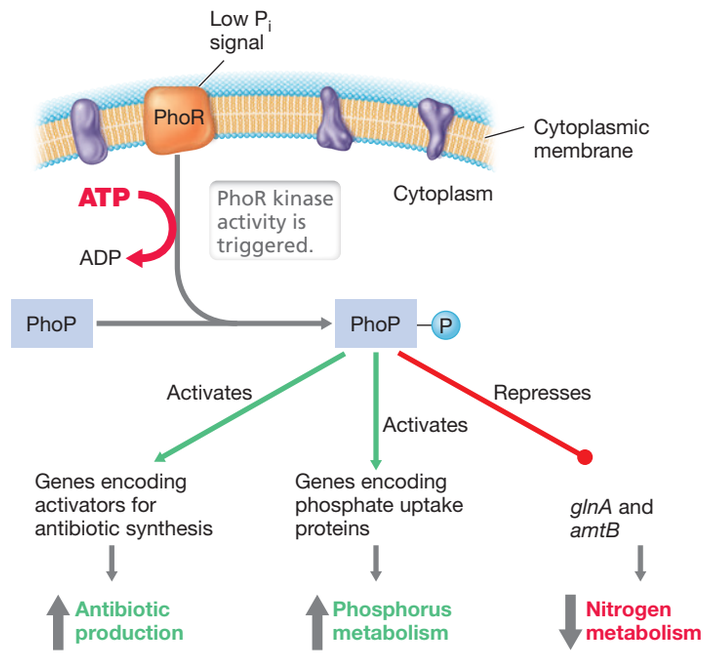


Figure 7.26 The phosphate (Pho) regulon of *Streptomyces*. If inorganic phosphate (P_i) concentrations are low, the kinase PhoR is signaled to phosphorylate PhoP. PhoP-P recognizes and binds to several promoters, functioning as either an activator or a repressor. This regulation results in activating phosphate uptake and antibiotic synthesis genes and decreasing nitrogen metabolism by repressing genes regulating glutamine synthetase (*glnA*) and ammonium transport (*amtB*), respectively.

signals the major sigma subunit of *Streptomyces* RNA polymerase to bind, and *activates* transcription of genes needed for P_i uptake. The same event occurs with promoters for some genes that encode products necessary for antibiotic production. By contrast, when P_i is present in excess, PhoP is dephosphorylated, and this leads to the removal of PhoP from Pho boxes and a decreased transcription of genes encoding proteins of phosphate metabolism and antibiotic synthesis.

While the Pho regulon is the mechanism many *Bacteria* use to respond to P_i starvation, why is this regulon considered an example of *global* control? Besides its role as an activator of P_i uptake and antibiotic synthesis genes in *Streptomyces* and of P_i genes in many other *Bacteria*, PhoP-P also binds some promoters weakly without recruiting a sigma subunit. This binding blocks RNA polymerase and thus leads to repression of the linked gene. Somewhat surprisingly, PhoP-P appears to actually repress more genes than it activates. Genes regulated by P_i limitation and PhoP-P that are unassociated with P_i uptake in *Streptomyces* include those whose products are necessary for nitrogen metabolism (glutamine synthetase, *glnA*; ammonium transport, *amtB*) (Figure 7.26). The physiological rationale here is that if P_i levels are growth limiting, the cell's production of key biosynthetic intermediates (such as amino acids) should be reduced until P_i levels increase.

Pathogenesis and the Pho Regulon

The Pho regulon can also control some aspects of pathogenesis. Pathogenic bacteria are exposed to both P_i-limiting and P_i-rich environments within a host depending on the site of infection. The genome of Shiga toxin–producing *Escherichia coli*, such as the notorious foodborne pathogen *E. coli* O157:H7 (▶ Section 33.11), contains a Pho regulon of over 40 genes. In its normal habitat—the intestinal tract—*E. coli* often experiences phosphate limitation, and this condition activates the cytoplasmic-membrane-bound PhoR. This protein then transfers a phosphate group to the cytoplasmic transcriptional regulator and DNA-binding protein PhoB (the equivalent to the *Streptomyces* PhoP, Figure 7.26). PhoB-P triggers increased expression not only of the genes for phosphate metabolism, but also of genes encoding the acid shock protein Asr and Shiga toxin. Increased production of Asr leads to protection of periplasmic proteins from acid stress, which would be encountered by *E. coli* cells present in the stomach en route to the intestine. Once the pathogen is inside an intestinal cell, its Shiga toxin production is responsible for the severe diarrhea associated with the infection, an event that effectively transmits the pathogen to a new host. Thus, phosphate limitation leads to increased fitness and survivability of pathogenic strains of *E. coli*.

While the mechanisms remain unclear, the Pho regulon has also been implicated in regulating biofilm formation (◀ Section 4.9 and ▶ Section 8.10), antimicrobial resistance (▶ Section 28.7), and toxin production (▶ Section 25.6) in other pathogenic bacteria such as *Vibrio cholerae* and *Pseudomonas* species. Thus, although the Pho regulon is the major barometer of P_i starvation, its activities impact a wide range of unrelated genes that control several distinct cellular functions.

We move on now to the last section of Part III to consider globally regulated systems found in all domains of life that respond to major temperature shifts, the phenomenon of heat shock.

Check Your Understanding

- What category of regulatory system is the Pho regulon?
- What types of bacterial processes are activated and repressed by phosphate limitation?

7.11 The Heat Shock Response

Most proteins are relatively stable, even to small increases in temperature. However, some proteins are less stable at elevated temperatures and tend to unfold (denature, ◀ Section 6.7). Other stress conditions besides heat can lead to protein denaturation. These include exposure to high levels of certain solvents (for example, ethanol), osmotic stress, and high doses of ultraviolet (UV) light. Because protein stability is critical for survival, cells employ a global control mechanism called the **heat shock response** to protect cells from protein denaturation. This response features many of the regulatory modes illustrated in Figure 7.1.

Heat Shock Proteins

Temperature and various stress conditions can generate large amounts of inactive proteins that need to be either refolded (and in the process, reactivated) or degraded to release free amino acids for the synthesis of new proteins (enzymes called *proteases* facilitate the latter process). Consequently, heat stress and other conditions that lead to protein unfolding trigger the synthesis of a set of proteins known as **heat shock proteins**. Heat shock proteins help counteract protein damage and assist the cell in recovering from stress.

In most *Bacteria* and *Archaea* there are five major classes of heat shock protein: Hsp100, Hsp90, Hsp70, Hsp60, and Hsp10. We have encountered some of these proteins before, although not by these names (◀ Section 6.11 and Figure 6.40*a*). The Hsp70 protein of *Escherichia coli* is DnaK, which prevents aggregation of newly synthesized proteins and stabilizes unfolded proteins. Major representatives of the Hsp60 and Hsp10 families in *E. coli* are the proteins GroEL and GroES, respectively. These are *molecular chaperones* that catalyze the correct refolding of misfolded proteins. Another group of heat shock proteins (Hsp100) includes various proteases that degrade denatured or irreversibly aggregated proteins. Because heat shock proteins perform vital functions in the cell, a low level of these proteins is always present, even under optimal conditions. However, genes encoding many bacterial heat shock proteins also make up a regulon (Section 7.3) whose expression is increased by the presence of an alternative sigma factor known as *RpoH*.

The Alternative Sigma Factor RpoH

In many bacteria, such as *E. coli*, the heat shock response is controlled by the alternative RNA polymerase σ factor (◀ Section 6.5) RpoH (σ^{32}) (**Figure 7.27**). RpoH controls expression of heat shock proteins and is normally degraded within a minute or two of its synthesis. However, when cells suffer a heat shock (or other stressor that leads to protein denaturation), degradation of RpoH is inhibited and its level therefore increases. As a consequence, transcription of those operons whose promoters are recognized by RpoH increases.

The rate of RpoH degradation depends on the level of free DnaK, which inactivates RpoH. In unstressed cells, the level of free DnaK

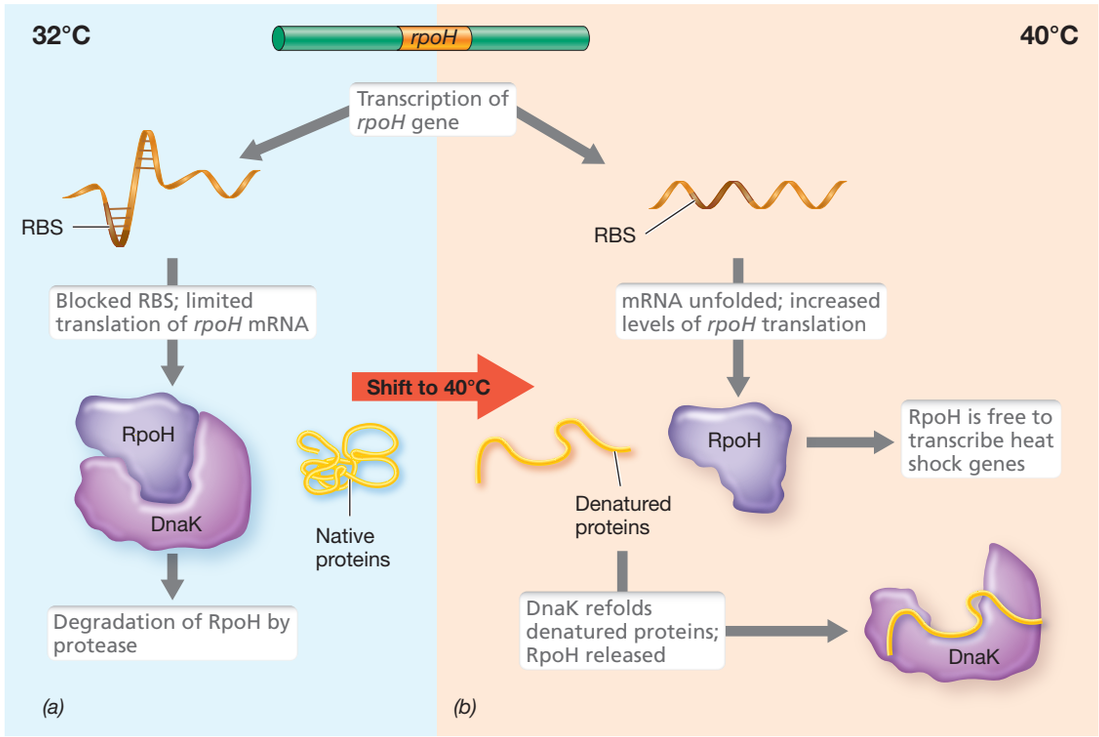


Figure 7.27 **Control of heat shock in *Escherichia coli*.** *(a)* The mRNA that encodes the alternative sigma factor RpoH folds under normal conditions, thus blocking the ribosome-binding site (RBS) and limiting translation. The RpoH protein that is made is broken down rapidly by proteases at 32°C, and binding of the DnaK chaperone to RpoH stimulates this degradation. *(b)* At 40°C, the mRNA encoding RpoH unfolds and more RpoH protein is made. The shift in temperature also denatures some proteins and DnaK recognizes, binds, and refolds these unfolded polypeptide chains. This removes RpoH from DnaK, which slows the degradation rate of RpoH. When the level of RpoH rises, the heat shock genes are transcribed.

is relatively high and the level of intact RpoH is correspondingly low (Figure 7.27*a*). However, if heat begins to unfold proteins, DnaK binds preferentially to unfolded proteins and so is no longer free to degrade RpoH (Figure 7.27*b*). Thus, the more denatured proteins there are, the lower the level of free DnaK and the higher the level of RpoH; the result is heat shock gene expression. When the environmental stressor has passed, for example upon a temperature downshift, RpoH is once again inactivated by DnaK and the synthesis of heat shock proteins is reduced. Thus, DnaK functions as a cellular "thermometer" for the heat shock response and its inactivation of RpoH is considered a form of post-translational regulation, which we will discuss in Section 7.16.

Not only is the activity of the RpoH protein controlled, but translation of its corresponding mRNA is also regulated. Under normal cellular conditions, the *rpoH* mRNA base-pairs within itself (Figure 7.27*a*). This folding results in a secondary structure that decreases the ribosome's ability to bind to the ribosome-binding site (RBS) of the mRNA (◀ Section 6.9). However, when the cell's internal temperature increases, the mRNA encoding RpoH unfolds. This unfolding allows the ribosome easier access and thus more overall RpoH protein production under the heat shock response (Figure 7.27*b*).

There is also a heat shock response in *Archaea*, even in species that grow optimally at very high temperatures. An analog of the bacterial Hsp70 is found in many *Archaea* and is structurally similar to that present in gram-positive species of *Bacteria*. However, other types of heat shock proteins unrelated to the stress proteins of *Bacteria* are also present in *Archaea*, Moreover, a heat shock response also exists in eukaryotes. Thus, this critically important mechanism for salvaging damaged yet recoverable proteins is present in all domains of life.

Check Your Understanding

- What is the heat shock response?
- How do levels of RpoH control the heat shock response?
- How is translation of the *rpoH* mRNA controlled?

IV • RNA-Based Regulation

In the course of evolution, prokaryotic cells have missed out on few if any potential regulatory mechanisms. For example, controls employing RNAs (rather than proteins) to monitor the cell's status and trigger specific gene expression are known and are widespread in *Bacteria* and *Archaea*.

Thus far we have focused on regulatory mechanisms in which proteins sense signals or bind to DNA. In some cases, a single protein does both; in other cases, separate proteins carry out these two activities. Nonetheless, all of these mechanisms rely on regulatory *proteins*. However, some regulatory mechanisms employ *RNA* to regulate gene expression, both at the level of transcription and at the level of translation, and we consider these now.

7.12 Regulatory RNAs

RNA molecules that are not translated to give proteins are collectively known as **noncoding RNA (ncRNA)**. This category includes the rRNAs and tRNAs that participate in protein synthesis and the RNA present in the signal recognition particle that catalyzes some types of protein secretion (◀ Sections 6.8, 6.10, and 6.12). Noncoding RNA also includes small RNA molecules necessary for RNA processing, especially the splicing of mRNA in eukaryotes (◀ Section 6.6). *Small RNAs* (sRNAs) that range from 40 to 400 nucleotides long and regulate gene expression are widely distributed in both prokaryotic cells and eukaryotes. In *Escherichia coli*, for example, a number of sRNA molecules regulate several aspects of cell physiology in response to environmental or cellular signals by binding to other RNAs or in some cases to other small molecules; the end result is control of gene expression.

Small RNAs (sRNAs) exert their effects by base-pairing directly to other RNA molecules, usually mRNAs, in regions of complementary sequence. This binding immediately modulates the rate of target mRNA translation because a ribosome cannot translate double-stranded RNA. Thus, sRNAs provide an additional mechanism to regulate the synthesis of a protein once its corresponding mRNA has already been made.

Mechanisms of sRNA Activity

Small RNAs alter the translation of their mRNA target by four different mechanisms (**Figure 7.28**). Some sRNAs base-pair to their target mRNA, changing its secondary structure to either block a previously accessible ribosome-binding site (RBS) (◀ Section 6.9) or to open up a previously blocked RBS, allowing access for the ribosome. These two events decrease or increase expression of the protein encoded by the target mRNA, respectively. The other two mechanisms of sRNA interaction affect mRNA stability; binding of the sRNA to its target either increases or decreases degradation of the transcript by bacterial ribonucleases, thus modulating protein expression. Increased degradation of an mRNA prevents the synthesis of new protein molecules encoded by that mRNA. Alternatively, increasing the stability of mRNA will lead to higher corresponding protein levels in the cell (Figure 7.28).

Types of Small RNA

Small RNAs are important modulators of various cellular processes including oxidative, iron limitation, and glucose–phosphate stresses as well as quorum sensing, biofilm formation, and stationary phase events. Transcription of sRNAs is often enhanced under conditions in which their target genes need to be silenced. For example, the RyhB RNA of *Escherichia coli* is transcribed when iron is limiting for growth. RyhB sRNA binds to several distinct target mRNAs that encode proteins needed for iron metabolism or that use iron as cofactors. Binding of RyhB sRNA blocks the RBS of the mRNA and thus inhibits translation (Figure 7.28*a*). The base-paired RyhB/mRNA molecules are then degraded by ribonucleases, in particular, ribonuclease E, before translation of the mRNA can occur. Small RNA control forms only part of the mechanism by which *E. coli* and related bacteria reduce the production of iron-requiring proteins in response to iron limitation. Other responses include transcriptional controls by repressor and activator proteins (Section 7.2) that decrease or increase, respectively, the capacity of cells to take up iron or to tap into intracellular iron reserves.

Similarly, SgrS is an sRNA in *E. coli* that is expressed during glucose–phosphate stress to avoid accumulation of glucose 6-phosphate. High levels of glucose 6-phosphate within the cell can block the glycolytic pathway (◀ Section 3.6) and ultimately lead to

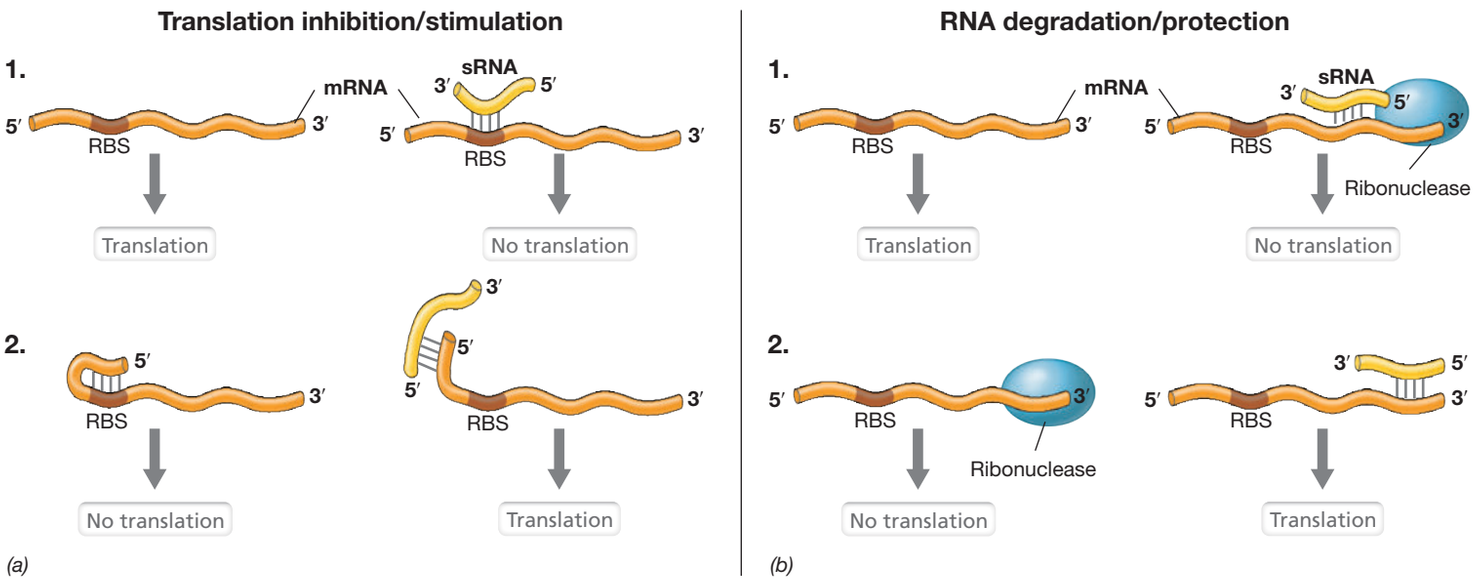


Figure 7.28 Small RNA mechanisms for modulating the translation of mRNA. *(a)* Binding of a ribosome to mRNA requires that the ribosome-binding site (RBS) of the mRNA be single-stranded. Binding of an sRNA to the RBS (shown in 1) can prevent translation while the binding of an sRNA to an mRNA whose RBS has secondary structure (shown in 2) can stimulate translation. *(b)* Ribonuclease degrades RNA. Ribonuclease binding to partially double-stranded RNA results in RNA degradation (shown in 1), while sRNA binding at the ribonuclease binding site (shown in 2) can protect the mRNA from degradation. Compare the mechanisms for controlling translation by small RNAs shown in this figure with the mechanism of controlling translation by a riboswitch shown in Figure 7.31*a*.

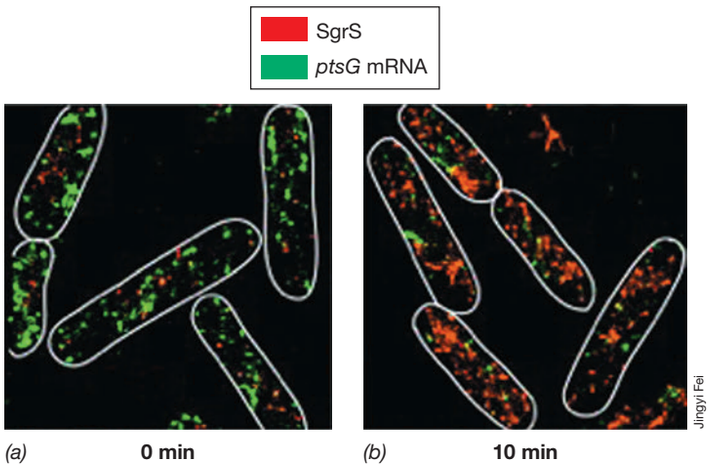


Figure 7.29 The interaction of SgrS sRNA and *ptsG* mRNA during glucose–phosphate stress. Three-dimensional super-resolution image of fluorescently labeled SgrS sRNA (red) and *ptsG* mRNA (green) in cells of *Escherichia coli*. *(a)* Before glucose–phosphate stress at time 0, *ptsG* mRNA is more abundant than SgrS sRNA. *(b)* After 10 min of glucose–phosphate stress, SgrS levels are higher, which leads to degradation of *ptsG* mRNA.

cell death if not overcome. When SgrS is expressed, the sRNA molecule binds to the *ptsG* mRNA encoding a glucose transporter. This binding results in a double-stranded RNA complex that triggers degradation of *ptsG* mRNA by ribonuclease E, as shown in living cells in **Figure 7.29**. This degradation decreases the amount of *ptsG* mRNA that is translated, and thus lower levels of the glucose transporter protein are made. This in turn results in decreased cellular levels of glucose 6-phosphate (recall that glucose is transported into *E. coli* by the phosphotransferase system, which converts glucose into glucose 6-phosphate during the transport process, ◀ Section 2.2 and Figure 2.6). By reducing glucose 6-phosphate levels, the glycolytic pathway remains active.

Because many sRNAs, including RyhB and SgrS, are encoded in intergenic regions and can be spatially separated from their mRNA target, they are called *trans-sRNAs*. As such, these sRNAs usually have limited complementarity to their target molecule and may only base-pair with a 5- to 11-nucleotide stretch. The binding of trans-sRNAs to their targets often depends on a small protein called *Hfq* (**Figure 7.30**) that binds to both mRNA and its corresponding sRNA to facilitate their interaction. Hfq forms hexameric rings with

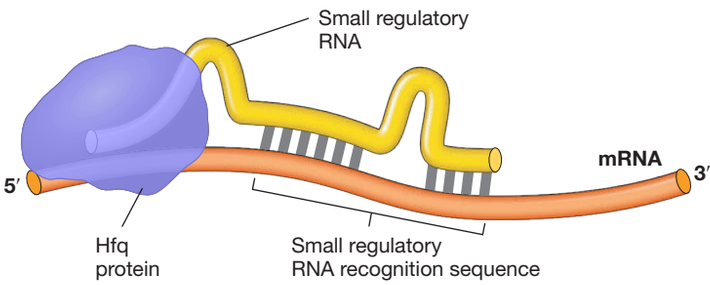


Figure 7.30 The RNA chaperone Hfq holds RNAs together. Binding of sRNA to mRNA often requires assistance from the Hfq protein. Small RNA molecules usually have several stem–loop structures. One consequence is that the complementary base sequence that recognizes the mRNA is noncontiguous.

RNA-binding sites on both surfaces. Hfq and functionally similar proteins are called *RNA chaperones*, as they help small RNA molecules, including many sRNAs, maintain their correct structure (Figure 7.30).

Small RNAs do not always work by affecting mRNA. For example, replication of the ColE1 plasmid in *Escherichia coli* is regulated by an sRNA that primes DNA synthesis on the plasmid and its antisense partner that blocks initiation of DNA synthesis. The level of the antisense RNA determines how often replication is initiated. Some sRNAs also bind directly to proteins and modulate their activity. Thus, depending on the sRNA, these molecules can affect several different activities in the cell.

In the next section we examine a different form of RNA-based regulation from that we have considered here with the sRNAs.

Check Your Understanding

- How do sRNAs alter the translation of target mRNAs?
- Why do trans-sRNAs often require a chaperone protein?
- How does SgrS help *Escherichia coli* prevent a potential metabolic disaster?

7.13 Riboswitches

RNA can carry out many roles once thought to be limited to proteins. In particular, RNA can specifically recognize and bind other molecules, including low-molecular-weight metabolites. It is important to emphasize that such binding does not require complementary base pairing (as does binding of the sRNAs described in the previous section) but results from the folding of the RNA into a specific three-dimensional structure that recognizes the target molecule, much as a protein enzyme recognizes its substrate in its active site. Catalytically active RNAs are called *ribozymes*. Other RNA molecules resemble repressors and activators in binding small metabolites and regulating gene expression; these are the **riboswitches**, our focus here.

Mechanisms of Riboswitches

Earlier in this chapter we discussed the regulation of gene expression by negative control of transcription (Section 7.3). In this process, a specific metabolite interacts with a specific repressor protein to prevent transcription of genes encoding enzymes for the biosynthetic pathway of the metabolite. In contrast, in a riboswitch there is no regulatory protein. Instead, the metabolite binds directly to a specific mRNA. This binding results in the riboswitch controlling gene expression at either the transcriptional or translational level, depending on the type of riboswitch.

Riboswitch RNAs have sequences upstream of their coding regions (5′-UTR; Figure 7.1) that can fold into specific three-dimensional structures that bind small molecules. These recognition domains are the *aptamer region* or "switch" in the riboswitch and exist as two alternative secondary structures, one with the small molecule bound and the other without (**Figure 7.31**). Alternation between the two forms of the riboswitch thus depends on the presence or absence of the small molecule, which in turn controls the secondary structure of the downstream RNA called the *expression platform* of the riboswitch. In one type of riboswitch, the secondary structure of the expression platform controls whether the ribosome binds to the

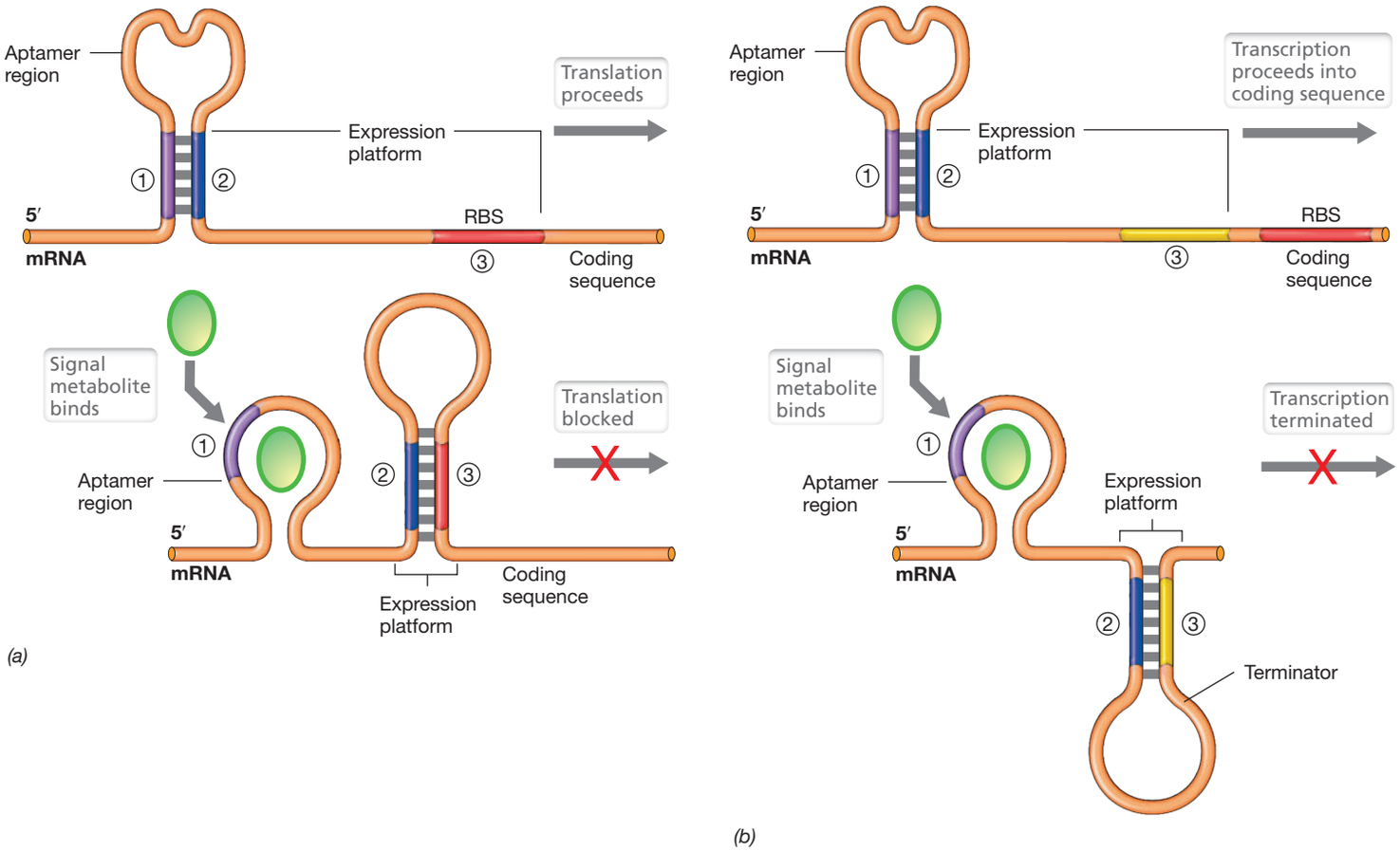


Figure 7.31 **Regulation by a riboswitch.** Riboswitches are located in the 5′ untranslated region (UTR) of mRNAs (Figure 7.1). The aptamer region of the riboswitch binds specifically to the metabolite. Numbers indicate regions within the riboswitch expression platform that can base-pair together, and RBS indicates the ribosome-binding site. *(a)* Translational regulation: Binding of a specific metabolite alters the secondary structure of the riboswitch domain, preventing translation. *(b)* Transcriptional regulation: Binding of a specific metabolite alters the secondary structure of the riboswitch domain, which results in the formation of a transcriptional terminator, preventing transcription of the coding region of the gene. Compare this process to that of attenuation shown in Figure 7.33*a*.

corresponding mRNA's ribosome-binding site (RBS) and begins translation (Figure 7.31*a*); in another type, it controls whether RNA polymerase continues to transcribe the mRNA (Figure 7.31*b*). Riboswitches exist in all three domains of life, with over 40 different types known in *Bacteria*. Riboswitches control not only the synthesis of enzymes in biosynthetic pathways for various vitamins, a few amino acids, some nitrogen bases of nucleic acids, and a peptidoglycan precursor, but also aspects related to biofilm formation, fluoride toxicity, and molybdenum/tungsten cofactor metabolism (Table 7.3).

The metabolite that is bound by the riboswitch aptamer region is typically the product of a biosynthetic pathway whose constituent enzymes are encoded by the mRNAs that carry the corresponding riboswitches. For example, the thiamine riboswitch that binds the vitamin thiamine pyrophosphate lies upstream of the coding sequences for enzymes that participate in the thiamine biosynthetic pathway. When the pool of thiamine pyrophosphate is sufficient in the cell, this metabolite binds to the aptamer region of its specific riboswitch mRNA. This binding results in a secondary structure of the riboswitch that blocks the ribosome-binding site on the mRNA; this prevents translation (Figure 7.31*a*). If the concentration of thiamine pyrophosphate drops sufficiently low, the molecule dissociates from its riboswitch mRNA, and this unfolds the mRNA and exposes the ribosome-binding site, allowing the mRNA to bind to the ribosome and be translated.

In contrast to riboswitches that control *translation*, some riboswitches control *transcription*. This mechanism is similar to

TABLE 7.3 Riboswitches in biosynthetic pathways of *Escherichia coli*

Type	Example of biosynthetic pathway
Vitamins	Cobalamin (B_{12}), tetrahydrofolate (folic acid), thiamine
Amino acids	Glutamine, glycine, lysine, methionine
Nitrogen bases of nucleic acids	Adenine, guanine (purine bases)
Others	Flavin mononucleotide (FMN), *S*-adenosylmethionine (SAM), glucosamine 6-phosphate (peptidoglycan precursor), cyclic di-GMP (biofilm signaling molecule), molybdenum cofactor (Moco), fluoride resistance proteins

regulation by attenuation, to be discussed in Section 7.14. With transcriptional riboswitches, metabolite binding to the aptamer region results in a conformational change within the expression platform that causes RNA polymerase to prematurely terminate synthesis of the mRNA (Figure 7.31*b*). This occurs because metabolite binding converts the expression platform to a form that mimics a traditional transcriptional terminator (◀ Section 6.5). The SAM (*S*-adenosylmethionine) riboswitch associated with sulfur metabolism in gram-positive bacteria such as *Bacillus* is a good example of a riboswitch that controls transcription in this way. SAM is a cofactor in the methionine biosynthesis pathway and its availability is a signal that sufficient methionine levels exist in the cell. Binding of SAM to the SAM riboswitch aptamer forms a transcriptional terminator in the downstream expression platform, and this results in decreased levels of mRNAs encoding methionine biosynthesis enzymes and enzymes in other sulfur metabolism pathways that require SAM.

Conversely, some riboswitches that control transcription employ the opposite form of control—binding of the metabolite *removes* the stem–loop structure that terminates transcription. When this occurs, increased levels of the mature mRNA are eventually formed and, following translation, yield increased levels of the protein(s) it encodes.

Riboswitches and Evolution

How widespread are riboswitches and how did they evolve? Thus far riboswitches have been found only in some *Bacteria*, *Archaea*, and a few plants and fungi. Some scientists believe that riboswitches are remnants of the RNA world, a period eons ago before cells, DNA, and protein were present when it is hypothesized that catalytic RNAs were the only self-replicating "life forms" (▶ Section 13.1 and Figure 13.4). In such an environment, riboswitches may have been an early mechanism of metabolic control—a simple means by which RNA life forms could have controlled the synthesis of other RNAs. As proteins evolved and DNA became the genetic material of all cells, riboswitches might have been the first control mechanisms for RNA (and even protein) synthesis as well. If this is true, the riboswitches that we see today may be the last vestiges of this simple form of control because, as we have seen in this chapter, metabolic regulatory mechanisms in cells are almost exclusively carried out by regulatory *proteins*.

We end this part of our study of microbial control with the description in the next section of one additional mechanism, a fascinating process by which cells essentially "decide" on whether to complete translation of a protein even before transcription of the mRNA that encodes it has been completed.

Check Your Understanding

- What happens when a riboswitch binds the small metabolite that regulates it?
- What are the major differences between a repressor protein and a riboswitch in the control of gene expression?

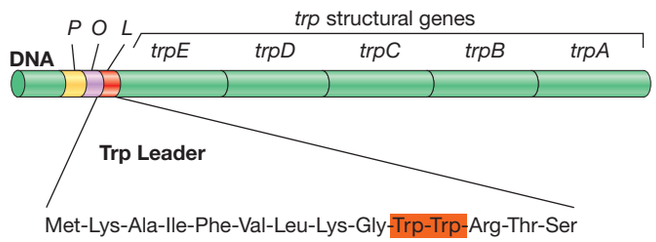
7.14 Attenuation

Attenuation is a form of transcriptional control in *Bacteria* and *Archaea* that functions by prematurely terminating mRNA synthesis. That is, in attenuation, control is exerted *after* the initiation of transcription but *before* its completion. Consequently, the number of *completed* transcripts from an operon is reduced, even though the number of *initiated* transcripts is not.

The basic principle of attenuation is that the first part of the mRNA to be made, called the *leader*, can fold into two alternative secondary structures. In this respect, the mechanism of attenuation resembles that of riboswitches that terminate transcription (Figure 7.31*b*). In attenuation, one mRNA secondary structure allows continued synthesis of the mRNA, whereas the other secondary structure causes premature termination. Folding of the mRNA depends either on events at the ribosome or on the activity of regulatory proteins, depending on the organism. The best examples of attenuation are the regulation of genes controlling the biosynthesis of certain amino acids in gram-negative *Bacteria*. The first to be described was in the tryptophan operon in *Escherichia coli*, and we focus on it here. Unlike the situation in prokaryotic cells, the processes of transcription and translation are spatially separated in eukaryotes (◀ Section 6.6), and thus attenuation control is absent from *Eukarya*.

Attenuation in the Tryptophan Operon

The tryptophan operon contains structural genes for five proteins of the tryptophan biosynthetic pathway plus the usual promoter and regulatory sequences at the beginning of the operon (**Figure 7.32**). Like many operons, the tryptophan operon has more than one type of regulation. Transcription of the entire tryptophan operon is controlled by a repressor protein and its corepressor (tryptophan; Section 7.3). However, in addition to the promoter and operator regions needed for negative control, there is a



(a)

Threonine	Met-Lys-Arg-Ile-Ser-Thr-Thr-Ile-Thr-Thr-Thr-Ile-Thr-Ile-Thr-Thr-Gly-Asn-Gly-Ala-Gly
Histidine	Met-Thr-Arg-Val-Gln-Phe-Lys-His-His-His-His-His-His-His-Pro-Asp
Phenylalanine	Met-Lys-His-Ile-Pro-Phe-Phe-Phe-Ala-Phe-Phe-Phe-Thr-Phe-Pro

(b)

Figure 7.32 Attenuation and leader peptides in *Escherichia coli*. Structure of the tryptophan (*trp*) operon and of the tryptophan leader peptide and other leader peptides in *E. coli*. *(a)* Arrangement of the *trp* operon. Note that the leader (*L*) encodes a short peptide containing two tryptophan residues near its terminus (there is a stop codon following the Ser codon). The promoter is labeled *P*, and the operator is labeled *O*. The genes labeled *trpE* through *trpA* encode the enzymes needed for tryptophan synthesis. *(b)* Amino acid sequences of leader peptides of some other amino acid biosynthesis operons. Because isoleucine is made from threonine, it is an important constituent of the threonine leader peptide.

sequence in the operon called the *leader sequence* that encodes a short polypeptide—the *leader peptide*—that functions as a translational barometer. The leader sequence contains tandem tryptophan codons near its terminus and is the "attenuator" of the attenuation system (Figure 7.32).

The basis of control of the tryptophan attenuator is as follows. If tryptophan is plentiful in the cell, there will be plenty of charged tryptophan tRNAs and the leader peptide will be synthesized. Synthesis of the leader peptide results in termination of transcription of the remainder of the *trp* operon, which includes the structural genes for the biosynthetic enzymes. By contrast, if tryptophan is scarce, the tryptophan-rich leader peptide will not be synthesized. If synthesis of the leader peptide is arrested by a shortage of tryptophan, the rest of the operon is transcribed.

Mechanism of Attenuation

How does translation of the leader peptide regulate transcription of the tryptophan genes downstream? Consider that in prokaryotic cells transcription and translation are simultaneous processes; as mRNA is released from the DNA, the ribosome binds to it and translation begins (◀ Section 6.1 and Figure 6.6). That is, while *transcription* of downstream DNA sequences is still proceeding, *translation* of transcribed sequences is already under way (**Figure 7.33**).

Transcription is attenuated because a portion of the newly formed mRNA folds into a unique stem–loop that inhibits RNA polymerase activity. The stem–loop structure forms in the mRNA because two stretches of nucleotides near each other are complementary and can thus base-pair. If tryptophan is plentiful, the ribosome translates the leader sequence until it comes to the leader stop codon. The remainder of the leader sequence then forms a stem–loop, a transcription pause site, which is followed by a uracil-rich sequence that actually triggers termination (Figure 7.33*a*).

If the concentration of tryptophan in the cell is limiting, transcription of genes encoding tryptophan biosynthetic enzymes is needed. Thus, during transcription of the leader, the ribosome pauses at a tryptophan codon because of a shortage of charged tryptophan tRNAs. The presence of the stalled ribosome at this position allows a stem–loop to form (sites 2 and 3 in Figure 7.33*b*) that differs from the terminator stem–loop. This alternative stem–loop is not a transcription termination signal but instead prevents the terminator stem–loop (sites 3 and 4 in Figure 7.33*a*) from forming. This allows RNA polymerase to move past the termination site and begin transcription of tryptophan structural genes. Thus, in attenuation, we see a situation in which the rate of *transcription* is ultimately influenced by the rate of *translation*, a situation that contrasts with typical cellular events where the rate of translation is controlled by the rate of transcription.

We finish up this chapter on regulation by examining in Part V two major mechanisms that cells have evolved to control the activities of proteins they have already made.

Check Your Understanding

- Why does attenuation control not occur in eukaryotes?
- Explain how the formation of one stem–loop in the RNA can block the formation of another.

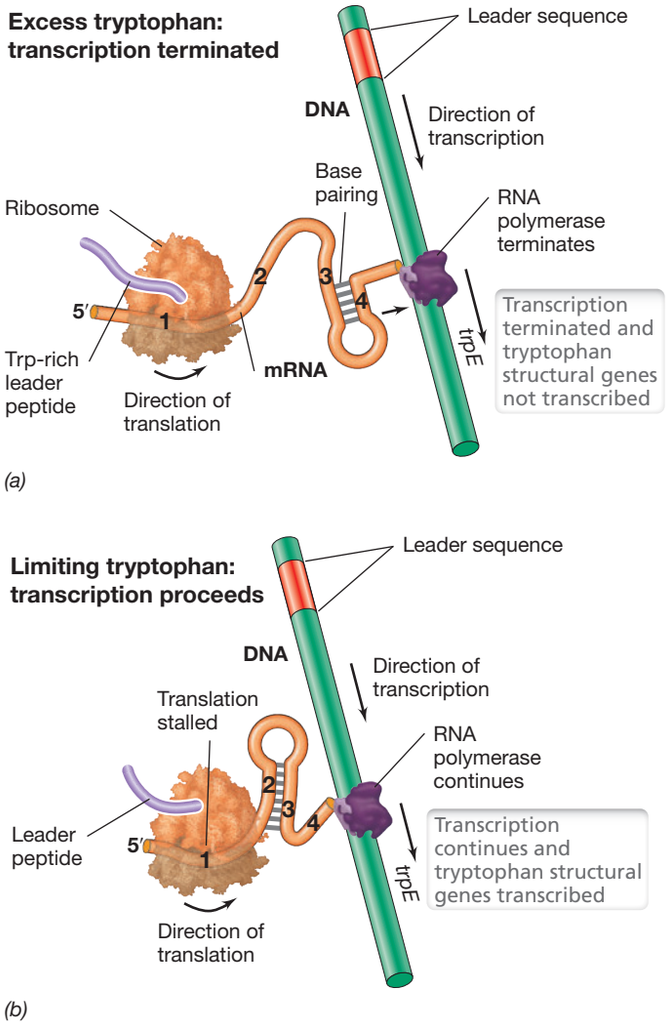


Figure 7.33 Mechanism of attenuation. Control of transcription of the *Escherichia coli* tryptophan (*trp*) operon structural genes by attenuation. The leader peptide is encoded by regions 1 and 2 of the mRNA. Two regions of the growing mRNA chain are able to form double-stranded loops, labeled as 3:4 and 2:3. *(a)* When there is excess tryptophan, the ribosome translates the complete leader peptide, and so region 2 cannot pair with region 3. Regions 3 and 4 then pair to form a loop that terminates transcription. *(b)* If translation is stalled because of tryptophan starvation, a loop forms by pairing of region 2 with region 3, loop 3:4 does not form, and transcription proceeds past the leader sequence.

V • Regulation of Enzymes and Other Proteins

Once a protein is made, its activity may still be subject to control. This ensures that cellular metabolites are synthesized in a balanced fashion and that energy is not expended making metabolites already present in excess of biosynthetic needs.

We have just explored some of the key mechanisms for regulating the *amount* (or even the complete presence or absence) of an enzyme or other protein within a cell. Here we focus on the

mechanisms that cells employ to control the *activity* of enzymes already present in the cell through processes such as feedback inhibition and post-translational regulation.

7.15 Feedback Inhibition

A major means of controlling enzymatic activity is by **feedback inhibition**. This mechanism temporarily shuts off the reactions in an entire biosynthetic pathway. The reactions are shut off because an excess of the end product of the pathway inhibits activity of an early (typically the *first*) enzyme of the pathway. Inhibiting an early step effectively shuts down the entire pathway because no intermediates are generated for subsequent enzymes in the pathway (**Figure 7.34*a***). Feedback inhibition is reversible, however, because once levels of the end product become limiting, the pathway again becomes functional.

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Art Activity: Figure 7.34*a*, *b* Inhibition of enzyme activity

How can the end product of a pathway inhibit the activity of an enzyme whose substrate is quite unrelated to it? This occurs because the inhibited enzyme has two binding sites, the *active site* (where substrate binds, ◀ Section 3.5), and the *allosteric site*, where the end product of the pathway binds. When the end product is in excess, it binds at the allosteric site, changing the conformation of the enzyme such that the substrate can no longer bind at the active site (Figure 7.34*b*). When the concentration of the end product in the cell begins to fall, however, the end product no longer binds to the allosteric site, so the enzyme returns to its catalytic form and once again becomes active.

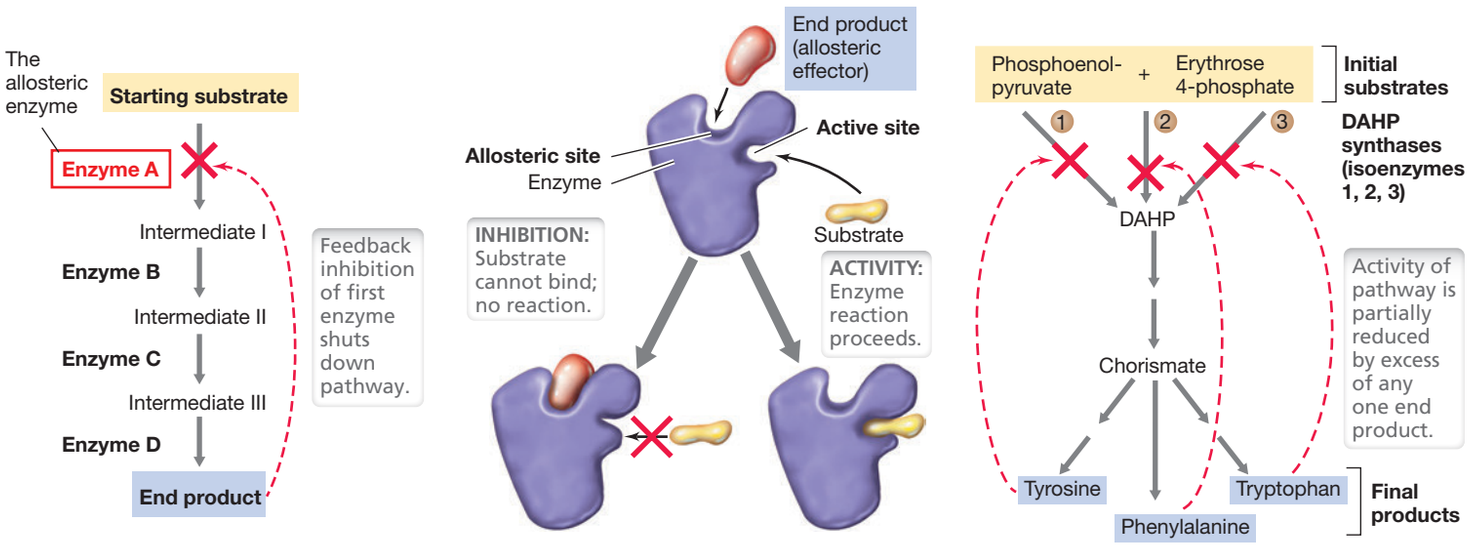
Some biosynthetic pathways controlled by feedback inhibition employ *isoenzymes* ("iso" means "same"). Isoenzymes are different proteins that catalyze the same reaction but are subject to different regulators, such as those required for the synthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine in *Escherichia coli*, a branched biosynthetic pathway (Figure 7.34*c*). Three isoenzymes catalyze formation of DAHP, an early intermediate of the pathway, and each is regulated independently by a different end product amino acid. In this way, synthesis of DAHP is inhibited *incrementally*, an excess of all three amino acids being necessary to completely shut down the pathway. This form of feedback inhibition prevents the excess of one or two of the amino acids from shutting down the entire pathway and thereby starving the cell for the third amino acid (Figure 7.34*c*).

Check Your Understanding

- What is feedback inhibition?
- What is the difference between an allosteric site and an active site?

7.16 Post-Translational Regulation

We have already discussed *phosphorylation* and *methylation*, two very common mechanisms for regulating a protein post-translationally, when we considered two-component regulatory systems and chemotaxis, respectively (Sections 7.5 and 7.6). Biosynthetic enzymes can also be regulated by the attachment of other small molecules, such as the nucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP), and uridine monophosphate (UMP). Throughout this chapter we have also encountered avenues in which the cell regulates the activity of proteins by protein–protein interactions. In contrast to these, some enzymes are regulated by *covalent modification*, typically the attachment or removal of a small molecule to or from the enzyme that subsequently affects its catalytic activity, and we consider this regulatory mechanism here.



(a) **Feedback inhibition** *(b)* **Allosteric inhibition** *(c)* **Isoenzyme inhibition**

Figure 7.34 Inhibition of enzyme activity. *(a)* In feedback inhibition, the activity of the first enzyme of the pathway is inhibited by the end product, thus shutting off the production of the three intermediates and the end product. *(b)* The mechanism of allosteric inhibition by the end product of a pathway. When the end product binds at the allosteric site, the conformation of the enzyme is so altered that the substrate can no longer bind to the active site. However, inhibition is reversible, and end product limitation will once again activate the enzyme. *(c)* Inhibition by isoenzymes. In *Escherichia coli*, the pathway leading to the synthesis of the aromatic amino acids contains three isoenzymes, each of which form a key intermediate, DAHP. Each isoenzyme is feedback-inhibited by a different aromatic amino acid. An excess of all three amino acids is required to completely shut off synthesis of DAHP and thus shut down the pathway.

Regulation of PII Signal Transduction Proteins

PII proteins are a widespread family of signal-transducing proteins (Section 7.5) found in *Bacteria*, *Archaea*, and the plastids of plants. PII proteins play an important role in nitrogen metabolism by regulating a diverse range of transcription factors, enzymes, and membrane transport proteins (we have already seen one instance of PII-based regulation with the Ntr system, Figure 7.14*b*). The activity of PII proteins on their regulatory cascade depends on whether they have been covalently modified or not. These modifications range from *uridylylation* (addition of a UMP group, Figure 7.14*b*), *adenylylation* (addition of AMP), or even *phosphorylation* in some organisms. Because these covalent modifications affect protein activity and occur *after* the PII proteins have been fully synthesized, the regulation is *post-translational*. Here we consider PII and its uridylylation by GlnD, a bifunctional enzyme that possesses both uridylyltransferase activity and uridyl-removal activity in response to levels of the amino acid glutamine (**Figure 7.35**).

One of the main ways the cell determines if ammonia (NH_3) assimilation is necessary is through glutamine sensing by GlnD. If the cellular glutamine pool is low, it signals that NH_3 assimilation is necessary. GlnD transduces this signal through the binding of glutamine itself. When GlnD is not bound to glutamine, the enzyme possesses uridylyltransferase activity and adds a UMP group to PII, yielding PII-UMP by post-translational modification.

One protein target of PII-UMP within the nitrogen metabolism pathway is glutamine synthetase adenyltransferase (Figure 7.35*a*). Once uridylylated, PII-UMP stimulates glutamine synthetase adenyltransferase to remove adenyl groups from glutamine synthetase (GS). GS is a key energy-requiring enzyme in ammonia (NH_3) assimilation (◀ Section 3.14) and must be tightly regulated to conserve energy if cellular levels of nitrogen are high, as sensed by the glutamine pool. As GS becomes less adenylylated, its activity *increases* and NH_3 assimilation increases (Figure 7.35*c*). Once glutamine levels are sufficient, the cell no longer needs to assimilate NH_3. This results in GlnD binding to available glutamine, which stimulates the protein to remove uridyl groups from PII-UMP (Figure 7.35*b*). PII in its unmodified form stimulates glutamine synthetase adenyltransferase to adenylate GS, forming GS-AMP. Fully adenylylated GS is *less active* and thus NH_3 assimilation by way of GS diminishes.

Why does all this elaborate regulation surround the enzyme GS? It is basically a matter of conserving energy. Because it has such a high affinity for its substrate, GS can assimilate ammonia present at very low levels, but this capability comes at a cost—the expenditure of ATP. However, when NH_3 is present at high levels in the cell, it can be assimilated into amino acids by enzymes that do not consume ATP; under these conditions, GS in the cell remains inactive. Then, when NH_3 levels are low, GS activity increases. By having GS active only when NH_3 is limiting, the cell conserves ATP that could be used to drive other energy-consuming processes in the cell.

Inactivation of Sigma Factors

In Section 7.11 we described how the σ factor RpoH is inactivated by DnaK under normal temperature conditions in the heat shock response (Figure 7.27). Proteins known as *anti-sigma* factors can

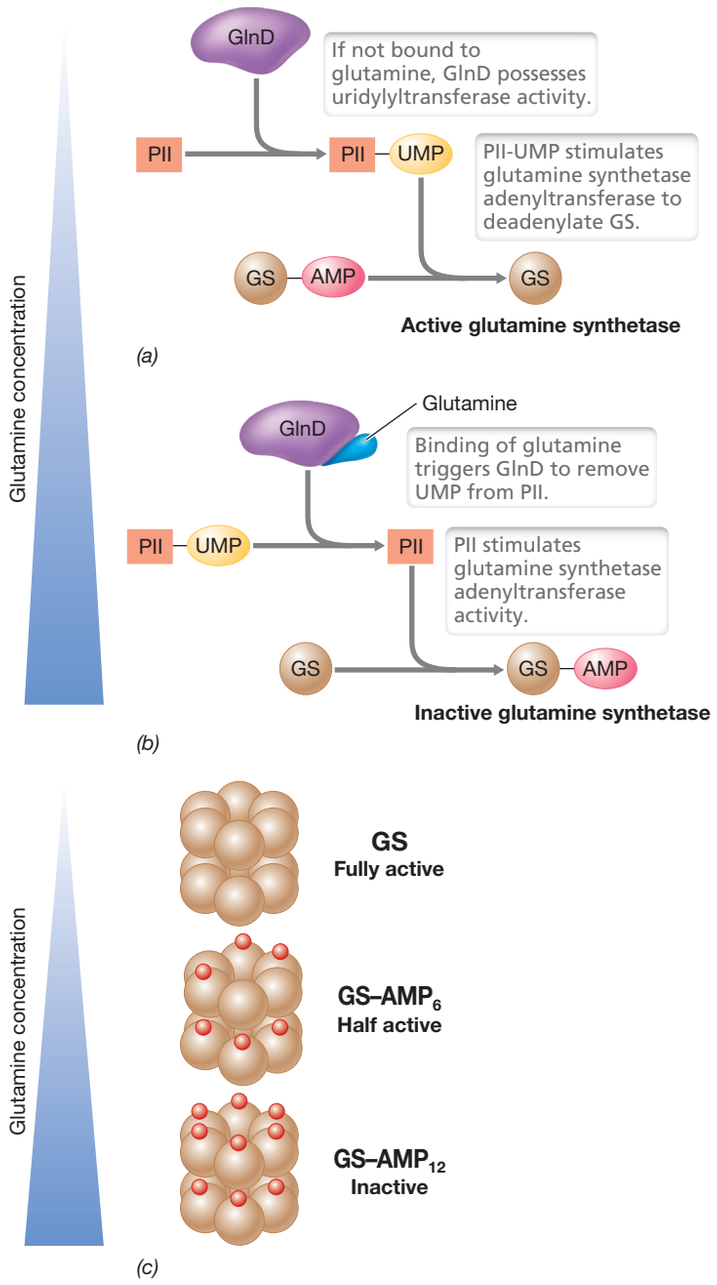


Figure 7.35 Post-translational regulation of PII and glutamine synthetase. Glutamine synthetase is a key enzyme of ammonia incorporation, especially when ammonia is present at low concentrations, in all *Bacteria* and *Archaea* (◀ Section 3.14). *(a)* Uridylylation of PII and activation of glutamine synthetase (GS), a multi-subunit enzyme. In response to low cellular pools of glutamine, the uridylyltransferase activity of GlnD is stimulated. This converts PII to PII-UMP, which triggers glutamine synthetase adenyltransferase to activate GS by removing AMP(s). *(b)* Removal of UMP from PII and inactivation of GS. In response to high cellular pools of glutamine, the UMP removal activity of GlnD is activated. This converts PII-UMP to PII, which triggers glutamine synthetase adenyltransferase to inactivate GS through adenylylation. *(c)* Overall control of GS activity. Adenylylated GS subunits are catalytically inactive, so the overall GS activity increases progressively as glutamine levels decrease and more subunits are deadenylylated. Conversely, GS activity decreases progressively as glutamine levels increase and more subunits are adenylylated.

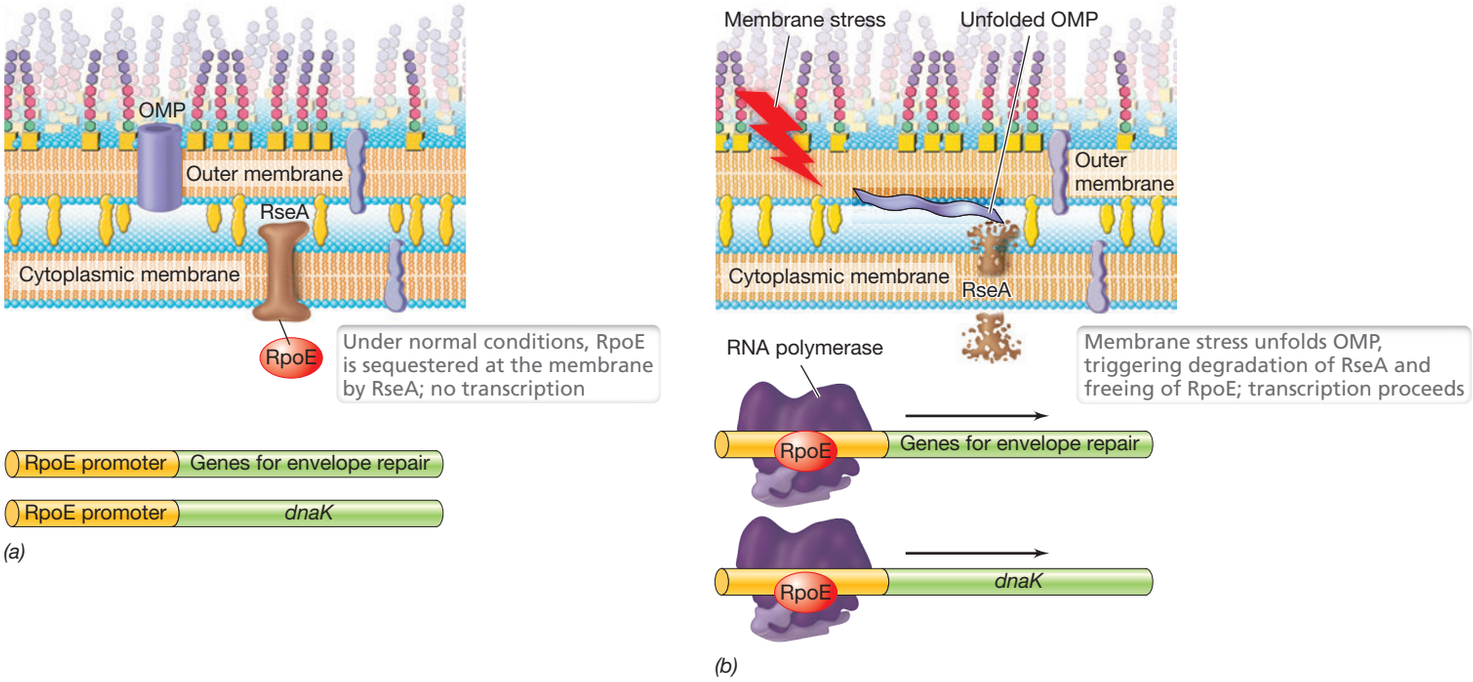


Figure 7.36 **Anti-sigma–sigma factor interactions.** *(a)* Sequestration of the RpoE sigma factor by the anti-sigma factor RseA. When the outer membrane is not stressed, cytoplasmic membrane–bound RseA binds to RpoE. This binding prevents the sigma factor from binding to promoters of genes for envelope repair and *dnaK*. *(b)* Inactivation of RseA and release of the RpoE sigma factor. When the outer membrane is exposed to stress, outer membrane proteins (OMPs) are denatured. These unfolded OMPs trigger proteolytic degradation of RseA. Without RseA, the RpoE sigma factor can bind to its promoter regions and transcribe genes for envelope stress and *dnaK* to help repair the membrane and refold proteins.

also bind to sigma factors, inactivating them in a form of post-translational regulation.

RpoE (σ^{24} or σ^{E}, ◀ Table 6.3) is a sigma factor widespread in *Bacteria* that responds to extracytoplasmic stress by recognizing promoters for genes that encode products necessary for proper folding, expression, and turnover of proteins that reside in the outer membrane (◀ Section 2.4). One of these promoters is for *dnaK*. Under nonstressful conditions, the membrane-bound anti-sigma factor RseA sequesters RpoE from binding to the promoters it targets (**Figure 7.36*a***). However, if a cell encounters a condition stressful to the membrane such as heat or osmotic stress, outer membrane proteins are denatured, and this *activates* a protease specific for RseA (Figure 7.36*b*). Once RseA has been degraded, RpoE is free to activate transcription of genes necessary for cell envelope repair such as refolding outer membrane proteins and the synthesis of phospholipids and lipopolysaccharides. Release of RpoE also includes increasing the level of DnaK produced so that this chaperone (◀ Section 6.11) can assist with folding and turnover of outer membrane proteins as well.

Once the membrane has been repaired, RseA is no longer targeted for degradation and reassociates with the cytoplasmic membrane. In this form, RseA can resume its anti-sigma factor activity by binding and sequestering RpoE from promoter regions. This leads to a *decreased* expression of both genes encoding products needed to respond to membrane stress and *dnaK*. Another well-studied example of regulation by anti-sigma–sigma interactions occurs during the formation of endospores in *Bacillus* when the anti-sigma factor SpoIIAB binds to σ^{F}, thereby preventing its association with RNA polymerase; we pick up on this control process in Section 8.6 and Figure 8.18.

Regardless of the mechanism, in the final analysis it should be clear that regulating the synthesis and activities of a cell's RNAs and proteins is (1) very important to its biology, (2) possible in many different ways, and (3) a major genetic investment. But the costs to the cell are worth it and are grounded in billions of years of evolutionary refinement. At every turn in a highly competitive world, a microbe's growth and very survival may well depend on its ability to conserve its resources and maximize its progeny.

Check Your Understanding

- What types of covalent modifications commonly alter the activity of proteins?
- How does GlnD signal to the cell that NH_3 assimilation is necessary?
- Explain the role of an anti-sigma factor.

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CHAPTER REVIEW

I • DNA-Binding Proteins and Transcriptional Regulation

7.1 Proteins can bind to DNA when specific protein domains bind to specific regions of the DNA molecule. In most cases the interactions are sequence-specific.

Q Explain how a protein ensures that it binds specifically to only a certain region of DNA and not to another.

7.2 Transcription factors are DNA-binding proteins that control gene expression. Activator and repressor proteins are common transcription factors whose DNA binding is controlled by the presence or absence of effector molecules.

Q What is an allosteric protein?

7.3 The amount of a specific enzyme in the cell can be controlled by regulatory proteins that bind to DNA and increase (induce) or decrease (repress) the amount of mRNA that encodes the enzyme.

Q How is β-galactosidase induced in cells?

7.4 *Archaea* resemble *Bacteria* in using DNA-binding activator and repressor proteins to regulate gene expression at the level of transcription.

Q What are the two mechanisms used by archaeal repressor proteins to repress transcription?

II • Sensing and Signal Transduction

7.5 Signal transduction systems transmit environmental signals to the cell. In *Bacteria* and *Archaea*, signal transduction is typically carried out by a two-component regulatory system that includes a membrane-integrated sensor kinase and a cytoplasmic response regulator. The activity of the response regulator depends on its state of phosphorylation.

Q What are the two components that give their name to the signal transduction system in prokaryotic cells? What is the function of each of the components?

7.6 Chemotactic behavior allows cells to respond to attractants and repellents in their environment. The regulation of chemotaxis affects the activity of proteins rather than their synthesis. Adaptation by methylation allows the system to reset itself to the continued presence of a signal.

Q Adaptation allows the mechanism controlling flagellar rotation to be reset. How is this achieved?

7.7 Quorum sensing allows cells to monitor their environment for cells of their own kind. Quorum sensing depends on the sharing of specific small molecules known as autoinducers. Once a sufficient concentration of the autoinducer is present, specific gene expression is triggered.

Q How can quorum sensing be considered a regulatory mechanism for conserving cell resources?

III • Global Control

7.8 Global control systems regulate the expression of many genes simultaneously. Catabolite repression is a global control system that helps cells make the most efficient use of available carbon sources. The *lac* operon is under the control of catabolite repression as well as its own specific negative regulatory system.

Q Describe the mechanism by which cAMP receptor protein (CRP), the regulatory protein for catabolite repression, functions. Use the lactose operon as an example.

7.9 The stringent and general stress responses are deployed to survive nutrient limitation and stresses by decreasing expression of genes for macromolecule biosynthesis and activating stress survival pathways.

Q Explain the sequence of molecular events that leads to the synthesis of (p)ppGpp in *Escherichia coli* during the stringent response.

7.10 The phosphate (Pho) regulon employs a two-component regulatory system to sense cellular levels of phosphate. The cytoplasmic transcriptional regulator of this regulon binds to promoter regions called Pho boxes and functions as either an activator or a repressor depending on phosphate levels in the cell.

Q Explain how the binding of Pho transcriptional regulator activates transcription of some genes within the Pho regulon, but not others.

7.11 Cells respond to excessive temperature and other stressors that lead to protein denaturation by employing the heat shock response. This response results in expressing sets of genes whose products help the cell overcome stress, including an alternative sigma factor.

Q Describe the proteins produced when cells of *Escherichia coli* experience a heat shock. Of what value are they to the cell?

IV • RNA-Based Regulation

7.12 Cells can control genes in several ways by employing regulatory RNA molecules. One way is to take advantage of base pairing and use small RNAs to promote or prevent translation of mRNAs.

Q What are the mechanisms by which regulation by sRNA occurs?

7.13 Riboswitches are sequences at the 5′ ends of certain mRNAs that recognize small molecules and respond by changing their three-dimensional structure to affect translation or transcriptional termination of the mRNA. Riboswitches are mostly employed to control biosynthetic pathways for vitamins, amino acids, purines, and a few other metabolites.

Q What are the mechanisms by which riboswitches regulate translation and transcription?

7.14 Attenuation is a mechanism whereby transcription is controlled after initiation of mRNA synthesis. Attenuation mechanisms depend upon alternative stem–loop structures in the mRNA that result in either read-through or stalling of the ribosome.

Q Describe in detail how a tryptophan attenuator is controlled.

V • Regulation of Enzymes and Other Proteins

7.15 In feedback inhibition, an excess of the final product of a biosynthetic pathway inhibits an allosteric enzyme at the beginning of the pathway. Enzyme activity can also be modulated by isoenzymes.

Q Describe how feedback inhibition is reversible, and why are isoenzymes necessary for some forms of feedback inhibition?

7.16 Protein activity can be regulated after translation. Reversible covalent modification or interactions with other proteins can modulate protein activity.

Q How can post-translational modifications regulate the activity of an enzyme?

APPLICATION QUESTIONS

1. What would happen to regulation from a promoter under repression if the region where the regulatory protein binds was deleted? What if the promoter was under positive control? Promoters from *Escherichia coli* under positive control are not close matches to the promoter consensus sequence for *E. coli*. Why?
2. Most of the regulatory systems described in this chapter employ regulatory proteins. However, regulatory RNA is also important. Describe how one could achieve negative control of the *lac* operon using either of two different types of regulatory RNA.
3. Many amino acid biosynthetic operons under attenuation control are also under negative control. Considering that the environment of a bacterium can be highly dynamic, what advantage could be conferred by having attenuation as a second layer of control?
4. How would you design a regulatory system to make *Escherichia coli* use succinic acid in preference to glucose? How could you modify it so that *E. coli* prefers to use succinic acid in the light but glucose in the dark?

CHAPTER GLOSSARY

Activator protein a regulatory protein that binds to specific sites on DNA and stimulates transcription

Allosteric protein a protein containing an active site for binding substrate and an allosteric site for binding an effector molecule such as the end product of a biochemical pathway

Attenuation a mechanism for controlling gene expression that terminates transcription after initiation but before a full-length messenger RNA is produced

Autoinducer a small signal molecule that takes part in quorum sensing

Catabolite repression the suppression of alternative catabolic pathways by a preferred source of carbon and energy

Cyclic AMP a regulatory nucleotide that participates in catabolite repression

Domain region of a protein with specific structure and function

Enzyme induction production of an enzyme in response to a signal (often the presence of the substrate for the enzyme)

Enzyme repression prevention of the synthesis of an enzyme in response to a signal

Feedback inhibition a process in which an excess of the end product of a multistep pathway inhibits activity of the first enzyme in the pathway

Gene expression transcription of a gene followed by translation of the resulting mRNA into protein

Heat shock proteins proteins induced by high temperature (or certain other stresses) that protect against high temperature, especially by refolding partially denatured proteins or by degrading them

Heat shock response response to high temperature that includes the synthesis of heat shock proteins together with other changes in gene expression

Noncoding RNA (ncRNA) RNA that is not translated into protein; examples include ribosomal RNA, transfer RNA, and small regulatory RNAs

Operon two or more genes transcribed into a single RNA and under the control of a single regulatory site

Quorum sensing a regulatory system that monitors the population level and controls gene expression based on cell density

Regulatory nucleotide a nucleotide that functions as a signal rather than being incorporated into RNA or DNA

Regulon a series of operons controlled as a unit

Repressor protein a regulatory protein that binds to specific sites on DNA and blocks transcription

Response regulator protein one of the members of a two-component regulatory system; a protein that is phosphorylated by a sensor kinase and then acts as a regulator, often by binding to DNA

Riboswitch an RNA domain, usually in a messenger RNA molecule, that can bind a specific small molecule, altering its own secondary structure; this, in turn, controls translation of the mRNA

Sensor kinase protein one of the members of a two-component regulatory system; a protein that phosphorylates itself in response to an external signal and then transfers the phosphoryl group to a response regulator protein

Signal transduction see *two-component regulatory system*

Stringent response a regulatory mechanism that detects nutrient or environmental stresses and antibiotics

Transcription factor a regulatory protein that binds to specific sites on DNA and controls transcription; generally activator and repressor proteins

Two-component regulatory system a regulatory system consisting of two proteins: a sensor kinase and a response regulator

8 Molecular Aspects of Microbial Growth

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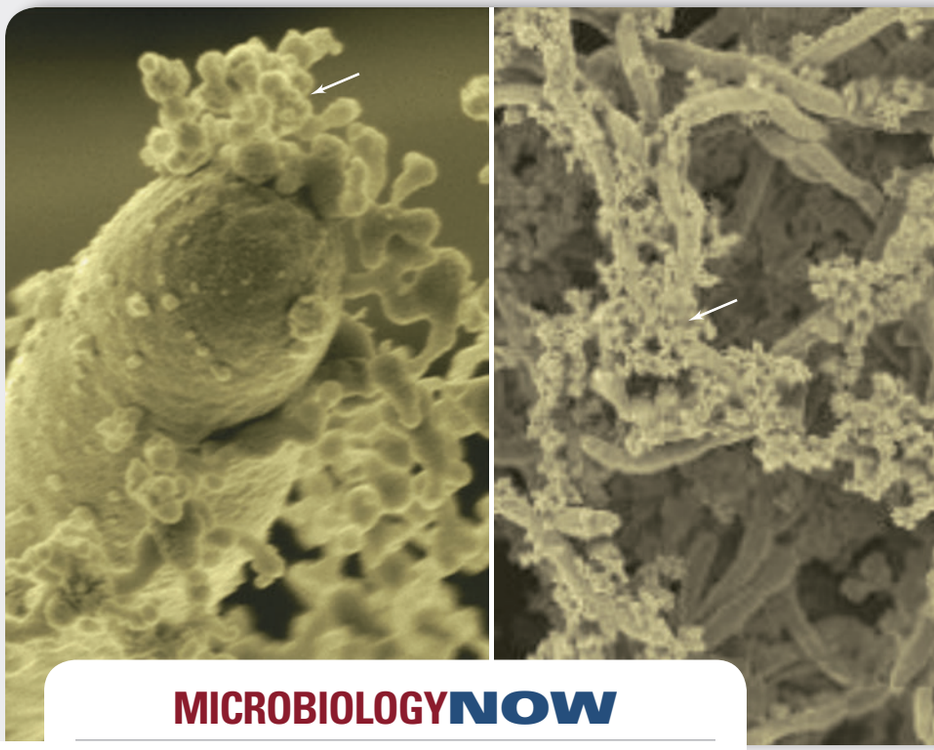
III Antibiotics and Microbial Growth 291



MICROBIOLOGYNOW

Membrane Vesicles: Nano Vehicles Transporting Important Cargo

Membrane vesicles are nano-sized structures (10–400 nm) released from cells of all three domains of life during membrane budding. These vesicles can contain a variety of biomolecules such as nucleic acids, carbohydrates, proteins, enzymes, toxins, and even viruses. Because of the lipid bilayer protecting the cargo, membrane vesicles are excellent vehicles for transferring substances between cells.

Remarkably, recent studies show that vesicle size and content are not random but are controlled by cellular signals. Thus, microbes are able to employ these tiny vehicles for an assortment of activities such as DNA exchange between cells, toxin delivery into host cells, reducing competition through antimicrobial release, and biofilm formation. The study here deals with the biofilm way of life.

Biofilms are slimy, three-dimensional microbial communities formed to withstand environmental challenges. One of the major requirements for biofilm formation is the release of sticky extracellular polymeric substances (EPS) to help hold the biofilm matrix together. The image on the left shows a scanning electron micrograph of the eukaryotic microbe *Candida albicans* (elongated cell) releasing membrane vesicles (arrow) containing such polysaccharides for biofilm construction.

Pathogenic strains of *C. albicans* often form biofilms on indwelling medical devices, resulting in yeast infections that evade the immune system and are resistant to antifungal drugs. Microbiologists investigating methods to treat these impervious biofilms also discovered specific enzymes within the vesicles that modify the EPS in a manner that makes the biofilm resistant to antifungal agents (image to the right, arrow denotes EPS). By contrast, vesicles from free-living cells contain completely different cargo. Strains of *C. albicans* genetically modified to limit their vesicle production produced yeast biofilms sensitive to antifungal drugs.

Research on membrane vesicles highlights the importance of ongoing studies of microbial growth and how new targets for attack by therapeutic agents can be exposed by basic research to understand the molecular aspects of microbial growth.

Source: Zarnowski, R., et al. 2018. *Candida albicans* biofilm-induced vesicles confer drug resistance through matrix biogenesis. *PLoS Biol. 16*(10): e2006872.

In previous chapters we discussed the basic biology of microbes (Chapters 1–3 and Chapter 5) and how prokaryotic cells grow and divide (Chapter 4), replicate their chromosome(s) (Chapter 6), and regulate gene expression (Chapter 7). Here we follow up on the major concepts that unfolded in Chapter 4 with a focus on key molecular processes that occur in the microbial growth cycle. We also consider cellular differentiation in some model bacteria, revisit the process of biofilm growth, and close by exploring how some common antibiotics affect microbial growth and the weapons bacteria deploy to counter such attacks.

I • Bacterial Cell Division

Prokaryotic cell division is preceded by chromosome replication and the synthesis of new cell wall material in a way that defines cell shape. Cell division is orchestrated in a carefully controlled fashion by protein complexes whose activities can be visualized by powerful light microscopic techniques.

Most cells divide by binary fission (◀ Section 4.6 and Figure 4.8), and this process occurs in a defined series of steps such that each daughter cell obtains a copy of the genome. During the division cycle, the cell must also produce new peptidoglycan and cytoskeleton elements to prevent bursting from osmotic forces. This cytoskeleton gives the cell its distinct morphology (◀ Figure 1.8). To successfully orchestrate all of these events, various regulatory cascades are put into play. In this first part of the chapter we focus on the molecular mechanisms employed by two well-studied gram-negative bacteria, *Escherichia coli* and *Caulobacter crescentus*, and introduce advanced microscopic techniques that have revealed the major molecular events that underlie cell division and cell morphology.

8.1 Visualizing Molecular Growth

In Chapter 1 we discussed transmission electron microscopy and electron cryotomography as powerful techniques to visualize macromolecules such as chromosomes, proteins, and membranes. In addition to these, **super-resolution microscopy** is a powerful form of *light* microscopy that employs a suite of fluorescent molecules to reveal even the tiniest details within a cell. Super-resolution techniques can resolve structures as small as 5–50 nm in living cells, allowing for dynamic cellular behaviors to be observed in real time.

Fluorescent Tagging

To visualize the localization of specific proteins and to monitor gene expression in cells, **reporter genes** are valuable tools. Reporter genes encode proteins that are easy to detect or assay and are used by fusing them to genes of interest in such a way that both the reporter gene and the gene of interest are coexpressed. For visualizing molecular events, reporter genes that encode fluorescent products such as the **green fluorescent protein (GFP)** are routinely used (**Figure 8.1**). Through the use of fluorescence microscopy (◀ Section 1.8) and multiple fluorescent reporter proteins, the activity and localization of different macromolecules can be determined simultaneously.

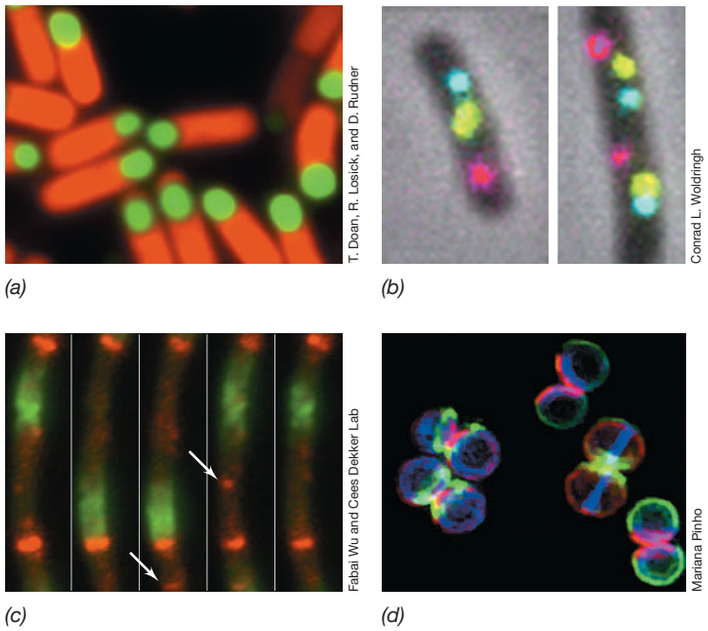


Figure 8.1 Fluorescence micrographs illustrating molecular growth characteristics. *(a)* During endospore formation in *Bacillus*, alternative sigma factors are localized to specific regions of the cell (Section 8.6). σ^F linked to GFP indicates activity of the σ^F protein in the developing endospore (at one end of each cell). σ^E linked to a reporter protein that fluoresces red indicates expression and activity of the σ^E protein throughout the mother cell prior to endospore formation. Regions correspond to the model depicted in Figure 8.18*b*. *(b)* Location and orientation of the *Escherichia coli* chromosome during cell cycle progression (Section 8.2). Regions are visible due to tagged DNA-binding proteins targeting the *oriC* (green), left arm (red), and right arm (cyan). Modified from Woldringh, C.L., Hansen, F.G., Vischer, N.O.E., and Atlung, T. 2015. *Front. Microbiol. 6:* 448. *(c)* Time-lapse series showing FtsZ (red) and MinD (green) in an elongated *E. coli* cell. Cell division was inhibited by antibiotic treatment and arrows indicate locations where unstable FtsZ proteins polymerize and depolymerize. Modified from Wu, F., Van Rijn, E., Van Schie, B.G.C., Keymer, J.E. and Dekker, C. 2015. *Front. Microbiol. 6:* 607. *(d)* Peptidoglycan synthesis in *Staphylococcus aureus*. The incorporation of amino acids is visible because a different fluorophore is linked to each D-amino acid present in peptidoglycan. The colors show that peptidoglycan synthesis begins at the septum region and continues in a concentric fashion around the growing cell. Modified from Pereira, A.R., et al., 2016. *MBio 7*(5): doi:10.1128/mBio.00908-16.

Figure 8.1*a* demonstrates use of GFP and another fluorescent protein to determine the cellular locations of σ^F and σ^E, two spore-specific sigma factors (◀ Section 6.5) that play key roles in endospore development in *Bacillus* (◀ Section 2.8 and Section 8.6).

Fluorescent tagging can also be used to resolve different genetic elements in a cell, such as a chromosome and a plasmid (see Figure 8.7). In fact, even different loci within an individual nucleic acid molecule can be observed in a living cell. While DNA or RNA loci are not linked directly to a reporter gene like GFP, genes encoding nucleic acid–binding proteins specific for target loci can be fused to a reporter gene. If necessary, the corresponding DNA or RNA binding sites can be introduced into the nucleic acid region of interest using recombinant techniques (Chapter 12). Figure 8.1*b* illustrates how separate "arms" of the *Escherichia coli* chromosome can be visualized in a living cell by engineering a strain to express different fluorescently tagged proteins that bind to different regions of the DNA.

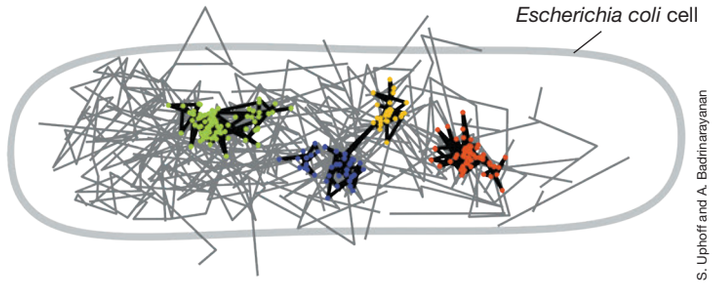
Advances in protein labeling and microscopy techniques have yielded new fluorescent variant proteins that fold quickly and allow for visualization of both spatial and temporal protein dynamics in live cells. Figure 8.1*c* illustrates the spatiotemporal dynamics of MinD and FtsZ (proteins of the divisome, see Section 8.3) in a growing cell over a time-lapse series. Other cellular aspects can be visualized by linking individual fluorophores (small fluorescent chemical compounds) to specific molecules such as amino acids. For example, Figure 8.1*d* illustrates the dynamic synthesis of peptidoglycan in growing *Staphylococcus aureus* cells through the incorporation of three separately fluorophore-labeled amino acids present in the cell wall.

Super-Resolution Techniques

Although fluorescence microscopy enables observation of tagged internal cellular structures, resolution is insufficient to observe the molecular events occurring during the cell cycle. By contrast, super-resolution techniques can reveal and quantify elements as small as *single molecules* in living cells. Super-resolution techniques employ photoactivated probes that switch from bright to dark emission states depending on the wavelength of light that strikes them; this allows for only one fluorescent molecule (or target) to be excited at a time. By recording the position of individual molecules over time, a super-resolution image is generated (**Figure 8.2*a***).

Super-resolution techniques can reveal the details of cellular superstructures, for example by precisely mapping the diffusion and movement of individual proteins. Figure 8.2*a* illustrates the use of *photoactivated localization microscopy*, a form of super-resolution microscopy, to map the movement of individual MukB molecules in a living *E. coli* cell. MukBEF is a protein complex that binds to the nucleoid and localizes to distinct regions in the cell to assist with unknotting daughter chromosomes after replication (Section 8.2). Not only can super-resolution microscopy be used to observe structural changes to cellular complexes over time, the micrographs can also be modified to construct three-dimensional (3D) images. Figure 8.2*b* illustrates the use of a super-resolution method called *3D subdiffraction* imaging to observe the arrangement of different protein complexes in the budding bacterium *Caulobacter crescentus*.

By providing spatiotemporal information about individual proteins, super-resolution techniques have been instrumental in proving that like their eukaryotic counterparts, prokaryotic cells contain a highly dynamic subcellular structural organization whose activities during the cellular growth process can be tracked at the molecular level. We now focus on some of these key molecular events as we revisit the growth cycle of prokaryotic cells in detail.



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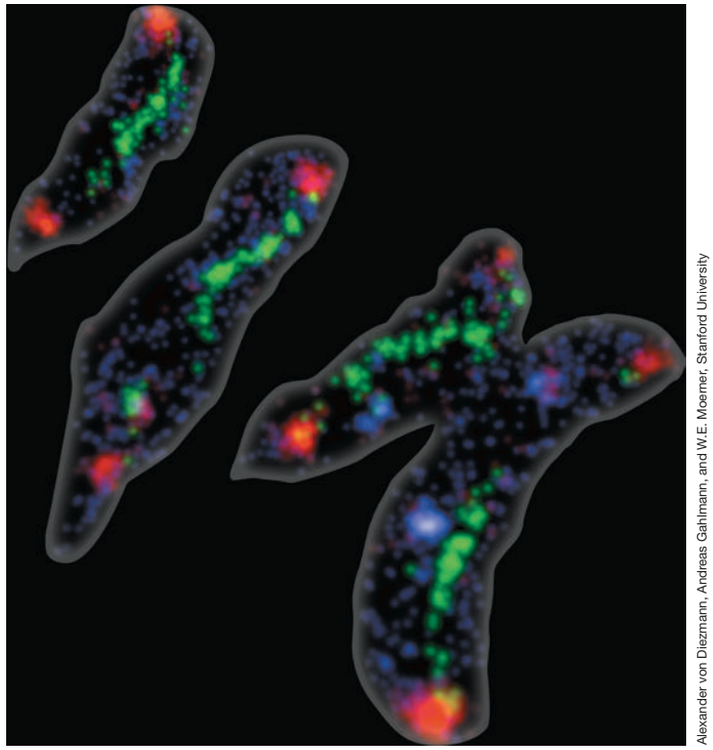


(b)

Figure 8.2 Super-resolution imaging of molecular growth characteristics. *(a)* Photoactivated localization micrograph (PALM) mapping the movement of the nucleoid-binding protein MukB in an individual *Escherichia coli* cell. The four different colors represent clusters of nucleoid-bound MukB molecules, while tracks corresponding to their movement are represented by black lines. Gray lines represent tracks of free MukB (Section 8.2). *(b)* Three-dimensional quantitative multicolor imaging of *Caulobacter* cellular structures. PopZ is tagged with a red fluorescent reporter, while crescentin (Section 8.4) is tagged with a green fluorescent reporter.

Check Your Understanding

- What is the utility of a reporter gene?
- What are the advantages of using super-resolution microscopy versus standard fluorescence microscopy?

8.2 Chromosome Replication and Segregation

A prerequisite for bacterial growth is replication of the genome. Chromosome replication must be tightly regulated to coincide with cell division. After genome replication and prior to cell division, molecular mechanisms must also ensure that the daughter chromosomes are segregated (**Figure 8.3**). Because the process of cell division in bacterial and archaeal cells requires both temporal and spatial control elements, the process is controlled by a cell cycle (Figure 8.3). Temporally, two copies of the cell's genome must exist prior to cell division. Spatially, the two copies must be equally segregated and the septum must form at the correct location in the cell for a successful cell cycle to occur.

Regulation of Chromosome Replication Initiation

How does the cell ensure that the genome is completely replicated before cell division while also preventing multiple rounds of replication? Several different proteins play a role in initiating and

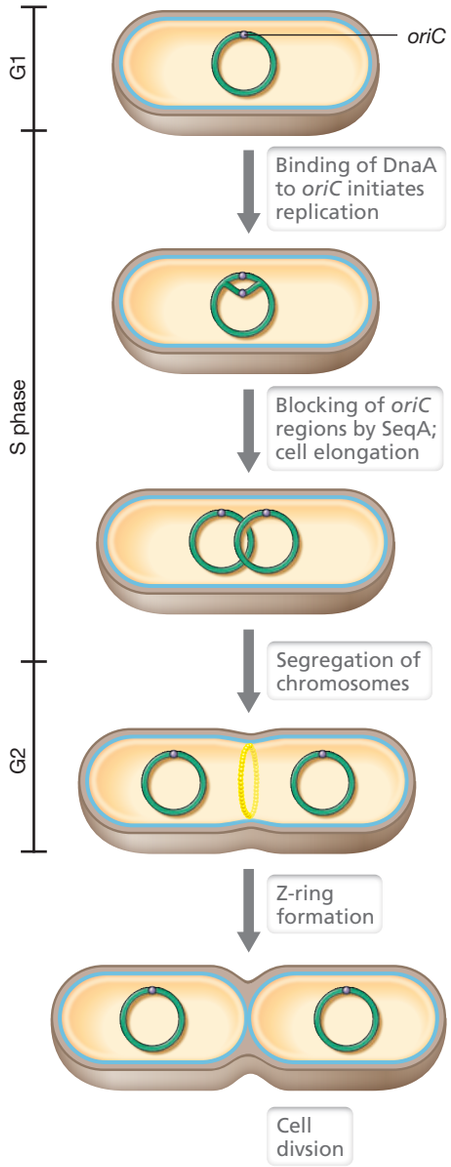


Figure 8.3 Overview of the bacterial cell cycle. Phases analogous to the growth 1 (G1), synthesis (S), and growth 2 (G2) phases of the eukaryotic cell cycle are represented.

inhibiting chromosome replication in *Escherichia coli*. Here we focus on a key protein in this regard, DnaA, along with a handful of accessory proteins necessary to complete replication initiation.

As we discussed in Section 6.3, binding of DnaA to specific DNA sequences within the *oriC* region of the chromosome leads to unwinding of the DNA and loading of the replisome for chromosome replication in *Escherichia coli* (Figure 8.3; to review DNA replication, see Figure 6.16). DnaA is most active when it is linked to a molecule of ATP, forming DnaA–ATP. To tightly control chromosome replication, multiple regulatory mechanisms come into play to inactivate DnaA–ATP once replication has initiated. These mechanisms include competition for *oriC* binding, repression of *dnaA* expression, titration of DnaA–ATP away from *oriC*, and inactivation of DnaA–ATP.

Before DNA replication initiates, both strands of the chromosome contain methyl groups on the adenine residue of –GATC– sequences within the chromosome. However, immediately after replication has initiated, only the parental strand remains methylated. This results in *hemimethylated DNA* and facilitates a competition for origin binding between DnaA–ATP and a DNA-binding protein called SeqA (**Figure 8.4**). Because hemimethylated *oriC* sequences are strongly bound by SeqA, DnaA–ATP is blocked from binding and reinitiating chromosome replication (Figures 8.3 and 8.4). Approximately 10 min after replication has initiated, GATC sequences within the newly synthesized daughter strand are methylated by a DNA adenine methylase.

As a result of the chromosomal proximity of the *dnaA* gene to *oriC* and of the promoter region (◀ Section 6.5) of *dnaA* possessing a GATC sequence, binding of SeqA also plays a role in repressing the expression of *dnaA*. Once replication has initiated, the promoter region for *dnaA* is quickly hemimethylated, and the binding of SeqA prevents RNA polymerase from binding and transcribing *dnaA* (Figure 8.4). Subsequently, expression of *dnaA* is also *autoregulated* by its corresponding protein binding to DnaA boxes within its promoter region.

The final mechanism of preventing DnaA–ATP from binding *oriC* is to decrease the ratio of DnaA–ATP to DnaA–ADP. How does the cell increase the level of DnaA–ADP when ATP dominates over ADP in a growing cell? This is controlled by the ATPase HdaA, which associates with DNA in the proximity of the replisome and specifically targets DnaA–ATP. This enzyme promotes the hydrolysis of ATP associated with DnaA in a replication-dependent manner and thus

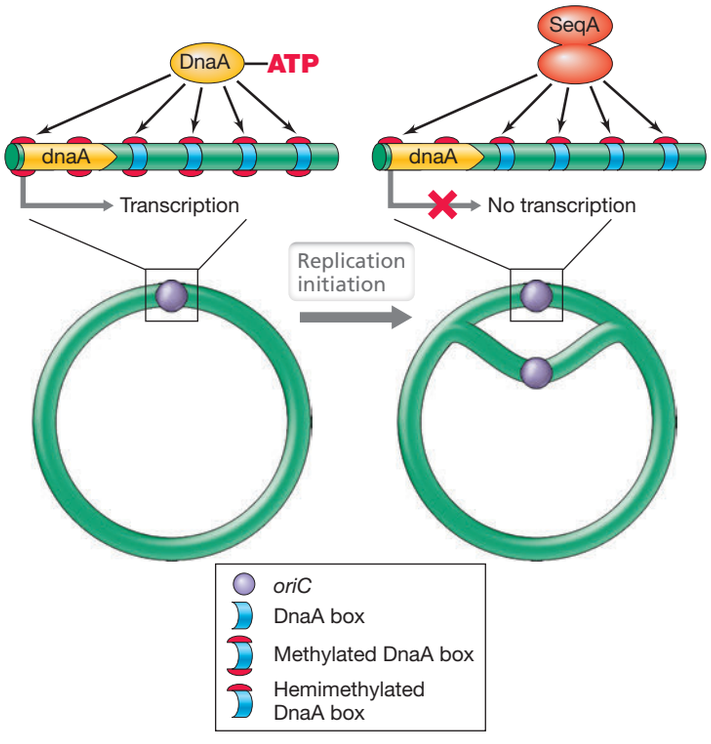


Figure 8.4 Binding of the *Escherichia coli oriC* by DnaA and SeqA proteins. DnaA–ATP only binds to fully methylated DnaA boxes, while SeqA binds to hemimethylated DNA. The binding of SeqA upstream of the *dnaA* gene leads to repression of transcription.

functions as a final method of regulating the initiation of chromosome replication. As a result of these combined mechanisms, the cellular level of DnaA–ATP oscillates during the cell cycle, reaching the maximum active amount when initiation of chromosome replication is needed and waning thereafter.

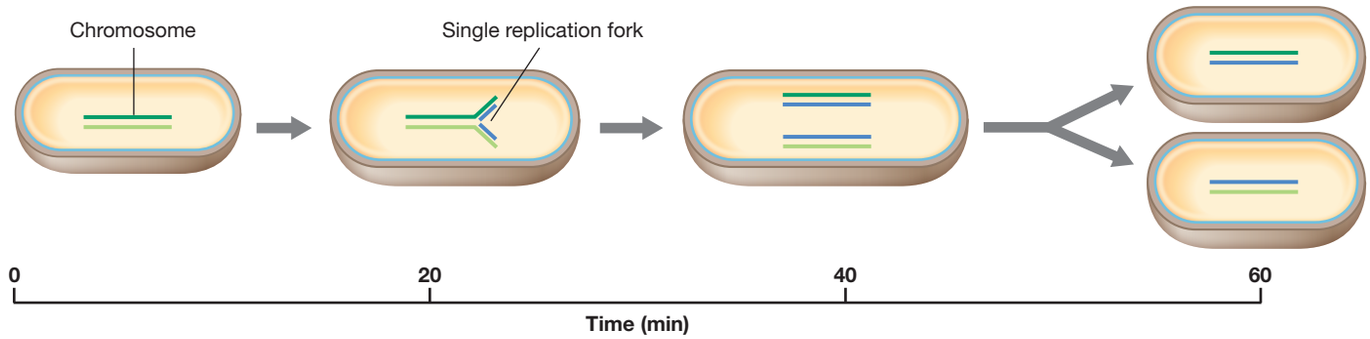
Genome Replication in Fast-Growing Cells

As we learned in Chapter 6, the circular nature of the chromosome of *E. coli* (and that of most other *Bacteria* and *Archaea*) creates an opportunity for speeding up DNA replication. This is because replication of circular genomes is *bidirectional* from the origin of replication. During bidirectional replication, synthesis occurs in both leading and lagging directions on each template strand, and this allows DNA to replicate as rapidly as possible (◀ Figure 6.15). Studies of chromosome replication in *E. coli* have shown that about 40 min is required for genome replication and that this value is independent of the generation time, which in *E. coli* can be as little as 20 min. If an *E. coli* cell is growing at twice the rate that its chromosome is replicating, how does the cell resolve this conundrum?

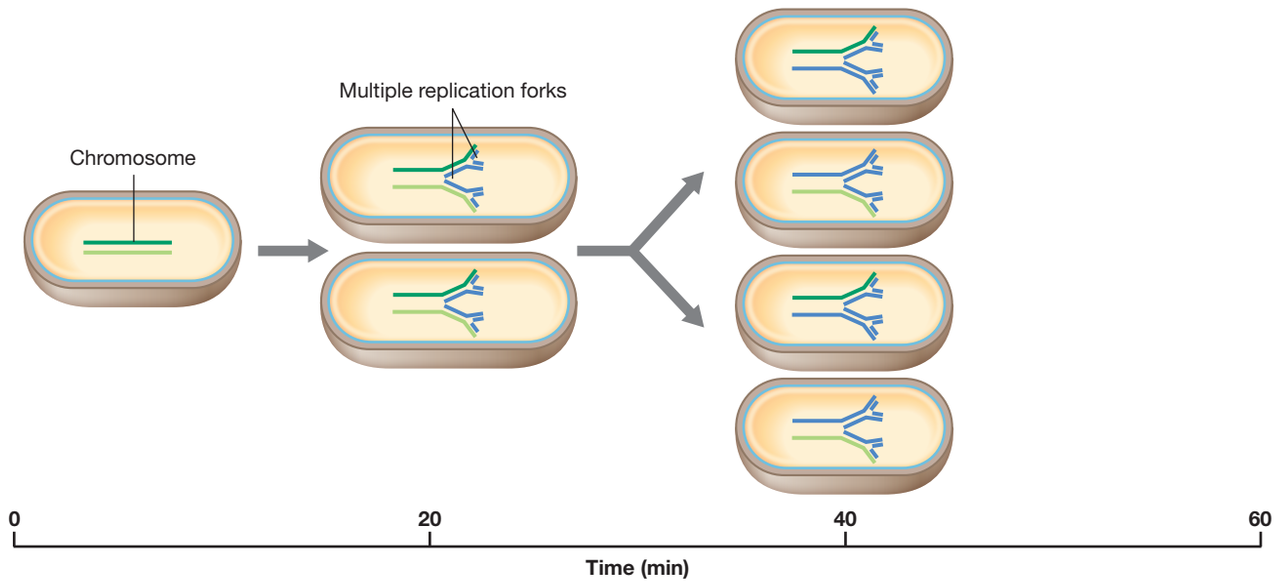
In cells growing at doubling times shorter than 40 min, *multiple DNA replication forks* are present in each cell. That is, a new round of DNA replication begins before the last round has been completed (**Figure 8.5**); as a result, some genes are present in more than one copy. This can occur after the DNA in the *oriC* region of the newly synthesized DNA has been methylated, which releases SeqA from the DNA and allows DnaA–ATP to be recruited to reinitiate another round of replication (Figure 8.4). This ensures that at generation times shorter than the time required for replication (a process that occurs at a constant and maximal speed), each daughter cell receives a complete genome at the time of septum formation.

Chromosome Segregation

During cell division, segregation of the chromosomes to the cell poles is needed not only to ensure that each daughter cell gets a copy of the genome, but also to allow for septum formation (Figure 8.3). If the two copies of the nucleoid remained in the center of the cell, nucleoid occlusion (a process that prevents the cell from dividing across the nucleoids) would impede proper cell division. In eukaryotes, mitotic spindles separate replicated chromosomes in mitosis (◀ Section 2.13). In many bacteria including the budding bacterium



(a) **Generation time, 1 h; replication time, 40 min.**



(b) **Generation time, 20 min; replication time, 40 min.**

Figure 8.5 Genome replication in cells of *Escherichia coli* growing at 60-min or 20-min generation times. In cells doubling every 20 min, multiple replication forks ensure that each daughter cell gets a complete copy of the genome, which takes 40 min to replicate. In cells doubling every 60 min, multiple replication forks are unnecessary.

Caulobacter, a similar mechanism called the *Par* (partitioning) *system* is used to distribute chromosomes and plasmids equally to progeny cells during growth (**Figure 8.6**). This system is composed of the ParA ATPase, the ParB chromosome-binding protein, and the PopZ complex, as well as a centromere-like *parS* sequence located near *oriC*.

Unlike eukaryotic mitotic spindles, the Par system does not segregate fully replicated chromosomes. Instead, PopZ proteins localize to the stalked pole of the cell and facilitate anchoring of the chromosome to this location by interacting with ParB bound to the *parS* sequence (Figure 8.6). Once chromosome replication initiates, the newly synthesized *parS* sequence binds to another molecule of ParB, which is then pulled to the stalked pole by the ATPase activity of ParA. Not only does PopZ help anchor *parS* of the parent chromosome to the stalked pole of the cell, it also helps recruit ParA to transfer the newly synthesized *parS* sequence bound to ParB to the nonstalked cell pole (Figure 8.6).

While *E. coli* lacks the Par system, the daughter chromosomes must still be segregated prior to cell division. After replication the resulting circular chromosomes remain interlinked or tangled, much like the links in a chain. This linkage is broken by the *structural maintenance of chromosome* complex, which is composed of a topoisomerase (IV) (◀ Section 6.1) and MukBEF proteins. Super-resolution images show that the MukBEF proteins move to discrete locations within the nucleoid (Figure 8.2*a*) and recruit a topoisomerase to separate replicated

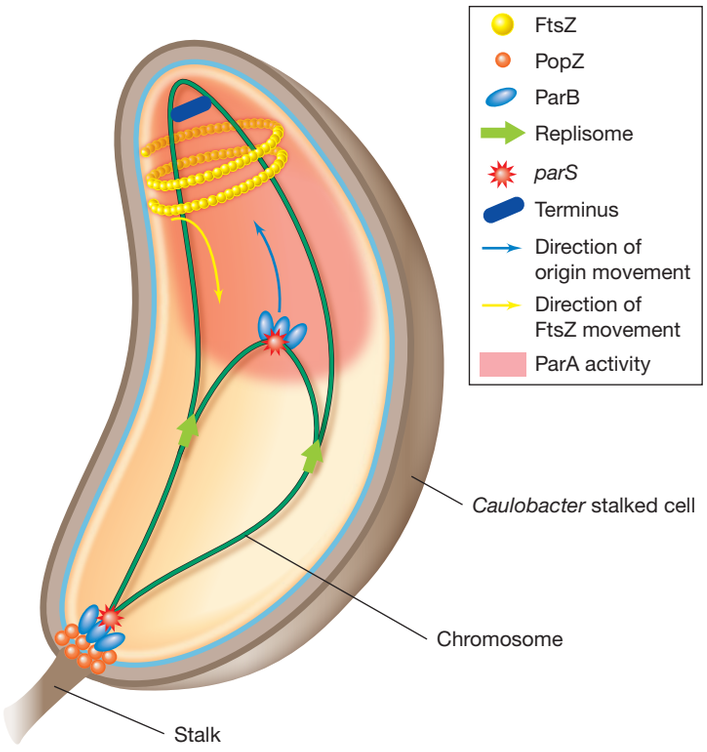


Figure 8.6 Chromosome partitioning in *Caulobacter*. After replication initiates, PopZ localizes to the stalked pole of the cell. Next, ParB binds to the *parS* sequence on the parental chromosome and the complex associates with PopZ at the pole. As replication continues, the *parS* sequence on the daughter chromosome is bound by ParB and moved to the nonstalked cell pole by the activity of diffuse ParA molecules. The location of FtsZ during partitioning is indicated.

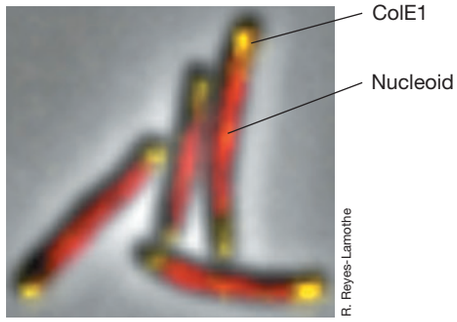


Figure 8.7 Cellular location of ColE1 plasmid in *Escherichia coli* during replication. The plasmid (yellow) localizes to the cell poles, while the nucleoid remains in the center of the cell during DNA replication. The separate DNA molecules are visible due to fluorescently labeled DNA-binding proteins.

sister chromosomes (a process called *decatenation*) prior to segregation. While the actual segregation process is still not completely understood, the chromosome "arms" appear to remain distinctly separated from one another during replication and the chromosomal loci are pushed to the cell poles in their order of replication (Figure 8.1*b*). This separation of daughter chromosomes in *E. coli* thus appears to be independent of specific proteins and proceeds instead by the physical action of replication and DNA accumulation.

How are extrachromosomal genetic elements segregated between daughter cells? Although plasmids are not considered essential for cell survival under all conditions, they are replicated using the same cellular machinery as the chromosome (**Figure 8.7**). Various control mechanisms exist to ensure that a relatively constant copy number of a given plasmid is disseminated to progeny cells. Replication of large ColE1-type plasmids occurs at the poles instead of in the center of the cell where the nucleoid exists (Figure 8.7). This location helps ensure that efficient transfer to daughter cells occurs during cell division and that stable inheritance is achieved over generations. Other mechanisms for segregating plasmids include partitioning systems similar to the Par system in *Caulobacter* (Figure 8.6).

Check Your Understanding

- How does SeqA prevent DnaA–ATP from binding to the *oriC* regions immediately after chromosome replication initiates?
- Explain how the minimum generation time for the bacterium *Escherichia coli* can be less than the time needed to replicate its chromosome.

8.3 Cell Division and Fts Proteins

Cell division is both spatially and temporally controlled to ensure that each daughter cell has a copy of the genome before the septum seals off the two cells (Figure 8.3). Here we consider regulation of septum formation and a series of key proteins that identify the site of cell division and control the overall process.

The Divisome

Several essential proteins play roles in cell division in *Bacteria*. Collectively, these proteins are called *Fts proteins* and a key one, **FtsZ**, plays a crucial role in binary fission. FtsZ is related to tubulin, the

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Art Activity: Figure 8.8*a* The FtsZ ring and cell division

important cell-division protein in eukaryotes (◀ Section 2.15) and is also found in virtually all *Archaea*. Other Fts proteins are found only in *Bacteria* and not in *Archaea*, so our discussion here will be restricted to the *Bacteria*. The gram-negative *Escherichia coli* and the gram-positive *Bacillus subtilis* have been the model bacterial species for the study of cell-division events.

Fts proteins interact in the cell to form a division apparatus called the *divisome*. In rod-shaped cells, formation of the divisome begins with the attachment of molecules of FtsZ in a ring precisely around the center of the cell; this ring becomes the cell-division plane. In a cell of *E. coli*, about 10,000 FtsZ molecules polymerize to form the ring; once formed, the FtsZ ring attracts other divisome proteins, including *FtsA* and *ZipA* (**Figure 8.8**). ZipA is an anchor that connects the FtsZ ring to the cytoplasmic membrane and stabilizes it. FtsA, a protein related to actin, an important cytoskeletal protein in eukaryotes (◀ Section 2.15), both recruits FtsZ and other divisome proteins and helps connect the ring to the cytoplasmic membrane. The divisome forms about three-quarters of the way into the cell-division cycle. However, long before the divisome forms, the cell has already begun to elongate and DNA replication has begun (see Figure 8.9).

The divisome also contains Fts proteins needed for peptidoglycan synthesis, such as FtsI (Figure 8.8). FtsI is one of several *penicillin-binding proteins* present in the cell. Penicillin-binding proteins are so named because their activities are inhibited by the antibiotic penicillin (Section 8.5). The divisome orchestrates synthesis of new cytoplasmic membrane and cell wall material, called the *division septum*, at the center of a rod-shaped cell until the cell reaches twice its original length. Following elongation, the septum forms and the cell divides, yielding two daughter cells (Figure 8.3).

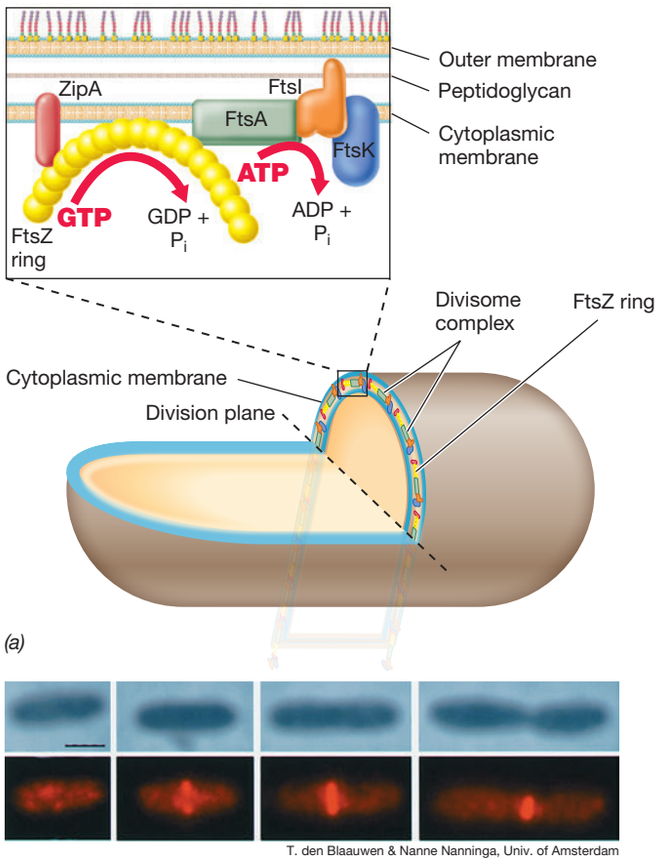


Figure 8.8 The FtsZ ring and cell division. *(a)* Cutaway view of a rod-shaped cell showing the ring of FtsZ molecules around the division plane. Blowup shows the arrangement of individual divisome proteins. ZipA is an FtsZ anchor, FtsI is a peptidoglycan biosynthesis protein, FtsK assists in chromosome separation, and FtsA is an ATPase. *(b)* Appearance and breakdown of the FtsZ ring during the cell cycle of *Escherichia coli*. Microscopy: upper row, phase-contrast; bottom row, cells stained with a specific reagent that binds to FtsZ. Cell division events: first column, FtsZ ring not yet formed; second column, FtsZ ring appears as nucleoids start to segregate; third column, full FtsZ ring forms as cell elongates; fourth column, breakdown of the FtsZ ring and cell division. Marker bar in upper left photo, 1 μm.

Min Proteins and Cell Division

DNA replicates before the FtsZ ring forms (**Figure 8.9**) because the ring forms in the space *between* the duplicated nucleoids; before the nucleoids segregate, they effectively block formation of the FtsZ ring in a process known as nucleoid occlusion (Section 8.2). The proteins MinC, MinD, and MinE interact to help guide FtsZ to the cell midpoint. MinD forms a spiral structure on the inner surface of the cytoplasmic membrane and helps to localize MinC to the cytoplasmic membrane. The MinD spiral oscillates back and forth along the

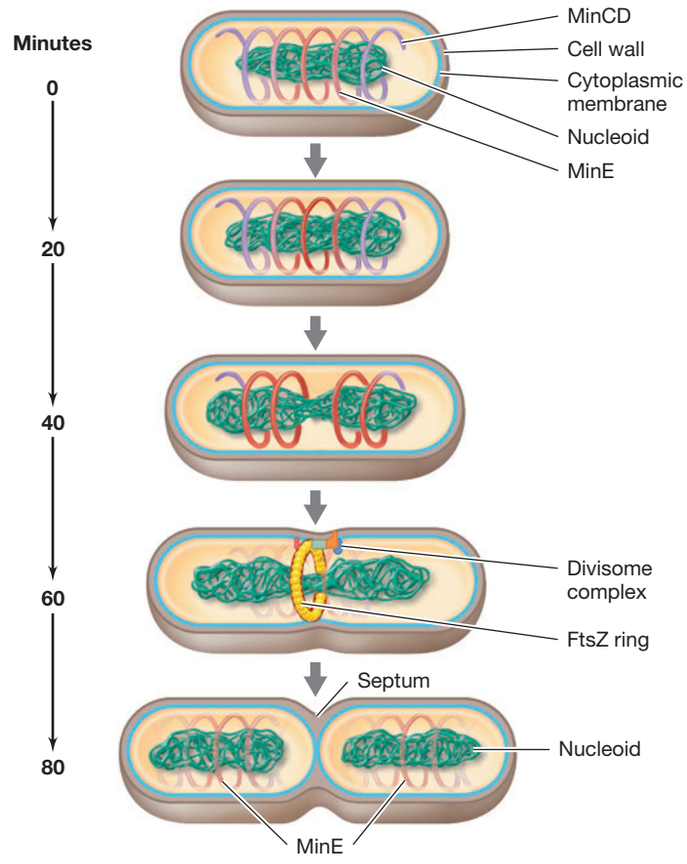


Figure 8.9 DNA replication and cell-division events. The protein MinE directs formation of the FtsZ ring and divisome complex at the cell-division plane. Shown is a schematic for cells of *Escherichia coli* growing with a doubling time of 80 min. MinC and MinD are most abundant at the cell poles during FtsZ ring formation.

long axis of the growing cell and functions to *inhibit* cell division by preventing the FtsZ ring from forming (Figure 8.9). Simultaneously, however, MinE also oscillates from pole to pole, and as it does, it functions to sweep MinC and MinD aside. Hence, because MinC and MinD dwell longer at the poles than anywhere else during their oscillation cycle, the center of the cell will have, on average, the lowest concentration of these proteins. As a result, the cell center becomes the most permissive site for FtsZ binding and so the FtsZ ring forms there. In this unusual series of events, the Min proteins ensure that the divisome forms only at the *cell center* and not at the cell poles (Figure 8.9).

As cell elongation continues and septum formation begins, the two copies of the chromosome are pulled apart, each to its own daughter cell (Figure 8.9). The Fts protein *FtsK* and several other proteins assist in this process. As the cell constricts, the FtsZ ring begins to depolymerize, triggering the inward growth of wall materials to form the septum and seal off one daughter cell from the other. The enzymatic activity of FtsZ also hydrolyzes guanosine triphosphate (GTP, an energy-rich compound) to yield the energy necessary to fuel the polymerization and depolymerization of the FtsZ ring (Figures 8.8 and 8.9).

In the budding bacterium *Caulobacter*, MinC and MinD are absent. Instead, a protein called MipZ (*mi*dcell *p*ositioning of Fts*Z*) controls the assembly site of FtsZ. During chromosome segregation, FtsZ localizes to the nonstalked pole of the cell with the chromosome terminus (Figure 8.6). MipZ forms a gradient throughout the cell by directly binding to the ParB–*parS* complex that facilitates chromosome segregation to the nonstalked cell pole as indicated in Figure 8.6. Once MipZ reaches the nonstalked pole, it displaces polar-localized FtsZ to the center of the cell for septum formation. The timeline for this process in *Caulobacter* is the same as for the Min system of *E. coli* (Figure 8.9).

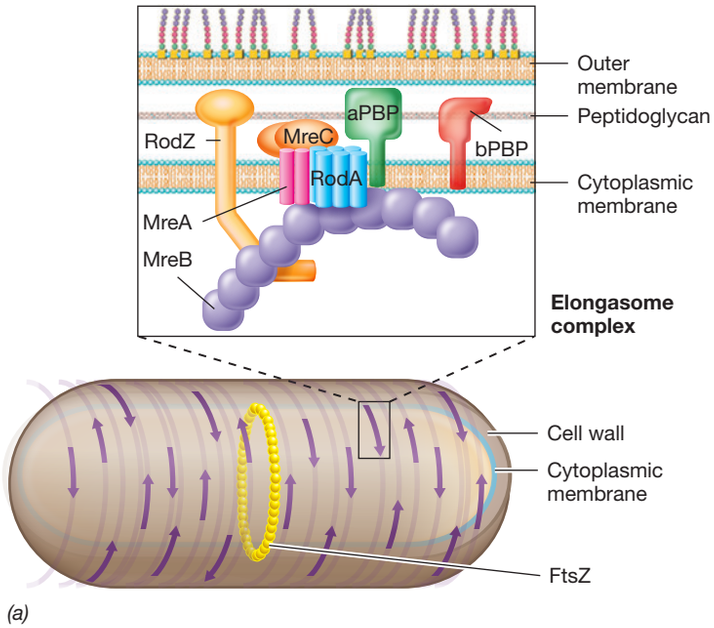
Why do we need to know such intricate details of the cell division process? Besides understanding the molecular biology of microbial growth for purely scientific reasons, there are significant practical reasons for understanding the details of bacterial cell division. Such knowledge could lead to the development of new drugs that target specific steps in the growth cycle of pathogenic bacteria. As with penicillin (a drug that targets bacterial cell wall synthesis), the discovery of drugs that interfere with the function of specific Fts or other cell-division proteins could have broad applications in clinical medicine. In a world where the efficacy of many widely used antibiotics is rapidly fading because of exploding pathogen resistance, the need for new antibacterial antibiotics becomes ever more critical.

Check Your Understanding

- What is the divisome?
- How does FtsZ find the cell midpoint of a rod-shaped cell?

8.4 Determinants of Cell Morphology

Just as specific proteins direct cell *division* in bacteria, other specific proteins form the cell *cytoskeleton*. The cytoskeleton is the scaffolding that directs cell *shape*, yielding the spheres, spirals, crescents, and other morphologies typical of bacterial cells (◀ Figure 1.8). Interestingly, these shape-determining proteins, known as *SEDS* (*s*hape, *e*longation, *d*ivision, and *s*porulation) *proteins*, show



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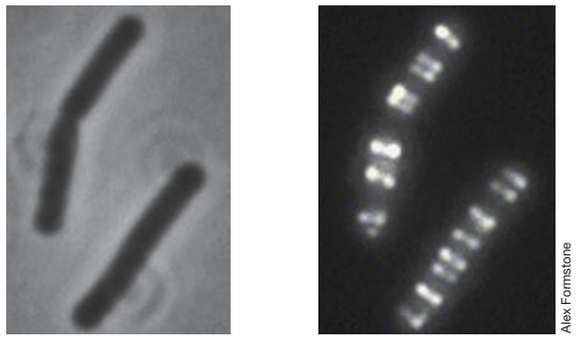

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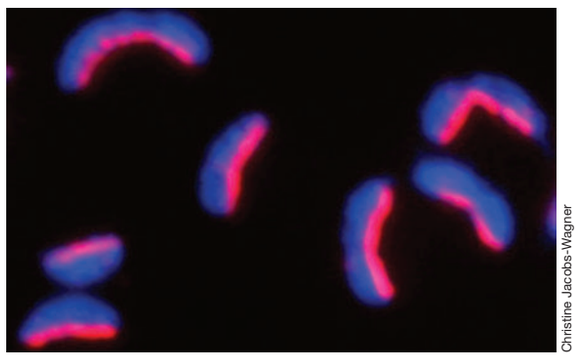


(c)

Figure 8.10 MreB and crescentin as determinants of cell morphology. *(a)* The cytoskeletal protein MreB is an actin analog that moves in tracks perpendicular to the long axis of a rod-shaped cell, making contact with the cytoplasmic membrane in several locations (indicated by arrows). The blowup shows the arrangement of individual elongasome proteins with MreB. RodZ anchors the complex, while MreA and MreC are structural proteins. RodA and class *A* and *B* *p*enicillin-*b*inding *p*roteins (aPBP and bPBP) coordinate to synthesize new peptidoglycan. *(b)* Photomicrographs of the same cells of *Bacillus subtilis*. Left, phase-contrast; right, fluorescence. The cells contain a substance that makes the MreB protein fluoresce, shown here as bright white. *(c)* Cells of *Caulobacter crescentus*, a naturally curved (vibrio-shaped) cell. Cells are stained to show the shape-determining protein crescentin (red), which lies along the concave surface of the cell, and with DAPI, which stains DNA and thus the entire cell (blue).

significant homology to key cytoskeletal proteins in eukaryotic cells (◀ Section 2.15). Thus, like eukaryotes, bacteria possess a dynamic and multifaceted cell cytoskeleton.

Cell Shape and MreB

The major shape-determining factor in *Bacteria* is the protein MreB that forms a simple cytoskeleton in *Bacteria* and in a few species of *Archaea*. MreB forms patchlike filaments just below the cytoplasmic membrane that follow the natural curves of the cell (**Figure 8.10**). The MreB cytoskeleton defines cell shape by recruiting other proteins that function in cell wall growth, such as RodA (peptidoglycan synthesis; Figure 8.10*a* and Section 8.5), to form the *elongasome*. Inactivation of the gene encoding MreB or other proteins in the elongasome causes rod-shaped bacteria to become coccus-shaped. Moreover, most naturally occurring coccoid bacteria lack the *mreB* and *rodZ* genes and thus do not make MreB or RodZ. This implies that the "default" morphology for a bacterium is a sphere. Variations in the arrangement of MreB filaments in cells of nonspherical bacteria are likely responsible for the different common morphologies of prokaryotic cells.

How does MreB define a cell's shape? The filament structures formed by MreB (Figure 8.10*a*) are not static, but instead passively move from one side to the other within the cytoplasm of a growing cell. MreB filaments and other associated proteins localize the synthesis of peptidoglycan (Section 8.5) at points where the rod-shaped filaments contact the cytoplasmic membrane (Figure 8.10*a*). This allows new cell wall to form at several points along the cell rather than from a single location at the FtsZ site outward, as in spherical bacteria (see Figure 8.13). By moving progressively in tracks perpendicular to the cell cylinder and initiating cell wall synthesis at multiple sites along the cell wall, MreB helps direct new wall synthesis in such a way that a rod-shaped cell elongates only along its long axis with cell wall synthesis dispersed at intervals (Figure 8.10*b*).

Crescentin

In the vibrio-shaped bacterium *Caulobacter*, a shape-determining protein called *crescentin* is present in addition to MreB. Copies of crescentin organize into filaments about 10 nm wide that localize onto the concave face of the curved cell (Figure 8.2*b*). The arrangement and localization of crescentin filaments impart the characteristic curved morphology to the *Caulobacter* cell (Figure 8.10*c*). *Caulobacter* is an aquatic bacterium that undergoes a life cycle in which swimming cells, called *swarmers*, eventually form a stalk and attach to surfaces. Attached cells then undergo cell division to form new swarmer cells that are released to colonize new habitats (see Section 8.8 and Figure 8.20). The steps in this life cycle are highly orchestrated at the genetic level, and because of this, *Caulobacter* has been used as a model system for the study of gene expression in cellular differentiation. Although crescentin seems to be unique to *Caulobacter*, proteins similar to crescentin have been found in other curved cells, such as the bacterium that causes peptic ulcers, *Helicobacter pylori*. This suggests that these proteins may be necessary for the formation of curved cells.

Evolution of Cell Division and Cell Shape

How do the determinants of cell shape and cell division in *Bacteria* compare with those in eukaryotes? While MreB functions similarly to the eukaryotic protein actin, FtsZ is both structurally and functionally related to the eukaryotic protein tubulin. Actin forms structures called *microfilaments* that function as scaffolding in the eukaryotic cell cytoskeleton and in cell division, whereas tubulin forms *microtubules* that are important in mitosis and other processes. In addition, the shape-determining protein crescentin in *Caulobacter* is related to the keratin proteins that make up *intermediate filaments* in eukaryotic cells. Intermediate filaments form part of the eukaryotic cytoskeleton, and genes encoding similar proteins have been found in some other *Bacteria*. It thus appears that several proteins that control cell division and the cell cytoskeleton in eukaryotic cells (◀ Section 2.15) have evolutionary roots in the *Bacteria*. However, with the exception of FtsZ, genes encoding homologs of these proteins appear to be absent from most *Archaea*.

While our focus has been on the filament proteins MreB and crescentin in bacteria that grow by synthesizing new peptidoglycan at the center of the cell (or dispersively), a diversity of morphologies exists in the bacterial world. This is especially evident in species of *Alphaproteobacteria* (**Figure 8.11**), gram-negative bacteria that show not only significant morphological diversity but also significant metabolic diversity (Chapters 14 and 16). Hence, *Alphaproteobacteria* fill a host of diverse ecological niches in nature, from soil and water to symbiotic plant and animal associations.

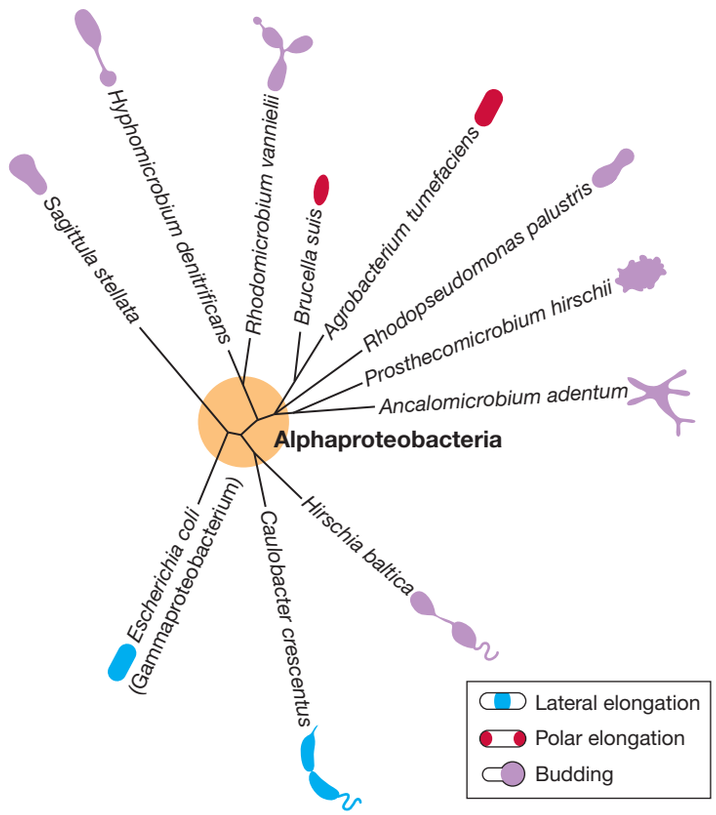
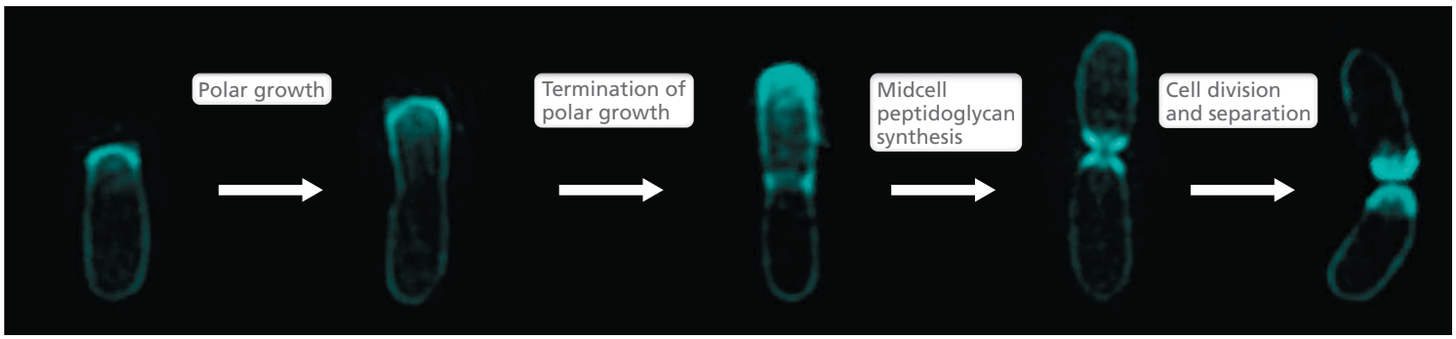


Figure 8.11 Phylogeny and morphology of select diverse *Alphaproteobacteria*. The tree was constructed from comparative sequences of *rpoC*, a gene encoding one of the subunits of RNA polymerase. *Escherichia coli*, a member of the *Gammaproteobacteria*, is included for comparison. Colored shading represents areas of new peptidoglycan synthesis as indicated. Note the extensive morphological diversity within the *Alphaproteobacteria*. Modified from Randich, A.M., and Brun, Y.V. 2015. *Front. Microbiol. 6:* 580.



Wanda Figueroa-Cuilan and Pamela Brown

Figure 8.12 **Polar growth of *Agrobacterium tumefaciens*.** A time series of fluorescent micrographs showing an *A. tumefaciens* cell following addition of a fluorescently labeled D-amino acid. The cell cycle progression shows addition of new peptidoglycan to one cell pole prior to septum formation and cell division.

Besides growing as rod-shaped cells and producing new cell material as we have seen in Figures 8.8–8.10, the *Alphaproteobacteria* contain species that can grow by synthesizing peptidoglycan at the poles (polar elongation). *Agrobacterium tumefaciens*, a plant pathogen, elongates at one pole in a process called unipolar elongation (**Figure 8.12**). Although rod-shaped, *Agrobacterium* lacks MreB. Instead peptidoglycan synthesis occurs at one pole before initiating at the midcell for cell division. For cell division to occur at the midcell, *Agrobacterium* does possess FtsZ. However, FtsZ resides in the growing pole during unipolar growth and then moves to midcell to recruit the peptidoglycan synthesis machinery and form the ring where the septum forms. It is thought that proteins within the divisome (Section 8.3) help sequester FtsZ in the growing pole during unipolar growth.

Other genera of *Alphaproteobacteria* grow in a process known as budding. Budding results in diverse cell morphologies (Figure 8.11; ◀ Section 4.10) and is a consequence of synthesizing peptidoglycan at various regions within the cell. However, the precise positioning of the peptidoglycan-synthesizing machinery in budding cells has yet to be discovered. Figure 8.11 also illustrates a principle we will see repeated many times in this book: The phylogenetic position of a bacterium cannot be predicted from its morphology and vice versa. With only rare exception (for example, the spirochetes, ▶ Section 15.17), morphology and phylogeny are unrelated characteristics.

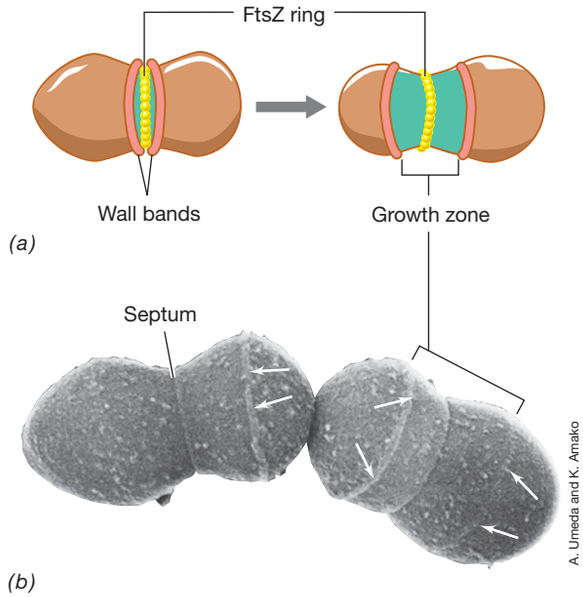
Check Your Understanding

- How does MreB control the shape of a rod-shaped bacterium?
- What protein is thought to control the shape of cells of *Caulobacter*?
- What relationships exist between cytoskeletal proteins in *Bacteria* and those in eukaryotes?

8.5 Peptidoglycan Biosynthesis

In cells of *Bacteria* that contain peptidoglycan—and virtually all species do—preexisting peptidoglycan has to be temporarily severed to allow newly synthesized peptidoglycan to be inserted during the growth process. As we have just seen in rod-shaped cells, new cell wall grows at several locations along the length of the cell (Figure 8.10*a*, *b*), and it is localized in those cells that divide by polar elongation or budding. By contrast, new cell wall material in cocci grows out in opposite directions from the FtsZ ring (**Figure 8.13**). Because cocci do not possess the SEDS proteins MreB and RodZ (Section 8.4), FtsZ plays a critical role in maintaining cell shape in cocci. **Figure 8.14** illustrates cell division and peptidoglycan synthesis in the gram-positive coccus *Staphylococcus aureus*, as well as the irregular elongation that occurs in an *S. aureus* FtsZ mutant. Without a fully functional FtsZ, *S. aureus* cells begin to elongate and form curved cytoskeletons that illustrate the importance of SEDS proteins in maintaining cellular morphology.

Regardless of the mechanism a bacterium uses to maintain its cytoskeleton, a growing bacterial cell must both synthesize new peptidoglycan and export it outside the cytoplasmic membrane to link up with existing cell wall material, and we consider this problem here.



A. Umeda and K. Amako

Figure 8.13 **Cell wall synthesis in gram-positive *Bacteria*.** *(a)* Localization of cell wall synthesis during cell division. In cocci, cell wall synthesis (shown in green) is localized at only one point (compare with Figure 8.10*a*). *(b)* Scanning electron micrograph of cells of *Streptococcus pyogenes* showing wall bands (arrows). A single cell is about 1 μm in diameter.

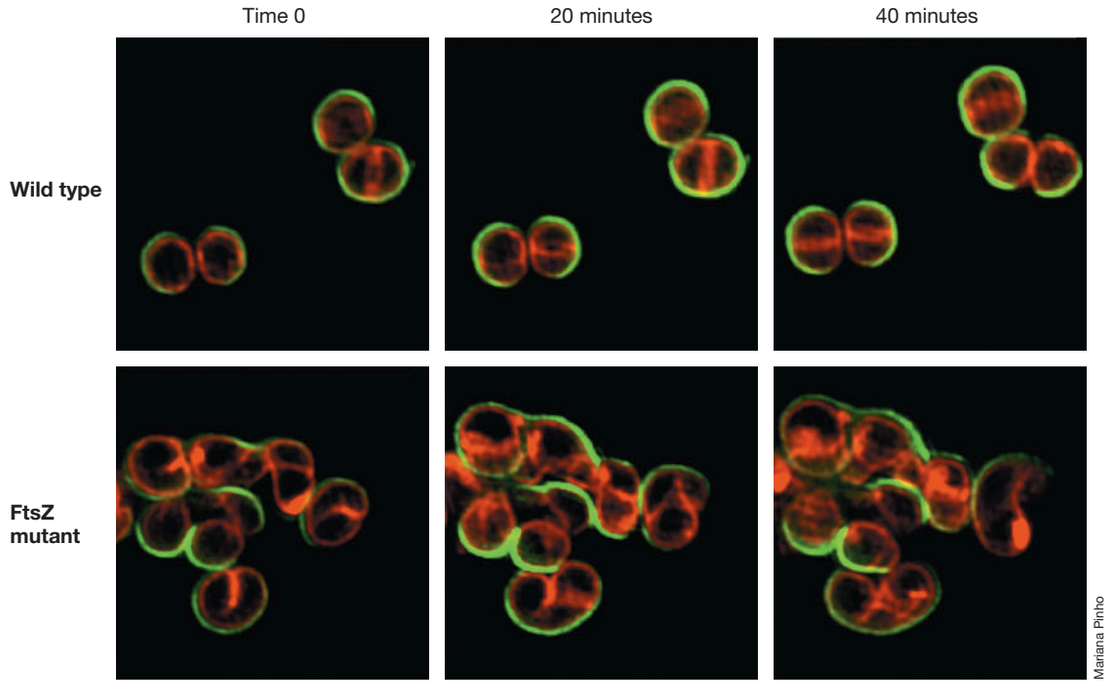


Figure 8.14 ***Staphylococcus aureus*** **cell division and FtsZ.** Time-lapse series of fluorescence micrographs illustrating cell division in the wild type and an FtsZ mutant strain. Cell wall material is stained green, while membrane material is stained red. Wild-type cells form a septum for cell division at midcell and retain a coccus shape, while the mutant cells form an irregular elongated morphology. Modified from Pereira, A.R., et al. 2016. *MBio* 7(5): doi:10.1128/mBio. 00908-16.

Insertion of New Peptidoglycan

Peptidoglycan can be thought of as a stress-bearing fabric, much like a thin sheet of rubber. Synthesis of new peptidoglycan during growth requires the controlled cutting of preexisting peptidoglycan along with the simultaneous insertion of peptidoglycan precursors. A lipid carrier molecule called *bactoprenol* plays a major role in the latter process. Bactoprenol is a hydrophobic C_{55} alcohol that is bonded to an *N*-acetylglucosamine/*N*-acetylmuramic acid/pentapeptide peptidoglycan precursor to form *lipid II* (**Figure 8.15**). Bactoprenol transports peptidoglycan precursors across the cytoplasmic membrane by rendering lipid II sufficiently hydrophobic to pass through a transmembrane ABC transporter (◀ Figure 2.6) known as *flippase* (**Figure 8.16*a***).

Mastering Microbiology
Art Activity: Figure 8.16 Peptidoglycan synthesis

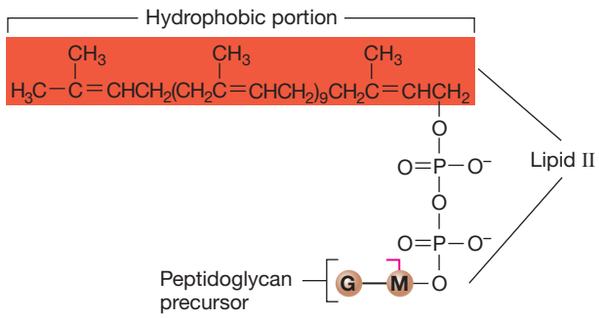


Figure 8.15 **Bactoprenol (undecaprenol diphosphate).** This highly hydrophobic molecule carries cell wall peptidoglycan precursors through the cytoplasmic membrane. The entire molecule is called lipid II.

Once outside the cell, lipid II interacts with peptidoglycan polymerases called *transglycosylases* that insert peptidoglycan precursors into a growing point in the cell wall and catalyze glycosidic bond formation (the remaining bactoprenol is then recycled to the cytoplasm to begin a new round of precursor transport). However, prior to insertion of the peptidoglycan precursor, small gaps in the existing peptidoglycan are made by enzymes called *autolysins*, enzymes that function to hydrolyze the bond that connects *N*-acetylglucosamine with *N*-acetylmuramic acid in the peptidoglycan backbone. The peptidoglycan precursor is then added across the gaps (Figure 8.16*a*). The junction between new and old peptidoglycan forms a ridge on the cell surface of gram-positive bacteria that can be observed by electron microscopy as a *wall band* (Figure 8.13*b*).

Peptidoglycan synthesis must be a highly coordinated process, and peptidoglycan precursors must be readily available during autolysin activity. This is because tetrapeptide units must be spliced into existing peptidoglycan immediately after autolysin activity in order to prevent a breach in the peptidoglycan fabric at the splice point; a breach could cause osmotic issues for the cell and lead to spontaneous cell lysis, called *autolysis*.

Transpeptidation

The final step in peptidoglycan synthesis is transpeptidation. Transpeptidation forms the peptide cross-links between muramic acid residues in adjacent glycan chains (◀ Section 2.3 and Figures 2.8 and 2.9). In gram-negative bacteria such as *Escherichia coli*, cross-links form between diaminopimelic acid (DAP) on one peptide and

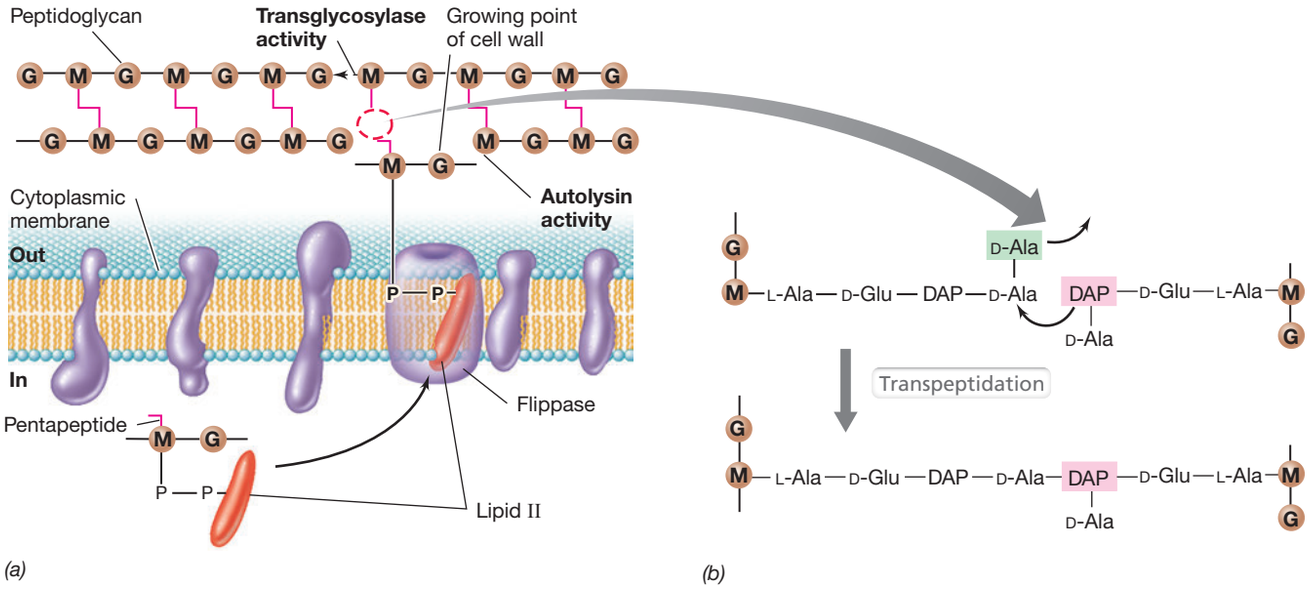


Figure 8.16 **Peptidoglycan synthesis.** *(a)* Flippase transports lipid II across the cytoplasmic membrane to the growing point of the cell wall. Autolysin breaks glycolytic bonds in preexisting peptidoglycan, while transglycosylase synthesizes them, linking old peptidoglycan with new. *(b)* The transpeptidation reaction that leads to the final cross-linking of two peptidoglycan chains. Penicillin inhibits this reaction. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid.

D-alanine on the adjacent peptide. Although there are two D-alanine residues at the end of the peptidoglycan precursor, only one remains in the final molecule, as the other is removed during transpeptidation (Figure 8.16*b*). This reaction is exergonic (energy-releasing, ◀ Section 3.1) and supplies the energy necessary to drive transpeptidation forward. In *E. coli*, the protein FtsI (Figure 8.8*a*) functions as the transpeptidase.

Transpeptidation is medically noteworthy because it is the reaction inhibited by the antibiotic penicillin. Several penicillin-binding proteins have been identified in bacteria, including FtsI (Figure 8.8*a*). When penicillin is bound to penicillin-binding proteins, the proteins are inactivated. If transpeptidation is blocked in an otherwise growing cell, the continued activity of autolysins (Figure 8.16*a*) so weakens the peptidoglycan that the cell eventually bursts. The absence of peptidoglycan in eukaryotes (such as humans) is the basis of the clinical efficacy of penicillin—the antibiotic destroys only growing bacteria and thus targets pathogenic bacteria that often grow rapidly in an active infection.

Morphology-Related Proteins and Peptidoglycan Synthesis

Many penicillin-binding proteins are bifunctional and possess not only transpeptidase activity but also transglycosylase activity (Figure 8.16). Unlike the protein domains in the transpeptidase, the transglycoslylase domains are not inhibited by β-lactam antibiotics such as penicillin (Section 8.11). These proteins, known as class *A* *p*enicillin-*b*inding *p*roteins (aPBPs), were previously considered the only proteins to perform peptidoglycan polymerization. However, studies of cell wall biosynthesis in both *Bacillus subtilis* and *Escherichia coli* have identified alternative SEDS proteins (Section 8.4) that help form new cell wall material through transglycosylase activity.

By genetically deleting all aPBP proteins in *B. subtilis* and *E. coli*, microbiologists have discovered that the elongasome proteins RodA and MreB, which were thought to only play roles in defining cell shape by localizing aPBPs to specific locations within growing cells for peptidoglycan synthesis (Figure 8.10*a*), can themselves add disaccharide precursors to the growing polypeptide chain. However, the SEDS family of proteins do not appear to have transpeptidase activity. Instead, class *B* *p*enicillin-*b*inding *p*roteins (bPBPs), which lack transglycoslyase activity, form the cross-links in peptidoglycan in the absence of aPBPs (Figures 8.10*a* and 8.16*b*).

Thus, and perhaps not surprisingly, bacteria have more than one distinct enzyme system available for synthesizing such a critical cellular component as the cell wall. Moreover, because SEDS are more widely distributed among *Bacteria* than are aPBPs, this explains how microorganisms lacking aPBPs can still synthesize peptidoglycan. It also highlights the possibility that SEDS proteins could be targets for new antibiotic development, as this family of proteins is not inhibited by β-lactams that bind and inactivate classical penicillin-binding proteins.

We move on now to Part II where we will explore developmental processes in bacteria. As in higher organisms where coordinated developmental changes result in new structures, some bacteria undergo transformations from one type of cell to another to either improve their chances of survival or allow them to be more successful in their habitat.

Check Your Understanding

- What is the function of bactoprenol?
- What is transpeptidation, and why is it important both to the cell and to clinical medicine?

II • Regulation of Development in Model *Bacteria*

The formation of endospores in *Bacillus* and cell differentiation in *Caulobacter* are excellent models of developmental events in individual cells. The transitions between swimming and biofilms in *Pseudomonas* and *Vibrio* are valuable models of developmental events in cell populations.

Now that we have an understanding of the basic molecular processes that occur during bacterial growth, we move on to explore how certain bacteria differentiate to form specialized cells and how bacteria can transition from growing in suspensions (planktonic growth) to form multicellular biofilms.

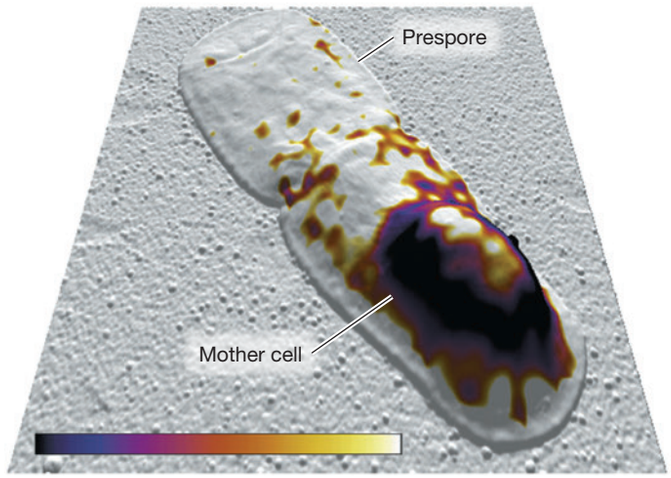
Development and differentiation are largely characteristics one associates with multicellular organisms. Because most *Bacteria* and *Archaea* grow as single cells, few species show differentiation. But a few important examples are known and are classical cases of differential gene expression yielding two genetically identical descendants whose functions differ. Here we discuss three well-studied examples of development and differentiation: the formation of endospores in the gram-positive soil bacterium *Bacillus*; the formation of two cell types—motile and stationary—in the gram-negative aquatic bacterium *Caulobacter*; and the formation of heterocysts in the nitrogen-fixing cyanobacterium *Anabaena*. We conclude by considering the formation of biofilms in the gram-negative and pathogenic bacteria *Pseudomonas aeruginosa* and *Vibrio cholerae*.

8.6 Regulation of Endospore Formation

Many microorganisms respond to adverse conditions by converting growing (vegetative) cells into rigid and resistant spores (◀ Section 2.8 and Figure 2.29). Once favorable conditions return, the spore germinates, and the organism returns to the vegetative state (see Figure 8.19 and Section 8.7). Among *Bacteria*, the genus *Bacillus* is well known for the formation of *endospores*, that is, spores formed inside a mother cell (**Figure 8.17**). Prior to endospore formation, the cell divides asymmetrically. The smaller cell develops into the endospore, which is surrounded by the larger mother cell (Figure 8.17). Once development is complete, the mother cell bursts, releasing the endospore.

Endospore Formation: Sporulation Factors

Endospore formation in *Bacillus subtilis*, a model species for endospore studies, is triggered by unfavorable conditions, such as starvation, desiccation, or growth-inhibitory temperatures. A cell of *B. subtilis* monitors its environment through a group of five sensor kinases. These enzymes function via a phosphotransfer relay system whose mechanism resembles that of a two-component regulatory system (◀ Section 7.5) but is considerably more complex (**Figure 8.18**). The net result of multiple adverse conditions is the successive phosphorylation of several proteins called *sporulation factors*, culminating with sporulation factor Spo0A. When Spo0A is highly phosphorylated, sporulation proceeds. Spo0A controls the expression of several sporulation-specific genes. The product of one of these, SpoIIE, removes the phosphate from SpoIIAA, and this triggers the latter to remove the anti-sigma factor SpoIIAB; this liberates the RNA polymerase sigma factor (◀ Section 6.5) σ^F, a key step in the sporulation process.



Wang, C., Stanciu, C., Ehrhardt, C.J., Yadavalli, V.K. 2015. *Journal of Microscopy* 258: 49–58

Figure 8.17 *Bacillus* endospore elasticity map. Atomic force microscopy combined with a nanochemical surface map illustrating rigidity and release of endospore from mother cell. Flat white regions are indicative of rigidity, while dark regions illustrate elasticity and near bursting of the mother cell. Inset: The heat map bar is a measure of elasticity with lower numbers (black) representing the highest level of elasticity.

Endospore Formation: Alternative Sigma Factors

Once a cell of *B. subtilis* commits to sporulation, endospore development is controlled by four different σ factors, two of which, σ^F and σ^G, activate genes needed inside the developing endospore (called the *forespore*) and two of which, σ^E and σ^K, activate genes needed in the mother cell surrounding the forespore (Figure 8.18*b*). The sporulation signal, transmitted via Spo0A, activates σ^F in the forespore (σ^F is already present in the forespore but is inactive because it is bound by an anti-sigma factor, Figure 8.18*a*). Once free, σ^F is active and can bind to RNA polymerase and promote transcription of genes whose products are needed for the next stage of sporulation (Figures 8.1*a* and 8.18*b*). These include the gene encoding the sigma factor σ^G and the genes for proteins that cross into the mother cell and activate σ^E.

Active σ^E is required for transcription of yet more genes inside the mother cell, including the gene for σ^K. The sigma factors σ^G (in the forespore) and σ^K (in the mother cell) are required for transcription of genes needed even later in the sporulation process (Figure 8.18*b*). Eventually, the many spore coats and other unique structures and conditions typical of the endospore (◀ Section 2.8 and Table 2.1) are formed, and the mature endospore is released.

Nutrients for Endospore Formation

Nutrient limitation is the major trigger of sporulation in *Bacillus* (◀ Section 2.8). But if this is the case, how do cells obtain sufficient nutrients to complete the formation of endospores? One fascinating aspect of the regulation of endospore formation is another regulatory event in which sporulating cells cannibalize cells of their own species. Those *Bacillus* cells in which Spo0A has already become activated secrete a toxic protein that lyses nearby *Bacillus* cells whose Spo0A protein has not yet become activated.

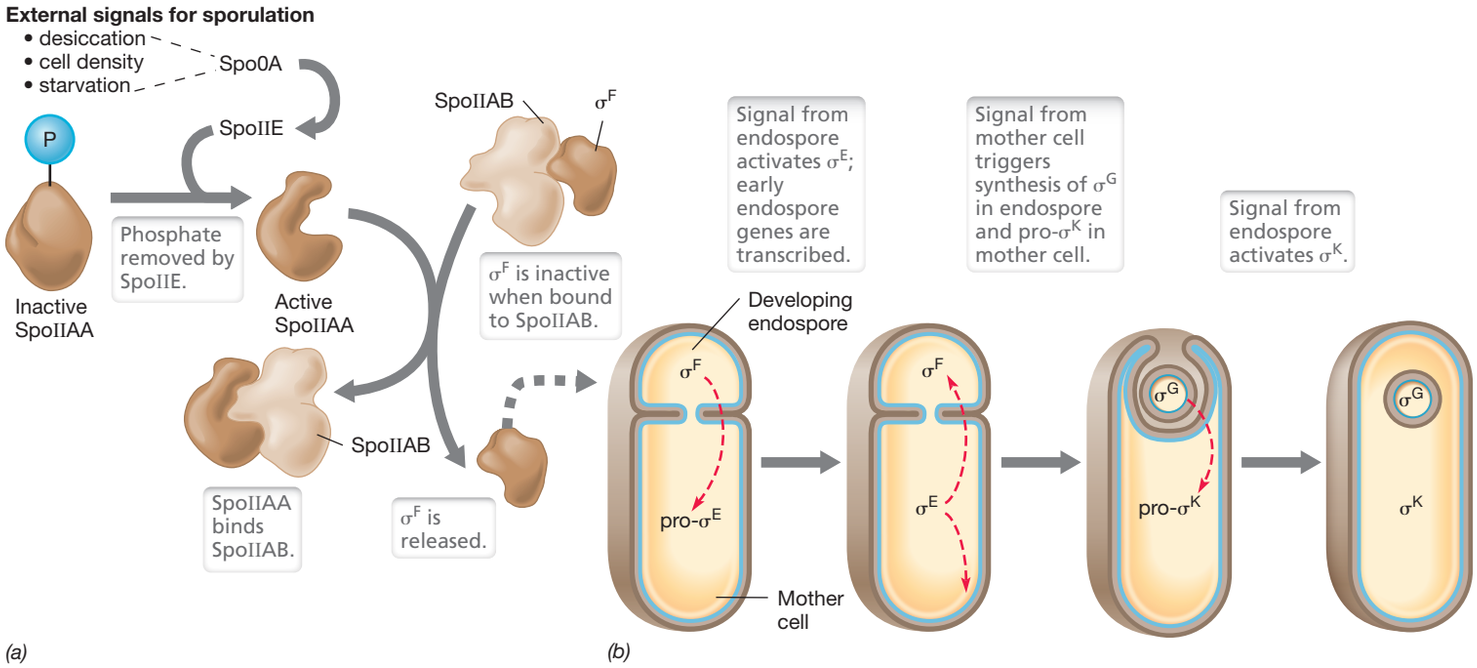


Figure 8.18 Control of endospore formation in *Bacillus*. After external signals for sporulation are received, a cascade of sigma (σ) factors controls differentiation. *(a)* Active SpoIIAA binds the anti-σ factor SpoIIAB, thus liberating the first σ factor, σ^F. *(b)* σ^F initiates a cascade of sigma factors, some of which already exist and need to be activated, others of which are not yet present and whose genes must be expressed. These σ factors then promote transcription of genes needed for endospore development.

This lytic protein is produced along with a second protein that functions to delay sporulation of neighboring cells. Cells committed to sporulation also make an antitoxin protein to protect themselves against the effects of their own toxic protein. When lysed, their sacrificed sister cells are used as a source of nutrients for developing endospores. Shortages of certain key nutrients, in particular phosphate, increase transcription of the gene that encodes the toxic protein.

In sporulation we thus see not only a strategy by which cellular differentiation allows the species to form cells that can withstand adverse conditions, but a strategy in which survival of a few (as opposed to all) cells of the species in a population is a priority and is facilitated by the sacrifice of other cells of the same species (see Figure 8.30 to visualize this strategy).

Check Your Understanding

- How are different sets of genes expressed in the developing endospore and the mother cell?
- What is an anti-sigma factor and how can its effect be overcome?

8.7 Regulation of Endospore Germination

Once cells have proceeded from cell to endospore, they are not committed to dormancy indefinitely. While endospores are not metabolically active and can remain dormant for years, they are still able to sense and respond to nutrients and favorable growth conditions. Such circumstances allow endospores to convert back to vegetative cells by triggering a highly ordered developmental process called *germination*. This process essentially reverses the steps in sporulation, and in *Bacillus*, it is orchestrated by over 600 proteins and occurs in three consecutive stages: activation, germination, and outgrowth (**Figure 8.19**).

Triggers for Activating Endospore Germination

Although an endospore is considered to be dormant, there are receptors called *germinant receptors* (GR) within the inner membrane surrounding the core of the endospore (Figure 8.19). GRs exist in clusters called the "germinosome," which functions to sense and bind nutrients such as amino acids, sugars, and cell wall peptides from vegetative cells. Germinants trigger stage I of endospore germination—activation. While it is unknown how specific GR binding can be, the protein GerA is known to bind the amino acid alanine, and GerB and GerK bind a mixture of asparagine, glucose, fructose, and potassium ions as germinants. Temperature increases also help trigger germination by altering endospore inner membrane properties and GR conformation, processes that lead to more efficient germinant binding.

How does binding of a germinant by a GR trigger activation of an endospore? This is not completely understood; however, it is known that a cascade of events occurs following germinant binding that results in the release of monovalent cations and calcium–dipicolinic acid (DPA, ◀ Section 2.8) from the spore core. This release is initiated by reversing sporulation factor activity (Figure 8.19), a process that allows for partial rehydration of the endospore core (the endospore core contains a small fraction of the water content of a vegetative cell, ◀ Table 2.1). Besides these physiological events, expression of genes encoding the cellular machinery for major molecular

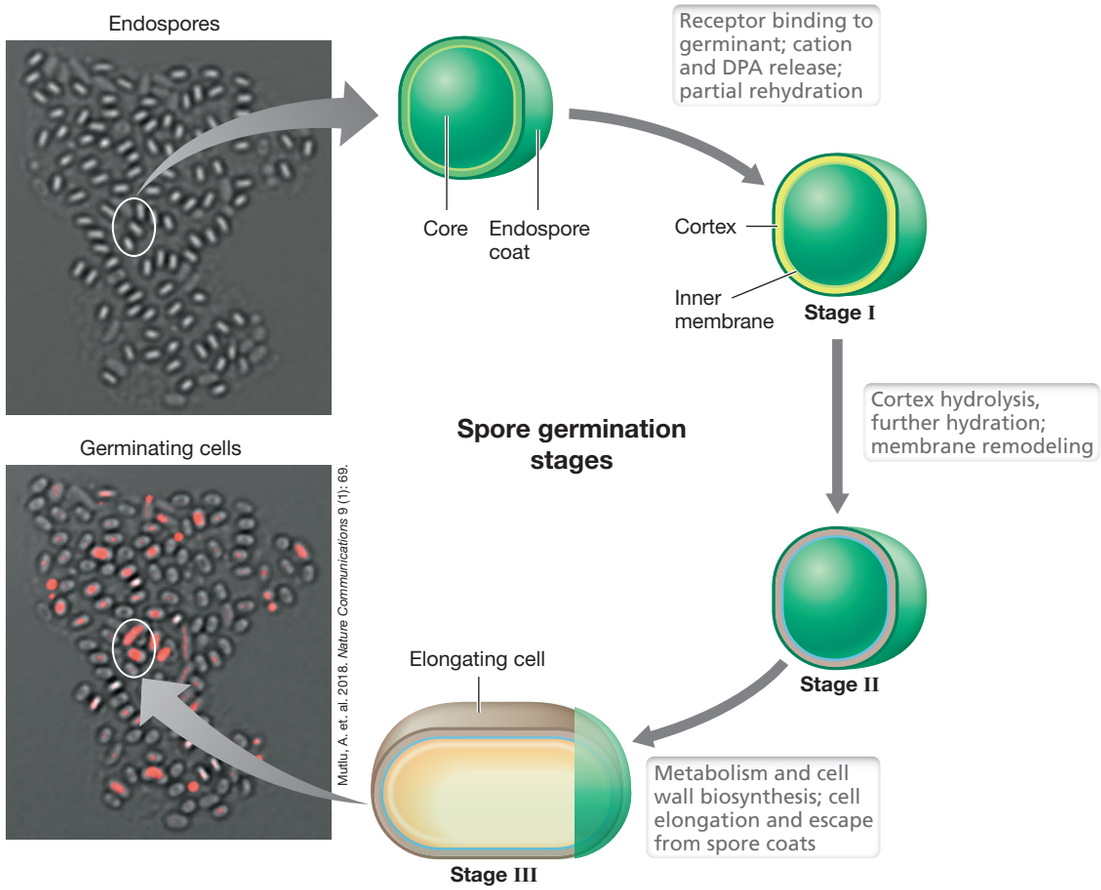


Figure 8.19 Germination of *Bacillus* endospores. The binding of nutrients to germinant receptors triggers three stages that result in conversion of an endospore to an actively dividing vegetative cell. Photos show time-lapse images of expression of a red fluorescent protein as endospores (green) become metabolically active (red). DPA, dipicolinic acid. Images adapted from Mutlu, A., et al. 2018. *Nat. Commun.* 9: 69.

processes such as transcription and translation are increased along with those encoding chaperones for protein folding (◀ Section 6.11) necessary for the cell to enter the germination and outgrowth stages, to be considered now.

Germination and Outgrowth Stages

Once stage I is completed, the next step to spore germination is the triggering of cortex lytic enzymes (CLE) to degrade peptidoglycan in the endospore cortex (Figure 8.19). Cortex peptidoglycan is modified from normal peptidoglycan such that CLE can recognize it and not degrade cell wall peptidoglycan produced during outgrowth of vegetative cells. This removal of the endospore cortex is the major event of stage II—*germination*. Once the endospore cortex has been degraded, complete hydration is achieved and this sets the stage for more active metabolism.

During stage III of endospore germination—*outgrowth*—germinated cells begin near-normal rates of metabolism and commence events necessary to initiate the cell cycle and binary fission (Figure 8.3). Cell elongation during outgrowth results in escape of the newly emerging vegetative cell from spore coats (Figure 8.19). Outgrowth requires the increased expression of genes encoding energy-generating processes, such as the citric acid cycle and respiratory enzymes (Chapter 3). Outgrowth is also triggered by the increased expression of genes encoding enzymes that biosynthesize peptidoglycan, teichoic and lipoteichoic acids (components of the gram-positive cell wall, ◀ Section 2.3 and Figure 2.10) and the cell division protein FtsZ (Section 8.3).

Check Your Understanding

- Where in the endospore are germinant receptors found, and what types of molecules do they detect?
- How does reversing the activity of sporulation factors lead to hydration of the endospore?
- What type of enzyme is necessary to remove the endospore cortex?

8.8 *Caulobacter* Differentiation

In addition to endospore formation in *Bacillus*, the gram-negative aquatic bacterium *Caulobacter* provides a second example of a cell that divides into two genetically identical daughter cells that are both structurally and functionally distinct and express different sets of genes.

Caulobacter is a genus of *Alphaproteobacteria* (Figure 8.11) that undergoes a simple life cycle and is a common bacterium in oligotrophic

(nutrient-poor) aquatic environments (▶ Section 15.18). In the *Caulobacter* life cycle, free-swimming (swarmer) cells alternate with cells that lack flagella and instead are attached to surfaces by a stalk with a holdfast at its end. The role of swarmer cells is strictly dispersal because swarmers cannot divide to form new swarmer cells nor replicate their DNA. Conversely, the role of the stalked cell is strictly reproduction. In order to divide, swarmer cells must first differentiate into stalked cells, and to swim, stalked cells must first produce swarmers; this is the nature of the *Caulobacter* life cycle (**Figure 8.20**; ◀ Section 7.9 and Figure 7.25*b*).

Regulatory Features

The *Caulobacter* cell cycle is controlled by three major regulatory proteins whose concentrations oscillate in succession. Two of these are the transcriptional regulators GcrA and CtrA. The third is DnaA, a protein that functions both in its normal role in initiating DNA replication (Section 8.2) and also as a transcriptional regulator. Each of these regulators is active at a specific stage in the cell cycle, and each controls many other genes needed at that particular stage in the cycle.

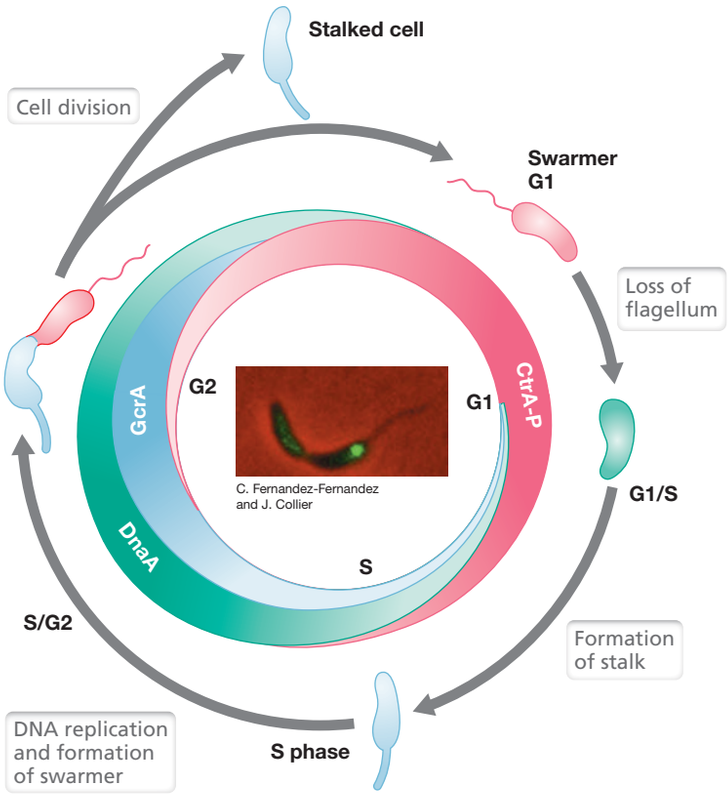


Figure 8.20 Cell cycle regulation in *Caulobacter*. Levels of three global regulators, CtrA, DnaA, and GcrA, oscillate through the cycle as shown. G1 and G2 are the two growth phases and S is the DNA synthesis phase. In G1 swarmer cells, CtrA represses initiation of DNA replication and expression of GcrA. At the G1/S transition, CtrA is degraded and DnaA levels rise. DnaA binds to the origin of replication and initiates replication (see inset photo). GcrA also rises and activates genes for cell division and DNA synthesis. At the S/G2 transition, CtrA levels begin to rise again and shut down GcrA expression. GcrA levels slowly decline in the stalked cell but are rapidly degraded in the swarmer (the daughter cell). CtrA is degraded in the stalked cell. Inset: Fused to the green fluorescent protein as a reporter, a subunit of DNA polymerase is localized in the end of the stalked *Caulobacter* cell where DNA replication occurs. Each cell of the dividing *Caulobacter* pair is about 2 μm long.

CtrA is activated by phosphorylation by a sensor kinase (◀ Section 7.5) in response to cell cycle signals. Environmental starvation signals such as carbon and ammonia limitation also trigger the stringent response and ppGpp production (◀ Section 7.9), which prevent degradation of CtrA-P in emerging swarmer cells. Once phosphorylated, CtrA-P activates genes that encode synthesis of the flagellum and other functions specific to swarmer cells. Conversely, CtrA-P represses the synthesis of GcrA and also inhibits the initiation of DNA replication in swarmer cells by binding to and blocking the origin of replication (Figure 8.20).

As the cell cycle proceeds, CtrA is degraded by a specific protease, and as a consequence, levels of DnaA begin to rise. The absence of CtrA-P allows access to the chromosomal origin of replication, and, as in all *Bacteria*, DnaA binds to the origin and triggers the initiation of DNA replication (Section 8.2). In addition, *Caulobacter* DnaA activates several other genes needed for chromosomal replication. The level of DnaA then falls due to protease degradation, and the level of GcrA rises. The GcrA regulator promotes the elongation phase of chromosome replication, cell division events, and the growth of the stalk on the immobile daughter cell. Eventually, GcrA levels fall and CtrA reappears (in the daughter cell destined to swim away) (Figure 8.20), and the cell cycle is repeated.

UNIT 2

Mastering Microbiology
Art Activity: Figure 8.20 Cell cycle regulation in *Caulobacter*

Caulobacter and the Eukaryotic Cell Cycle

Both external stimuli and internal factors such as nutrient and metabolite levels coordinate the events of the *Caulobacter* cell cycle (◀ Section 7.9). Since its genome has been sequenced and good genetic transfer systems are available, the *Caulobacter* cell cycle has been used as a model for studying cell developmental processes in other organisms as well. This focus is primarily due to the strict cell cycle followed by *Caulobacter*, which resembles the cell cycle of eukaryotic cells in many respects. In fact, the resemblance is so striking that terminology used to describe the eukaryotic cell cycle has been adapted to the *Caulobacter* system (Figure 8.20).

In eukaryotic cells, phase G1 of cell division is where growth and normal metabolic events occur, and in phase G2 the cell prepares for subsequent mitotic events, which occur in the M phase. Between G1 and G2 is the S phase, where DNA replication occurs. In the *Caulobacter* life cycle there is no mitosis, of course, but analogs of the G1, G2, and S phases are apparent (Figure 8.20), making this bacterium an excellent model for studying cell-division events in higher organisms.

We now consider one other well-studied cell differentiation process, this time in a metabolically robust oxygenic phototrophic bacterium in which photosynthetic O_2 production has driven the evolution of a specialized structure for nitrogen metabolism.

Check Your Understanding

- Why are the levels of DnaA protein controlled during the *Caulobacter* cell cycle?
- When do the regulators CtrA and GcrA carry out their main roles during the *Caulobacter* life cycle?

8.9 Heterocyst Formation in *Anabaena*

Cyanobacteria are oxygenic phototrophs; that is, their photosynthetic metabolism yields oxygen (▶ Sections 14.6 and 15.3). They are also able to perform nitrogen fixation—the reduction of N_2 to NH_3 as a nitrogen source (◀ Section 3.12). Nitrogen fixation is a highly energy-demanding process catalyzed by *nitrogenase*, an extremely oxygen-sensitive enzyme. How then is it possible for both nitrogen fixation and oxygenic photosynthesis to occur simultaneously in the same bacterium? To solve this problem, some filamentous cyanobacteria such as *Anabaena* and *Nostoc* undergo a developmental process to form specialized cells called **heterocysts** that are dedicated to nitrogen fixation.

Heterocyst Formation

Because heterocysts lack photosystem II—the pigment–protein complex that produces O_2 during oxygenic photosynthesis (◀ Section 3.11 and ▶ Section 14.6)—they are anoxic cells. This anaerobic lifestyle provides a hospitable environment for nitrogenase and thus nitrogen fixation. Heterocysts arise from the differentiation of vegetative cells that contain photosystem II and thus produce O_2, and typically develop in a regular pattern along a filament (**Figure 8.21*a***). As we will see, this patterning separates two incompatible metabolic processes while still allowing for necessary nutrient exchanges and growth.

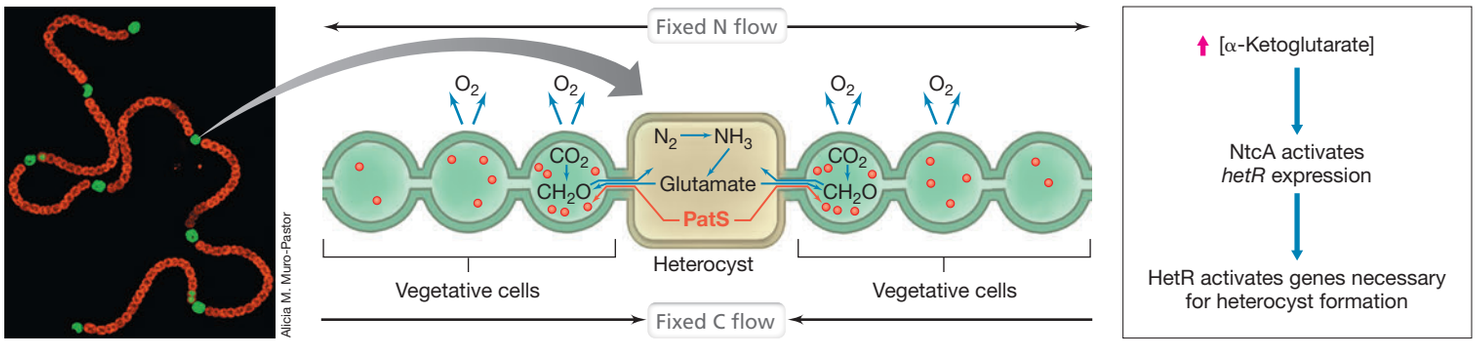
Heterocyst formation requires several morphological and metabolic changes that are regulated by a network of systems that sense both external conditions and intracellular signaling molecules. These changes include the formation of a thickened cell wall to prevent O_2 diffusion into the cell, inactivation of photosystem II, expression of nitrogenase, and the patterning of heterocyst differentiation along the filament (Figure 8.21*a*). Because nutrients can be exchanged between heterocysts and adjacent vegetative cells (Figure 8.21*b*), other regulatory steps are initiated to prevent nearby vegetative cells from undergoing the developmental conversion to heterocysts.

Regulation of Heterocyst Formation

The cascade of events leading to heterocyst formation is triggered by a limitation of "fixed" nitrogen (nitrate, ammonia, etc.); the limitation is sensed in the vegetative cell as an elevation in levels of α-ketoglutarate, the acceptor molecule for formation of the amino acid glutamate (◀ Section 3.14). When the cell is starved for fixed nitrogen, α-ketoglutarate accumulates and activates the transcriptional global regulator NtcA. NtcA then activates transcription of the gene *hetR*, which encodes HetR, the major transcriptional regulator controlling heterocyst formation. HetR activates a cascade of genes necessary for differentiation of the heterocyst, expression of cytochrome *c* oxidases to remove traces of O_2 that may still be present, and expression of the *nif* operon for the synthesis and regulation of nitrogenase (Figure 8.21*c*).

Only specific cells along the filament form heterocysts, and the consistent pattern observed (Figure 8.21*a*) is under strict control. Intercellular connections between cells in an *Anabaena* filament allow vegetative cells to provide fixed carbon to the heterocyst as an electron donor (for N_2 reduction by nitrogenase) in exchange for some of the NH_3 produced. However, the cell connections also allow for intercellular communication by regulatory molecules. In this regard, differentiating cells produce a small peptide called PatS that diffuses away from the developing heterocyst to form a gradient along the vegetative cells in the filament (Figure 8.21*b*). PatS inhibits differentiation in vegetative cells by preventing HetR from activating genes necessary for heterocyst formation. A second regulator called PatA, a response regulator analogous to the chemotaxis response regulator CheY (◀ Sections 7.5 and 7.6 and Figure 7.17), also participates in heterocyst pattern development. PatA promotes the activity of HetR, decreases the activity of PatS, and may also participate in cell division.

While other regulatory links in heterocyst formation remain active areas of study, the differentiation of vegetative cells to heterocysts in heterocystous cyanobacteria is a unique example of multicellular patterning and intercellular communication in prokaryotic cells. We



(a) **A filament of *Anabaena*** *(b)* **Heterocyst—vegetative cell interactions** *(c)* **Triggering heterocyst formation**

Figure 8.21 Regulation of heterocyst formation. *(a)* Fluorescence microscopy showing *Anabaena* filaments expressing the green fluorescent protein linked to heterocyst-specific genes; vegetative cells are red from chlorophyll *a* fluorescence. *(b)* Molecule dispersion in heterocysts. Fixed carbon from photosynthesis in the vegetative cells is transferred to the heterocyst, while fixed nitrogen produced in the heterocyst is shared with the vegetative cells. The protein PatS, which is synthesized by heterocysts, is also dispersed to neighboring vegetative cells where it inhibits expression of genes necessary for heterocyst formation. *(c)* Cascade of events in the activation of genes necessary for heterocyst formation. The cascade is initiated by an increase in levels of α-ketoglutarate, the carbon skeleton of the amino acids glutamate and glutamine.

now consider a final developmental model system, one in which cell populations—in contrast to individual cells—cooperate to dramatically change their growth characteristics.

Check Your Understanding

- Vegetative cells in *Anabaena* produce oxygen, while its heterocysts do not. Why?
- What is the major transcriptional regulator that controls heterocyst formation?

8.10 Biofilm Formation

In Section 4.9 we briefly discussed the basic characteristic of bacterial biofilms—attached polysaccharide matrices containing embedded bacterial cells—along with their environmental and medical significance. We revisit the concept of biofilms here with a focus on the molecular mechanisms that control biofilm development.

Biofilm formation can be controlled in different ways, and the process has been particularly well studied in two gram-negative *Bacteria*, *Pseudomonas aeruginosa* and *Vibrio cholerae.* However, the process is not limited to *Bacteria*, as biofilms have also been observed in some *Archaea* and yeasts. Biofilm formation can be considered a type of developmental cycle that has four basic stages: (1) attachment, (2) colonization, (3) development, and (4) dispersal. Random collision of cells with a surface accounts for the initial cell attachment, which is facilitated by cell surface structures such as flagella and pili or surface proteins. Attachment of a cell to a surface (**Figure 8.22**) is a signal for the expression of biofilm-specific genes. The latter include genes encoding proteins that produce intercellular signaling molecules and *extracellular polymeric substances* (abbreviated as *EPS*), which includes polysaccharide, DNA, and protein, that initiate matrix formation. Once committed to biofilm formation, a previously suspended (planktonic) cell typically loses its flagella and becomes nonmotile. However, biofilms are not static entities and cells can be released from the biofilm through an active process of dispersal.

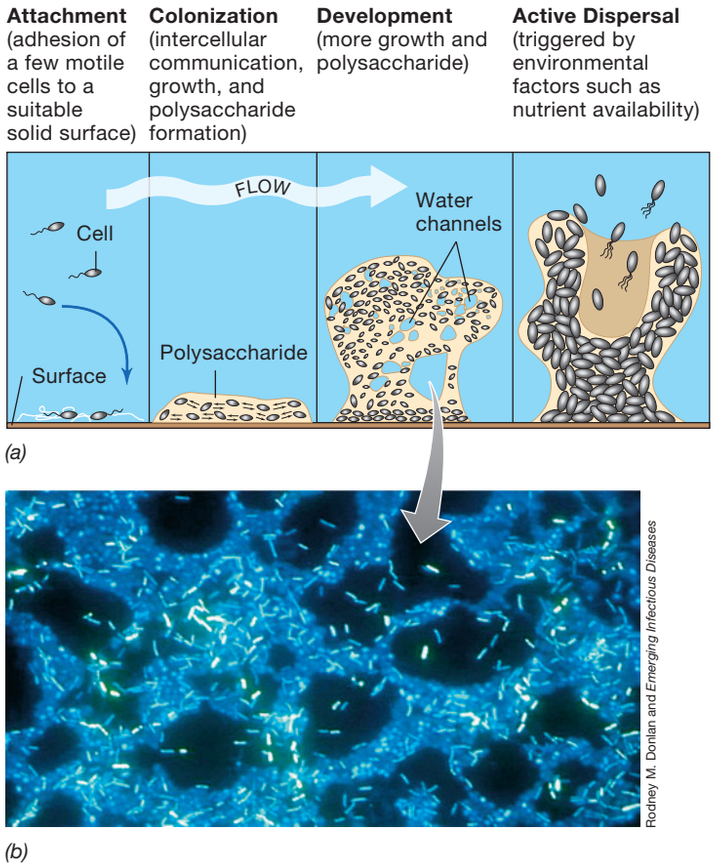


Figure 8.22 Biofilm formation. *(a)* Biofilms begin with the attachment of a few cells that then grow and communicate with other cells. The matrix is formed and becomes more extensive as the biofilm grows, eventually releasing cells. *(b)* Photomicrograph of a DAPI-stained biofilm that developed on a stainless steel pipe. Note the water channels.

Cyclic Di-GMP and Biofilm Formation

Several signals guide bacteria in transitioning from planktonic growth to life in a semisolid matrix. The actual switch from planktonic to biofilm growth in many bacteria is triggered by the cellular accumulation of the regulatory nucleotide *cyclic di-guanosine monophosphate* (c-di-GMP) (**Figure 8.23**). Although various nucleotides play important regulatory roles in all domains of life (◀ Section 7.8), c-di-GMP is widely distributed only in *Bacteria*. The synthesis or degradation of c-di-GMP depends on both environmental and cellular cues, and its synthesis either promotes or inhibits a variety of physiological events including cell differentiation, bacterial development, and biofilm-relevant functions (Figure 8.23).

Enzymes that synthesize c-di-GMP from GTP are called diguanylate cyclases, and these enzymes often possess an allosteric binding site for c-di-GMP. Thus, these enzymes are sensitive to feedback inhibition (◀ Section 7.15) and c-di-GMP production is reduced once elevated levels exist in the cell. Conversely, proteins with phosphodiesterase domains degrade the signaling nucleotide to the guanine derivatives pGpG and GMP (Figure 8.23). These two types of proteins coordinate antagonizing activities to yield cellular levels of c-di-GMP necessary to influence cellular activities. This regulatory molecule directly affects a host of cellular activities by binding to structural proteins and enzymes as well as to transcription factors and riboswitches (◀ Sections 7.2 and 7.13, respectively). In the specific context of biofilms, c-di-GMP binds to proteins that reduce the activity of the flagellar motor, regulates cell surface proteins required for attachment, and mediates the biosynthesis of extracellular matrix polysaccharides of the biofilm.

Pseudomonas aeruginosa and Biofilms

Pseudomonas aeruginosa can form a tenacious biofilm (◀ Section 4.9) containing specific polysaccharides that subsequently increase its pathogenicity and prevent the penetration of antibiotics. *P. aeruginosa* is a classic opportunistic pathogen and from its primary reservoir in soil can infect the blood, lungs, urinary tract, ears, skin, and other tissues of humans. The major symptoms of the human genetic disease cystic fibrosis are caused by thick biofilms of *P. aeruginosa* that develop in the lungs, and the bacterium is a significant nosocomial (hospital-acquired) pathogen.

Besides the intracellular activities triggered by c-di-GMP, intercellular communication by quorum sensing (◀ Section 7.7) is critical for the development and maintenance of *P. aeruginosa* biofilms. As

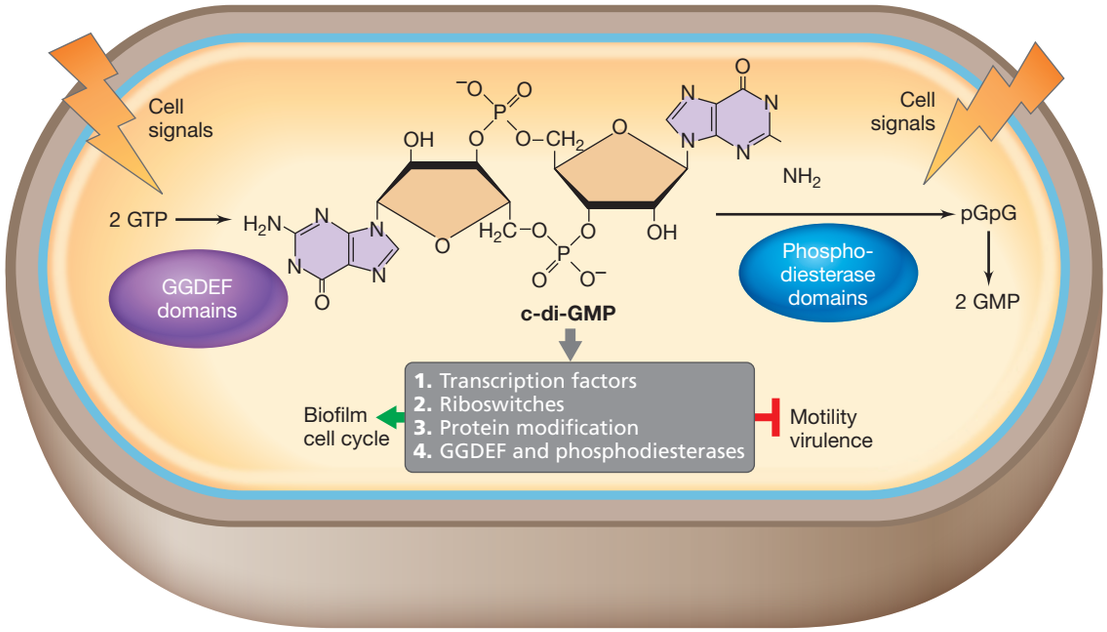
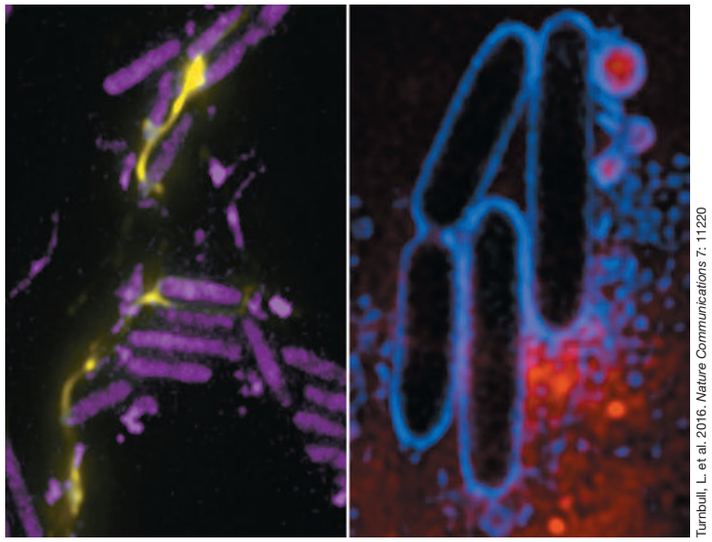


Figure 8.23 The signaling molecule cyclic di-guanosine monophosphate. This molecule is used as an intracellular signaling molecule by many bacteria to control specific physiological processes by binding to various effectors. Two molecules of GTP are converted to c-di-GMP by the activity of proteins with GGDEF (diguanylate cyclase) domains, while the activity of proteins with phosphodiesterase domains converts c-di-GMP to pGpG and GMP.

the regulatory molecules called *acyl homoserine lactones* (AHLs, ◀ Figure 7.18*b*) accumulate, they signal *P. aeruginosa* cells that the population of this species is enlarging and thus the opportunity for biofilm formation exists. The production of AHLs also triggers expression of a subset of the genes necessary for biofilm formation including those that increase extracellular polysaccharide and c-di-GMP synthesis.

Elevated c-di-GMP levels initiate the production of EPS, including a polysaccharide called Pel, which functions as both a primary scaffold for the microbial community and a mechanism for resisting the penetration of antibiotics; c-di-GMP also leads to decreased flagellar function. Over time in nutrient-rich conditions, *P. aeruginosa* cells can develop mushroom-shaped microcolonies that can be more than 0.1 mm high and contain millions of cells (Figure 8.22; ◀ Figure 4.16). The final architecture of a biofilm is determined by several factors in addition to signaling molecules, and these include both nutritional factors and the flow rate of the biofilm environment.

Besides the contribution of decreased flagellar movement and Pel production, DNA release by lysed cells can also promote biofilm formation in *P. aeruginosa* (**Figure 8.24*a***). This event—termed "explosive death"—occurs in a subpopulation of *P. aeruginosa* cells. Explosive death is caused by the expression of a lysis protein encoded in the *P. aeruginosa* genome by an inactive prophage; the protein is produced in response to stress conditions such as DNA-damaging reagents and antibiotics and is occasionally a random event under normal conditions. When this lysis enzyme is expressed, the bacterial cells break open and release their DNA (Figure 8.24*a*). This highly viscous DNA, now extracellular, is integrated into the EPS that forms the biofilm scaffold. Besides the release of DNA, explosive death also leads to shattered cell membrane fragments that curl together to form



(a) (b)

Turnbull, L. et al. 2016. *Nature Communications 7:* 11220

Figure 8.24 Explosive death and biofilm formation in *Pseudomonas aeruginosa*. This gram-negative gammaproteobacterium (▶ Section 16.4) exists in soil as a harmless saprophyte, but can also be a vicious opportunistic human pathogen that can cause infections of blood, burns, and related wounds, and ears, lungs, and eyes. *P. aeruginosa* is of particular concern in individuals suffering from the genetic disease cystic fibrosis because of the organism's ability to form biofilms that affect lung capacity. *(a)* Cell lysis and DNA release. Production of the lysis enzyme causes the bacterial cells (purple) to break open, releasing their DNA (yellow). This DNA is critical to forming the EPS that holds the biofilm together. *(b)* Cell bursting and biofilm formation. Cell lysis leads not only to the release of DNA, but also to shattered cell membrane fragments that curl together to form vesicles surrounding proteins and DNA that contribute to EPS formation (small blue circles with red centers). Images adapted from Turnbull, L., et al. 2016. *Nat. Commun. 7:* 1120.

vesicles surrounding cellular proteins and DNA. These vesicles also contribute to EPS formation and biofilm development (Figure 8.24*b*). Explosive death likely explains why antibiotic treatment of *P. aeruginosa* infections often enhances biofilm formation; this protective mechanism in bacterial cells thwarts antibiotic penetration.

Biofilm formation strategies can differ among species of the same genus. In *Pseudomonas fluorescens*, increases in c-di-GMP also promote biofilm formation. However, the biofilm machinery regulated by c-di-GMP in *P. fluorescens* differs from that of *P. aeruginosa*. In *P. fluorescens*, changes in c-di-GMP levels affect secretion and cell surface localization of a large adhesion protein called LapA that helps attach cells to the surface. For example, when environmental levels of a key nutrient such as phosphate are *high*, c-di-GMP levels are high, LapA localizes to the cell membrane, and biofilm synthesis is promoted. However, in response to *low* levels of phosphate, *P. fluorescens* cells maintain a low c-di-GMP level that prevents localization of LapA to the outer membrane, thereby disabling the attachment mechanism required to initiate biofilm formation. If phosphate levels continue to fall within a *P. fluorescens* biofilm, the associated reduction in c-di-GMP levels results in the release of a specific protease normally sequestered by a c-di-GMP-binding protein. This protease cleaves LapA, resulting in the release of attached cells and promoting their dispersal to explore for available nutrients in nearby habitats.

Vibrio cholerae and Biofilms

Vibrio cholerae, the causative agent of cholera (▶ Section 33.3), forms biofilms containing polysaccharide and the carbohydrate-binding matrix proteins RbmA, RbmC, and Bap1. **Figure 8.25** illustrates the impressive production of these matrix proteins during biofilm growth. Synthesis of these biofilm proteins is controlled by both intercellular and intracellular signaling. Two transcriptional activators, VpsR and VpsT, are required to activate expression of the matrix protein genes, while a repressor protein, HapR, negatively regulates biofilm formation by decreasing the activity of the two activator proteins VpsT and VpsR. The activity of these key transcription factors is controlled by a host of other factors including two-component regulatory systems, alternative sigma factors, catabolite repression, quorum sensing, and regulatory RNAs (Chapter 7).

While c-di-GMP is the inducer molecule that activates VpsT and thus expression of genes for biofilm formation (Figure 8.23), quorum sensing in *V. cholerae* acts in an opposite manner from that in

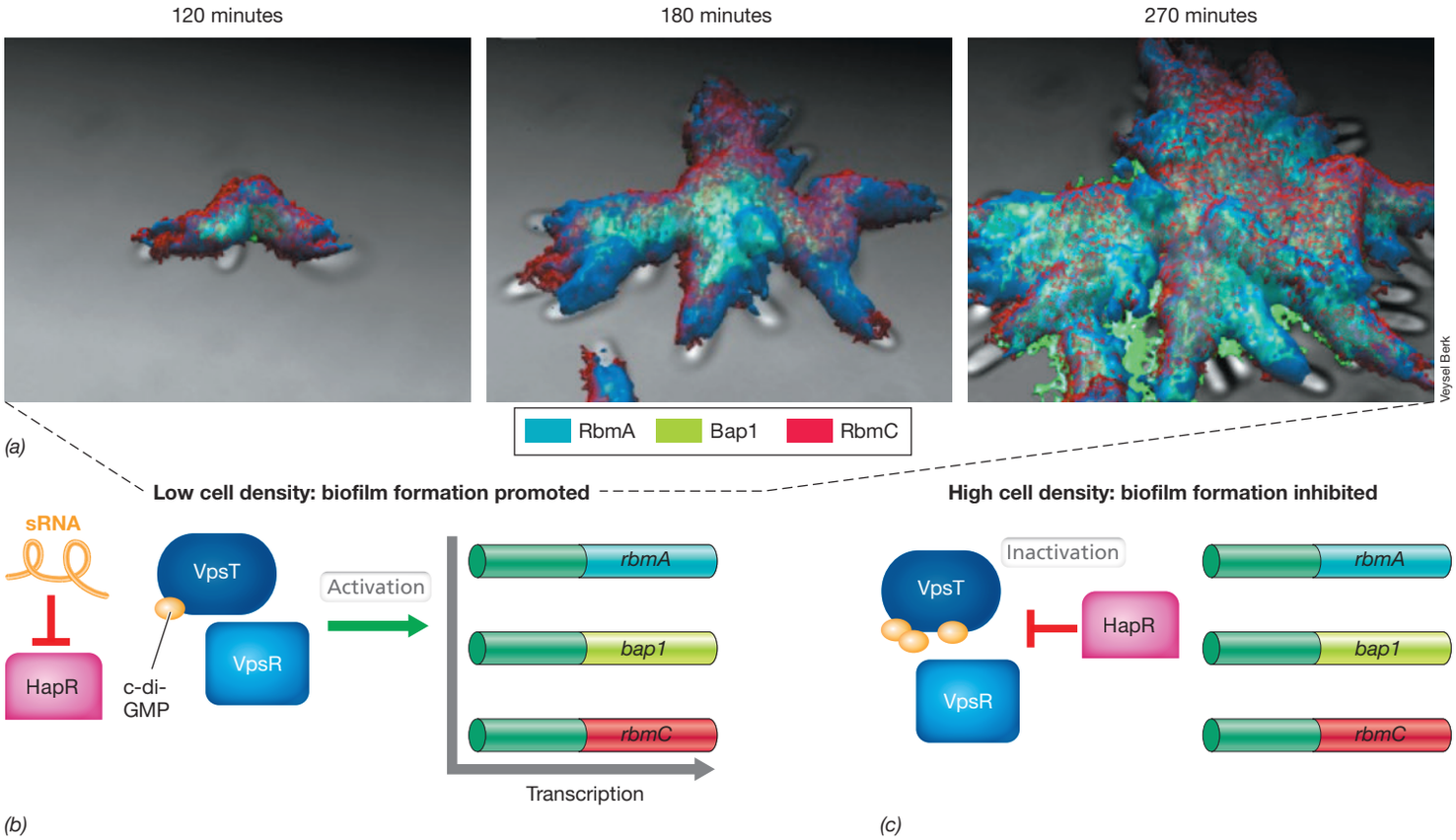


Figure 8.25 ***Vibrio cholerae*** **biofilm formation.** *(a)* Time-course multiresolution fluorescence micrographs illustrating the production and release of the carbohydrate-binding matrix proteins RbmA, RbmC, and Bap1 during biofilm formation. Each protein is detected by the binding of a specific fluorescently labeled antibody. *(b)* Low-cell-density-dependent activation of genes encoding carbohydrate-binding matrix proteins for biofilm formation. A low concentration of quorum-sensing molecules promotes transcription of a small regulatory RNA that binds to the mRNA encoding the HapR repressor. This results in lower levels of HapR protein and thus synthesis of the transcriptional activators VpsR and VpsT; these activate transcription of the genes *rbmA*, *rbmC*, and *bap1*. Note that VpsT is active when bound to c-di-GMP. *(c)* High-cell-density-dependent inhibition of biofilm formation. A high concentration of quorum-sensing molecules results in decreased expression of the sRNA that binds to the mRNA encoding HapR. Thus, high levels of HapR protein exist in the cell, inhibiting the activity of VpsR and VpsT. This signal cascade shuts off transcription of *rbmA*, *rbmC*, and *bap1*.

P. aeruginosa. At *low* cell densities, small regulatory RNA molecules bind to the mRNA encoding HapR, thus preventing its translation. As *V. cholerae* cell density *increases*, the level of quorum signaling molecules also increases and this results in *decreasing* levels of the regulatory RNAs that target HapR mRNA. The latter results in an increased concentration of the HapR repressor, decreased expression of the activator proteins VpsT and VpsR, and the dispersal of attached cells. Thus, unlike in *P. aeruginosa*, in *V. cholerae*, biofilm formation is triggered by *low* cell densities and repressed by *high* cell densities. Or, said another way, in *V. cholerae*, the accumulation of quorum sensing molecules triggers a regulatory cascade that ultimately results in the *repression* of biofilm formation genes and the *activation* of flagellar genes, just the opposite of what occurs in *P. aeruginosa*.

The ecological significance of this phenomenon is that biofilm formation is more likely to occur when *V. cholerae* is found in its natural marine environment where nutrients are typically scarce, thus leading to smaller populations than those found inside the close quarters of an intestinal cell where nutrients are more plentiful. Biofilm formation allows the *V. cholerae* cell to attach to marine surfaces such as plankton (▶ Section 33.3), crustaceans, and sediments for greater access to nutrients and protection from perturbations. The final step in biofilm formation, dispersal, ultimately aids in the transmission of *V. cholerae* to new host cells.

Because the biofilm lifestyle of many pathogenic bacteria often leads to either persister cells (Section 8.12) or antibiotic-resistant cells (or both), and because biofilms themselves function as barriers to the penetration of antimicrobial drugs, there is great medical interest in understanding the molecular biology of biofilm development and pursuing new classes of drugs that might interfere with biofilm formation.

Biofilms and *Archaea*

While our understanding of biofilms in *Archaea* is just beginning, at least some of the events in archaeal biofilm formation appear to share features with those of bacterial biofilms. Most of our knowledge comes from studying biofilm formation in the motile hyperthermophilic sulfur chemolithotroph *Sulfolobus acidocaldarius* (**Figure 8.26*a***).

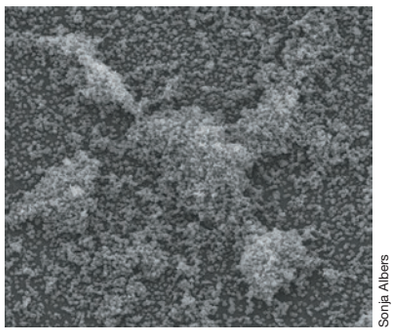
A transcription factor called AbfR1plays a major role in the *Sulfolobus* biofilm. In the phosphorylated state, AbfR1 activates biofilm formation through the increased expression of genes for EPS and pili synthesis and decreased expression of genes encoding archaella (◀ Section 2.9) synthesis (Figure 8.26*b*). This ultimately leads to increased cell attachment and decreased motility. The opposite occurs when AbfR1 is in the unphosphorylated state—archaella production is activated and pili and EPS synthesis repressed. While the signal and sensor kinase (◀ Section 7.5) responsible for phosphorylating AbfR1 remain unknown, biofilm formation in *Sulfolobus* appears to be a response to significant changes in pH or temperature.

Although the role that quorum sensing (◀ Section 7.7) may play in *Sulfolobus* biofilm formation is also unclear, *Archaea* have been shown to produce quorum-sensing-like molecules. *Archaea* do not produce c-di-GMP—a major regulator in *Bacteria* (Figure 8.23)—but genomic analysis suggests that many species can produce c-di-AMP. This regulatory molecule, which is also found in *Bacteria*, may therefore play a role in archaeal biofilm formation (Figure 8.26) and other events in *Archaea* where quorum-sensing molecules may participate.

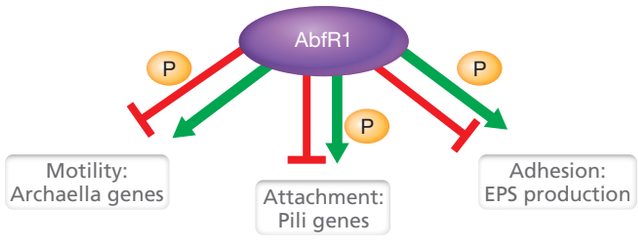
We will continue our discussion of biofilms and strategies to control their formation when we consider the ecology of biofilms in Chapter 20. But for now, we move on to Part III where we consider antibiotics and bacterial growth and focus on the alarming practical problem of antibiotic resistance.

Check Your Understanding

- What are the four basic stages of biofilm formation?
- Besides autoinducer synthesis, what intracellular molecule promotes biofilm formation in many *Bacteria*?



(a)



(b)

Figure 8.26 Biofilm formation in the crenarchaeon *Sulfolobus acidocaldarius*. *Sulfolobus* is an acidophilic and hyperthermophilic sulfur-oxidizing archaean (▶ Section 17.10). *(a)* Scanning electron micrograph of a 6-day-old biofilm. *(b)* Regulation of biofilm formation by the transcription factor AbfR1. Activation and repression are dependent on the phosphorylation state of AbfR1 with green arrows indicating activation and red lines indicating repression. As can be seen, when AbfR1 is phosphorylated, motility is repressed and attachment activated. Image adapted from Koerdt, A., et al. 2010. *PLoS ONE 5*(11): e14104.

III • Antibiotics and Microbial Growth

Antibiotics target specific bacterial growth processes and have been a boon to clinical medicine. However, antibiotic efficacy has been compromised by bacterial resistance mechanisms that include mutations that alter the targets, drug destruction or efflux, and entering a transient dormant state.

Throughout this chapter we have discussed how cells coordinate their molecular biology to optimize steps in microbial growth. But what about mechanisms that short-circuit these synchronized processes? Here we focus on how antibiotics target growth processes and the bacterial responses that can lead to resistance and persistence. In Chapter 28 we will focus on the clinical significance of antibiotics and consider their spectrum of activity and spread of resistance.

8.11 Antibiotic Targets and Antibiotic Resistance

Antibiotics are antimicrobial agents produced by microorganisms, primarily certain bacteria and fungi. These agents are characterized by their ability to either kill or inhibit the growth of bacteria, and all target molecular processes in the cell that are essential to growth and survival. In this section we consider how antibiotics work and some key resistance mechanisms that bacteria have evolved to counter their effects.

Antibiotics That Target Major Molecular Processes

Because all steps within the central dogma (the flow of genetic information; Chapter 6) are essential for growth, many antibiotics specifically target enzymes that catalyze DNA replication, RNA synthesis, and protein synthesis (Figure 8.27). For example, quinolones such as ciprofloxacin target DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive bacteria. Thus quinolones lead to cell death by interfering with DNA unwinding and replication (◀ Section 6.1). Likewise, when transcription is inhibited in a growing cell, mRNA cannot be made and new protein synthesis is interrupted. The antibiotics rifampin and actinomycin prevent RNA synthesis by either blocking the RNA polymerase active site (rifampin) or blocking RNA elongation by binding to the major groove in DNA (◀ Section 6.5).

Many antibiotics inhibit bacterial growth by targeting some aspect of protein synthesis (Figure 8.27). Recall that ribosomes in *Bacteria* are 70S structures while eukaryotic ribosomes are 80S (◀ Section 6.10). The antibiotic puromycin contains a region that mimics the 3′ end of a tRNA, and this structural mimicry results in specific binding of the antibiotic to the A site in the 70S ribosome; this induces chain termination and effectively shuts down protein synthesis. Aminoglycoside antibiotics such as streptomycin specifically target the 16S rRNA of the 30S ribosome and result in the ribosome misreading mRNAs, thus leading to error-filled proteins that accumulate in the cell and ultimately inhibit growth.

Antibiotics That Target the Cell Membrane and Wall

Other common antibiotic targets include the cell membrane, the cell wall, and specific metabolic processes (Figure 8.27).

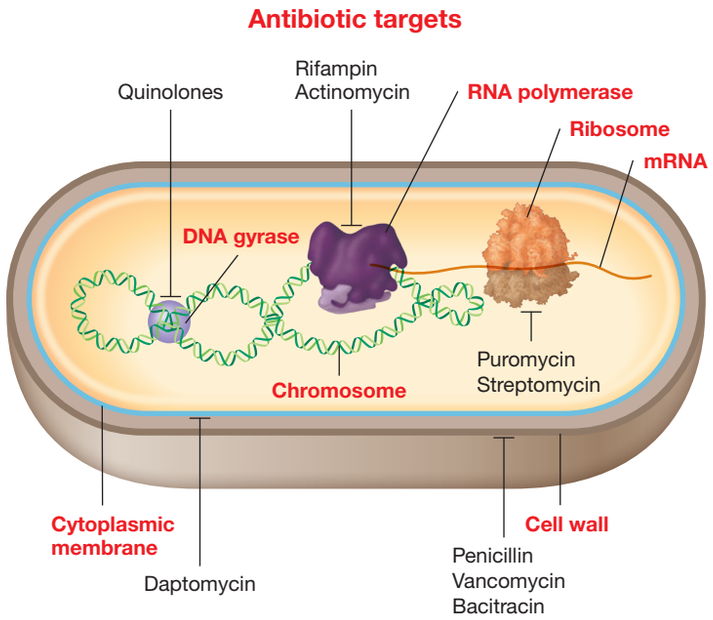


Figure 8.27 Growth targets of select antibiotics. Antibiotic targets are in red bold.

Daptomycin is a lipopeptide produced by certain *Streptomyces* species that specifically binds to phosphatidylglycerol residues of the bacterial cytoplasmic membrane (◀ Section 2.1); this leads to pore formation and depolarization of the membrane, ultimately resulting in cell death. Some antibiotics target the gram-negative cell outer membrane (◀ Section 2.4). For example, polymyxins are cyclic peptides whose long hydrophobic tails specifically target the LPS layer and ultimately disrupt membrane structure, causing leakage and cell death.

Several antibiotics target the synthesis of peptidoglycan in bacteria, including the β-lactams penicillin, cephalosporin, and their derivatives. These antibiotics inhibit growth by interfering with proteins that catalyze transpeptidation (penicillin-binding proteins, Section 8.5), the process by which cross-links are made between muramic acid residues that contribute to the structural strength of peptidoglycan (◀ Section 2.3). Similarly, the antibiotic vancomycin inhibits peptidoglycan synthesis in gram-positive bacteria by binding to the pentapeptide of peptidoglycan precursors and preventing the formation of peptide interbridges by transpeptidases. The topical antibiotic bacitracin prevents peptidoglycan synthesis by binding to the peptidoglycan precursor transport system (bactoprenol, Section 8.5) and preventing new peptidoglycan precursors from reaching the site of peptidoglycan synthesis. As autolysins continue to introduce small gaps in the existing peptidoglycan (Figure 8.16*a*), a shortage of precursors to patch the gaps weakens the cell wall and leads to cell lysis.

Many other antibiotics and related antimicrobials are known, some of which target other aspects of a bacterium's biology, such as particular metabolic reactions. But the bulk of clinically useful antibiotics strike one of the targets shown in Figure 8.27. We finish this section with a look at how bacteria have countered the activity of antibiotics by evolving major mechanisms of antibiotic resistance.

Antibiotic Resistance: Spontaneous Mutations and Antibiotic Modification

If bacteria are to survive the onslaught of antibiotics produced by other microbes (or their own antibiotics if they are an antibiotic-producing organism), they require resistance mechanisms of one sort or another. Resistance mechanisms are genetically encoded and fall into four classes: (1) modification of the drug target, (2) enzymatic inactivation, (3) removal from the cell via efflux pumps, and (4) metabolic bypasses (**Figure 8.28**).

Random chromosomal mutations can lead to antibiotic resistance. For example, spontaneous mutants of *Escherichia coli* or other bacteria resistant to the antibiotic rifampin—which inhibits the activity of RNA polymerase—can be obtained by simply exposing a large cell population to the antibiotic. Under such conditions, spontaneous mutants that produce an altered RNA polymerase unaffected by rifampin are strongly selected for.

Resistance genes can also exist on a variety of mobile genetic elements (Chapter 6), and such genes can be readily transmitted between bacteria of the same or different species by horizontal gene flow (Chapter 9). Unlike a spontaneous mutation that affects the target of the antibiotic, many of these mobile resistance genes, especially ones transmitted by R plasmids, encode enzymes that inactivate the antibiotic by altering its structure, either by chemical modification or cleavage. As examples, β-lactamase binds to β-lactam antibiotics and cleaves a key ring structure in the molecule, and an acetylating enzyme adds acetyl groups to free hydroxy groups of chloramphenicol; once cleaved or chemically modified, the drugs can no longer bind to their target and microbial growth can continue.

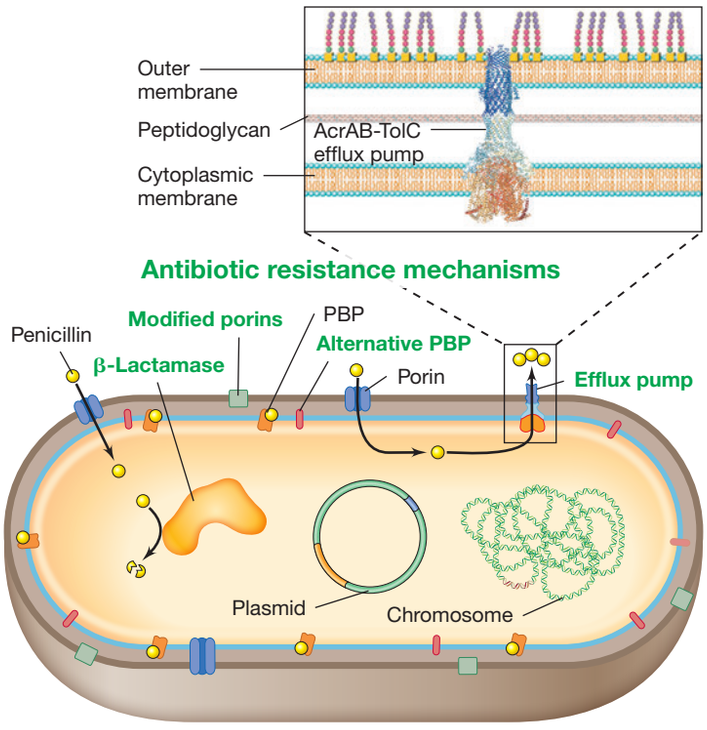


Figure 8.28 Mechanisms of antibiotic resistance. Penicillin (a β-lactam antibiotic) is used to illustrate the mechanisms of modified target (modified porin), drug inactivation (β-lactamase), efflux pump, and metabolic bypass (alternative penicillin-binding protein). Genes for efflux pumps and β-lactamase enzymes can be plasmid or chromosomally encoded, while the alternative penicillin-binding protein is encoded only on the chromosome. PBP: Penicillin-binding protein. Mechanisms of antibiotic resistance are in green bold. Insert: Electron micrograph of the ArcAB-TolC efflux system from *Escherichia coli* positioned in an illustration of a gram-negative cell envelope.

Antibiotic Resistance: Efflux Pumps and Metabolic Bypasses

Another effective resistance strategy is to pump out antibiotics that have entered the cell. *Efflux pumps* are ubiquitous in gram-negative bacteria and function by transporting various molecules, including antibiotics, out of the cell (Figure 8.28). Efflux lowers the intracellular concentration of an antibiotic and thus allows the cell to survive at higher external concentrations. Many efflux pumps act promiscuously by transporting different classes of antibiotics outside of the cell, which contributes to the problem of multidrug resistance. The AcrAB-TolC efflux system of *E. coli* is one of the best-characterized efflux pumps and can pump out several antibiotics including rifampin, chloramphenicol, and fluoroquinolones (Figure 8.28, inset).

Growth as a biofilm (Section 8.10) leads to increased antibiotic resistance, a characteristic that makes infections caused by biofilm-forming bacteria difficult to treat. Although the exopolysaccharide matrix of a biofilm decreases the permeability of antibiotics, efflux pumps also play a major role. For example, genes encoding the AcrAB-TolC efflux pump in *E. coli* are upregulated when cells enter a biofilm growth mode. *Pseudomonas aeruginosa*, a classic biofilm former, encodes several multidrug efflux pumps that are also more active when cells grow in an attached state.

A final form of antibiotic resistance occurs when the target of the antibiotic is no longer essential to the cell's metabolism or survival. A classic example of this is *methicillin-resistant Staphylococcus aureus* (*MRSA*), the causative agent of a variety of serious, even life-threatening, infections (▶ Section 31.9). Methicillin is a β-lactam antibiotic that, like other penicillins, targets the activity of penicillin-binding proteins. However, unlike many penicillin derivatives, methicillin is resistant to cleavage by β-lactamase. Although many strains of *S. aureus* are killed by methicillin, MRSA strains contain a 20- to 60-kilobase DNA chromosomal (or genomic) island (▶ Section 13.10) called the *Staphylococcus chromosomal cassette for methicillin resistance* (*SCCmec*); these genes encode an *alternative* penicillin-binding protein called MecA that is not recognized by methicillin or other β-lactams (Figure 8.28).

Interestingly, MRSA strains synthesize MecA *only* in the presence of methicillin or other β-lactams. This regulation is due to the presence of the repressor protein MecI and the β-lactam sensor MecR1. In the absence of a β-lactam antibiotic, MecI binds to the operator site (◀ Section 7.2) of *mecA* and represses transcription. If the cell is treated with methicillin or other β-lactams, it triggers the MecR1 protease to degrade the MecI repressor. This induces transcription of *mecA* (and thus the production of MecA), which allows MRSA strains to synthesize peptidoglycan in the presence of methicillin. The chromosomal island of MRSA strains also possesses a transposable element (◀ Section 6.2) that encodes resistance to the antibiotics erythromycin and spectinomycin. The island is thus likely subject

to horizontal transfer to confer multidrug resistance. This reality makes MRSA a serious problem for clinical medicine.

Check Your Understanding

- Describe two targets of antibiotics and discuss why the drugs are effective.
- Some antibiotics target peptidoglycan synthesis. What is a molecular growth target of an antibiotic that inhibits peptidoglycan synthesis?
- Why are efflux pumps capable of conferring multidrug resistance?

8.12 Persistence and Dormancy

Besides being *sensitive* or *resistant* to antibiotics, a third growth response is possible—**persistence**. Persistence occurs when a population of antibiotic-sensitive bacteria produces rare cells that *transiently* become tolerant to multiple antibiotics (**Figure 8.29*a***). These *persisters*, as they are called, are genetically identical to their antibiotic-susceptible siblings, and thus persistence is not inheritable or conferred by genetic transfer. Instead, persisters, which form at a rate of 10^{-6} to 10^{-4} in exponentially growing cultures, derive from a state of *dormancy* in which the cells are viable but do not grow.

Because antibiotics target active processes, the dormant state prevents the antibiotic from killing the cell. When antibiotic treatment is stopped, the dormant cells emerge from dormancy and grow. Persistence is believed to be the cause of recurring infections of *Mycobacterium tuberculosis* in those with chronic tuberculosis (▶ Section 31.4) and of *Pseudomonas aeruginosa* in those suffering from the genetic disease cystic fibrosis.

How does a subpopulation of cells go dormant while genetically identical daughter cells do not? For a cell to become a persister and be able to later reverse the process, coordinated molecular events that affect cell growth must take place. The keys to this unusual phenomenon are chromosomally encoded toxin–antitoxin modules, the stringent response (◀ Section 7.9), and a phenomenon called *phenotypic heterogeneity*. Sporulation is a classic example of cells in a population displaying different phenotypes (Section 8.6). This phenotypic heterogeneity occurs because gene expression is not equal in all cells of a population. As **Figure 8.30** illustrates, a subpopulation of vegetative cells remains metabolically active after other cells have committed to forming endospores.

Toxin–Antitoxin Modules

Toxin–antitoxin (TA) modules are genes that encode two components: a toxin whose production inhibits cell growth and an antitoxin that counteracts the activity of the toxin. TA modules are found in almost all *Bacteria* and in many *Archaea*. The identification of over 30 TA systems in *Escherichia coli* and 60 TA systems in *M. tuberculosis* suggests that TA modules play a role not only in normal physiology but also in pathogenicity. It is thought that toxin activity promotes cellular adaptation to ever-changing environments by slowing down cell growth to help ensure survival during stressful conditions.

The *hipAB* genes encode a TA module that has been shown to trigger persistence in *E. coli* (Figure 8.29*b*). In this module, HipA is a toxin that inhibits translation and HipB is an antitoxin that is susceptible to a protease called Lon. Under normal cellular conditions, the HipA toxin and HipB antitoxin form a stable complex that prevents HipA from exerting its toxicity. However, if Lon is activated by its signal molecule polyphosphate (PolyP), the HipB antitoxin is degraded.

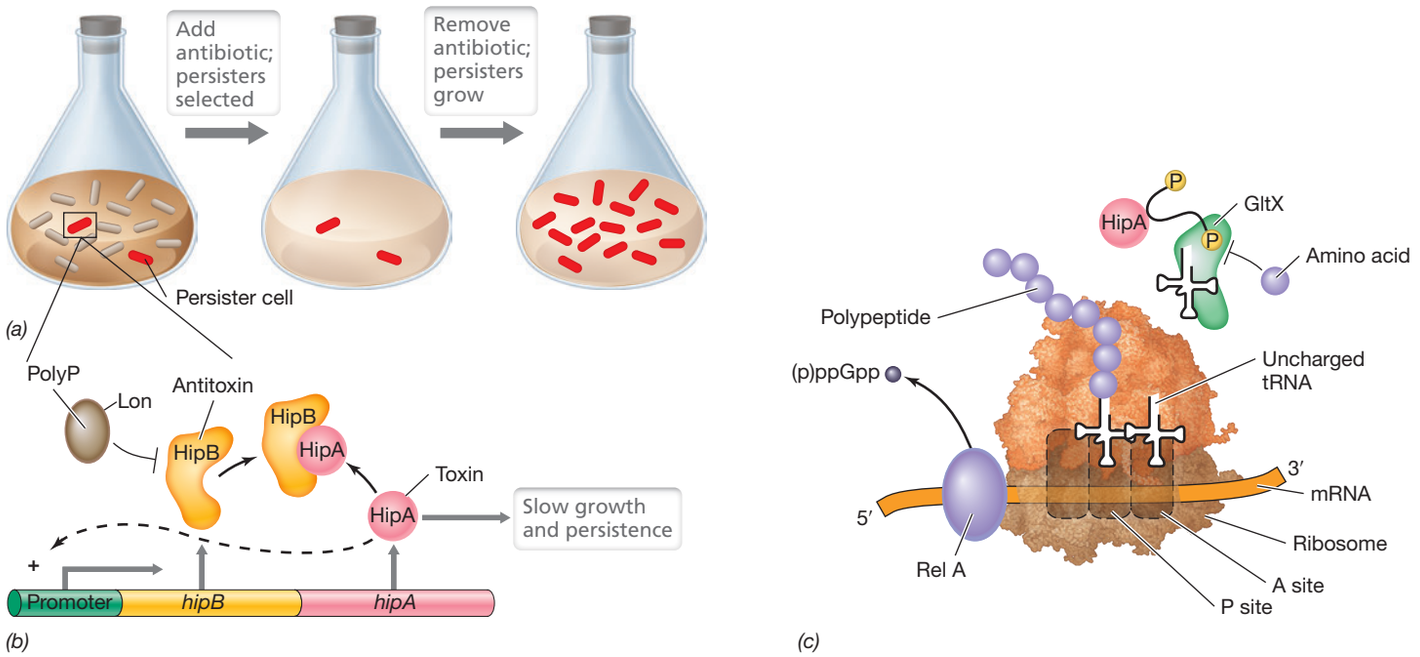
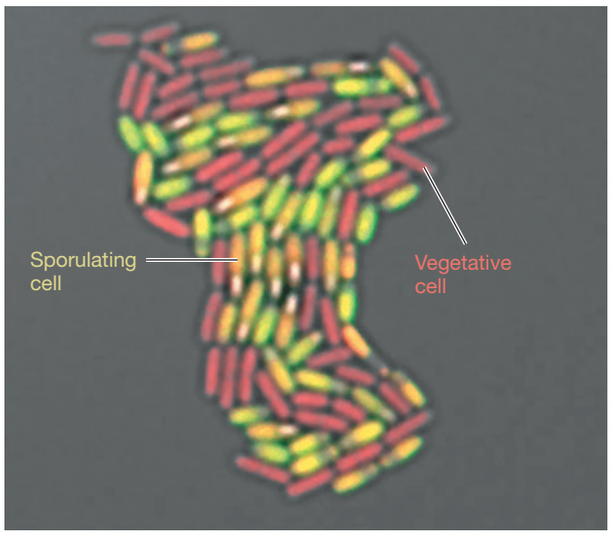


Figure 8.29 Persistence and the HipAB toxin–antitoxin module. *(a)* Antibiotic selection of persister cells and recovery from dormancy. *(b)* HipAB toxin–antitoxin module. During normal conditions, HipB sequesters the toxin HipA. The presence of Lon–PolyP leads to inactivation of the HipB antitoxin and free HipA toxin. HipA leads to slow growth and persistence and positively regulates the *hipAB* operon. *(c)* Mechanism of HipA toxin. HipA phosphorylates GltX, preventing the tRNA synthetase from charging amino acids. This leads to an uncharged tRNA entering the A site of the ribosome and the stimulation of RelA to produce (p)ppGpp.



Mutlu, A., Trauth, S., Ziesack, M., Nagler, K., Bergeest, J.P., Rohr, K., Becker, N., Höfer, T., and Bischofs, I.B. 2018. *Nat. Commun.* 9: 69

Figure 8.30 Phenotypic heterogeneity during sporulation. Fluorescence micrograph illustrating a population of *Bacillus subtilis* cells with some members committing to developing spores, while other cells remain vegetative and metabolically active. Cells yellow or green in color are sporulating cells expressing GFP linked to a promoter for the sporulation factor Spo0A (Figure 8.18) (developing endospores are green). Cells red in color are vegetative cells expressing a red fluorescent protein linked to tryptophan synthesis genes.

Without the HipB antitoxin present to neutralize the HipA toxin, translation is inhibited and cell growth is arrested (Figure 8.29*b*).

How does the HipA toxin inhibit translation? HipA acts as a kinase (phosphorylating protein) targeting glutamyl-tRNA synthetase (GltX). If HipA is free to phosphorylate GltX, GltX can no longer charge its respective tRNAs with glutamine. This leads to uncharged tRNAs entering the A site of the ribosome (◀ Section 6.10) and ultimately to stalling of the ribosome and activation of RelA (Figure 8.29*c*). Stalling, in turn, inhibits translation and thus protein synthesis. Free HipA also binds upstream of the *hipAB* operon, further activating transcription and thus increasing the concentration of cellular HipA (Figure 8.29*b*).

Steps to Dormancy

As we have seen, free HipA induces ribosome stalling. This stalling not only inhibits translation but also leads to the most important criteria for the development of persistence—production of the alarmone (p)ppGpp (guanosine tetraphosphate and guanosine pentaphosphate) by RelA and induction of the *stringent response pathway* (Figure 8.29*c*).

Recall that the stringent response leads to decreased rRNA and tRNA synthesis, and thus protein synthesis is inhibited (◀ Section 7.9). The pathway also leads to decreased DNA replication and cell division. Thus, those cells in a population that have triggered the stringent response no longer actively grow; they enter a state of dormancy. Although this cascade of events only occurs in a small subpopulation of cells, antibiotic treatment strongly selects and enriches for multidrug-tolerant persister cells. Once the antibiotic exposure has ended, the persisters can exit the stringent response pathway and resume producing antitoxin to neutralize the effect of the toxin. When this happens, protein synthesis returns to normal levels, allowing cells to grow.

Why does this cascade of events only occur in a *subpopulation* of cells without any sort of genetic direction? HipA-induced persistence occurs in cells that randomly produce higher amounts of the signal molecule PolyP. While it is often assumed that cells within a clonal population are homogeneous, cell-to-cell differences in gene expression and in metabolite and protein content do occur, resulting in phenotypic heterogeneity (Figure 8.30). These differences can be the result of RNA polymerase producing a subset of mRNAs through random interactions with DNA or of unequal distribution of macromolecules between daughter cells during cell division. These cell-to-cell variations can have many outcomes, but a major one for clinical medicine is the persistence of difficult-to-eradicate pathogenic bacteria whose dormant state can falsely signal a cure, only to be followed by reinfection by the same pathogen at a later date.

We move on now to a new chapter, one whose focus is on genetics and the mechanisms for genetic exchange that *Bacteria* and *Archaea* have exploited to rapidly obtain new metabolic and other properties that boost their chances of survival and reproduction.

Check Your Understanding

- Is persistence a heritable trait?
- What prevents the toxin component of TA modules from killing the cell under normal conditions?
- What occurs in the cell that results in freed HipA toxin?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Bacterial Cell Division

8.1 Macromolecules such as proteins and nucleic acids can be visualized in living cells using fluorescent tagging and microscopy techniques. Super-resolution microscopy can even resolve two individual molecules, generate 3D images, and track the movement of molecules throughout the cell.

Q What type of probes must be used for super-resolution microscopy and why?

8.2 Before cell division occurs in prokaryotic cells, two complete copies of the genome must be present. While multiple replication forks can exist to decrease generation times, the process must be regulated. The nucleoid must also segregate to the poles of the cell to allow the septum to form.

Q What is the role of PopZ in segregating the *Caulobacter* daughter chromosomes?

8.3 Cell division and chromosome replication are coordinately regulated, and the Fts proteins are keys to these processes. With the help of MinE, FtsZ defines the cell-division plane and helps assemble the divisome, the protein complex that orchestrates cell division.

Q What is the role of the penicillin-binding protein FtsI in cell division?

8.4 MreB helps define cell shape, and in rod-shaped cells, MreB forms patchlike filaments that direct cell wall synthesis along the long axis of the cell. The protein crescentin plays an analogous role in *Caulobacter*, leading to formation of a curved cell. The eukaryotic cell proteins actin and tubulin that affect shape and cell division have prokaryotic cell counterparts.

Q How does a rod-shaped bacterial cell differ morphologically if its MreB function is disrupted?

8.5 During bacterial growth, new cell peptidoglycan is synthesized by the insertion of peptidoglycan precursors into the preexisting peptidoglycan fabric. Bactoprenol facilitates transport of these units through the cytoplasmic membrane. Transpeptidation completes the process of cell wall synthesis by cross-linking adjacent ribbons of peptidoglycan at muramic acid residues.

Q What step is necessary prior to inserting a new peptidoglycan subunit into a growing cell wall?

II • Regulation of Development in Model *Bacteria*

8.6 Sporulation in *Bacillus* during adverse conditions is triggered by a complex phosphotransfer relay system that monitors multiple aspects of the environment. The sporulation factor Spo0A then sets in motion a cascade of regulatory responses under the control of several alternative sigma factors.

Q What is a common trigger for sporulation in *Bacillus*?

8.7 Endospore germination occurs when nutrients become available. This developmental process occurs in three stages: activation, germination, and outgrowth. Activation is triggered by nutrients binding to germinant receptors within the endospore. This binding triggers a cascade of events that ultimately leads to rehydration of the endospore, normal metabolism, growth, and cell division.

Q What type of genes are upregulated during the different stages of endospore germination?

8.8 Differentiation in *Caulobacter* consists of the alternation between motile cells and those that are attached to surfaces. Three major regulatory proteins—CtrA, GcrA, and DnaA—act in succession to control the three phases of the cell cycle. Each in turn controls many other genes needed at specific times in the cell cycle.

Q How is the *Caulobacter* life cycle similar to the eukaryotic cell cycle?

8.9 Heterocyst formation requires expression of the major regulatory protein HetR in the protoheterocysts. However, the protein must be inactivated in vegetative cells by diffusion of the PatS peptide along the filament.

Q What is meant by "patterning" during heterocyst formation?

8.10 During biofilm formation, cells make contact with a surface using cell structures such as flagella and pili. Once cells are attached to a surface, both intra- and intercellular signaling molecules lead to the production of an extracellular matrix and colonization. Biofilms are not static entities, and the final step in their development is dispersal and release of cells.

Q What are the regulatory roles of c-di-GMP in biofilm formation?

III • Antibiotics and Microbial Growth

8.11 Antibiotics are antimicrobial agents produced by microorganisms that target processes critical for growth such as DNA replication, transcription, protein synthesis, and cell wall synthesis. Specific mechanisms of antibiotic resistance include modification of the drug target, enzymatic inactivation, removal via efflux pumps, and metabolic bypass. These mechanisms are either chromosomally or plasmid encoded.

Q Why do antibiotics that target bacterial ribosomes not make humans sick?

8.12 Persistence is a dormant growth state in which cells are not susceptible to antibiotics. This lack of sensitivity is transient and thus not considered antibiotic resistance. Persistence is the result of toxin–antitoxin modules and the induction of the stringent response. Cells in the persistent state can resume growth once antibiotic exposure ceases.

Q What are the steps to inducing the stringent response during the development of persistence?

APPLICATION QUESTIONS

1. If DnaA was not regulated in *Escherichia coli* and multiple rounds of replication were completed before cell division, what would be the consequence to the daughter cell and why? Would the resulting cell still be considered haploid?
2. Explain how cells exhibiting different phenotypes under the same conditions can possess the same genotype. Give examples in your explanation.
3. Describe how you would genetically design a "superbug" resistant to β-lactams, methicillin, streptomycin, daptomycin, and trimethoprim.
4. Discuss why so many different sigma factors can be produced by bacteria such as *Bacillus*.

CHAPTER GLOSSARY

Antibiotic a chemical substance produced by a microorganism that kills or inhibits the growth of another microorganism

FtsZ a protein that forms a ring at the future site of septum formation in a prokaryotic cell

Green fluorescent protein (GFP) a protein that fluoresces green and is widely used in genetic analysis

Heterocysts specialized cells dedicated to nitrogen fixation

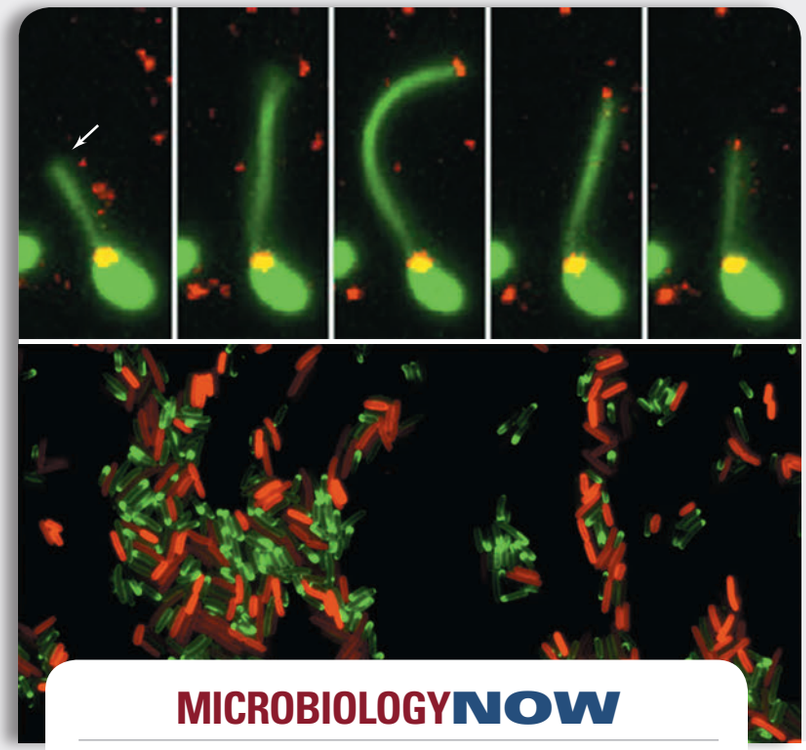
Persistence a dormant growth state in which a few members of a genetically identical population are transiently tolerant to multiple antibiotics

Reporter gene a gene used in genetic analysis because the product it encodes is easy to detect

Super-resolution microscopy a form of light microscopy that employs special fluorescent molecules to resolve structures as small as 5–50 nm

Toxin–antitoxin (TA) module a genetic locus encoding a toxin that inhibits growth and an antitoxin that neutralizes the activity of the toxin

Genetics of *Bacteria* and *Archaea* 9



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II Gene Transfer in *Bacteria* 306

III Gene Transfer in *Archaea* and Other Genetic Events 318

MICROBIOLOGYNOW

Live Cell Imaging Captures Bacterial Promiscuity

While often considered asexual, both *Bacteria* and *Archaea* evolve quickly through DNA exchange by horizontal transfer. Increased fitness resulting from genetic promiscuity has traditionally been monitored in the laboratory through changes in physiology. However, advances in microscopy and fluorescent labeling now make it possible to witness DNA exchange using live cell imaging.

One fascinating example is the DNA uptake system (natural competence) in the bacterium *Vibrio cholerae*. Using an appendage called a pilus, *V. cholerae* is able to reach outside of its cell, snatch pieces of free DNA, and transport them into the cell. Thus, its pili effectively function as "harpoons" fishing for free DNA. The top panel of the images shown here is a time series of the "harpoon" (white arrow) being cast from the cell into the environment, attaching to free DNA (red), and then retracting back to the cell.[1]

Transfer of antibiotic resistance can also be monitored using live cell imaging. The photo at the bottom shows a mixed population of *Escherichia coli* cells surrounded by the antibiotic tetracycline (labeled green). Cells possessing a plasmid encoding resistance to tetracycline appear red due to the labeled TetA resistance protein, whereas cells sensitive to tetracycline appear green due to their uptake of the labeled antibiotic.[2] Why are the sensitive cells still alive? Remarkably, an efflux pump (Chapter 8) helps the sensitive cells buy just enough time to obtain the plasmid from their resistant neighbors through a genetic event called conjugation. How long does this whole process take? Transfer of the plasmid only takes minutes, and sensitive cells can express TetA in under 2 hours.

These examples highlight the astounding power of horizontal gene transfer in prokaryotic cells as well as the variety of tools at their disposal to improve their fitness. Bacterial genetics is indeed an exciting and dynamic discipline and has given birth to many of the genetic concepts we know today.

Sources: [1]Ellison, C.K., et al. 2018. Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation of *Vibrio cholerae*. *Nat. Microbiol. 3*: 773.
[2]Nolivos, S., et al. 2019. Role of AcrAB-TolC multidrug efflux pump in drug-resistance acquisition by plasmid transfer. *Science 364*: 778.

The diversity and amazing ability of bacteria to survive ever-changing environmental conditions and a host of stressors is controlled by their genetic makeup. While their asexual reproduction would suggest that cells within *Bacteria* and *Archaea* do not exchange genes, the microbiologist Joshua Lederberg made a groundbreaking discovery in 1946—like plants and animals, bacteria can also exchange genes! Lederberg's groundbreaking work showcasing genetic recombination in *Bacteria* not only earned him a Nobel Prize but also helped launch the field of molecular biology and the use of *Bacteria* to study how genes work in higher organisms, such as plants and animals.

Understanding the varied mechanisms by which *Bacteria* and *Archaea* exchange genes has helped tackle the conundrum of how these microbes can exhibit so much diversity and persist in habitats where other forms of life cannot. Gene exchange between microbes, along with genetic innovations that arise from random changes in a cell's genetic blueprint, occur frequently and confer selectable advantages that ultimately drive cell survival and genetic diversity.

In this chapter we discuss the major themes of bacterial and archaeal genetics and the processes of gene transfer from one cell to another (**Figure 9.1**). We first describe how changes arise in the genome, which can be due to random errors in DNA replication or from DNA damage, and then we consider how **horizontal gene transfer** (Figure 9.1; see also the photomicrograph of actual gene transfer on page 297) moves genes from one cell to another by mechanisms uncoupled from reproduction. Microbiologists have leveraged aspects of these mutational and genetic exchange processes to identify genes encoding exciting microbial processes such as chemotaxis, quorum sensing, and persistence that we discussed in Chapters 7 and 8. While changes to the genome underlie microbial diversity and habitat adaptation, microorganisms also possess mechanisms to maintain genomic stability, and we end this chapter by considering these. Taken together, both genomic change and genomic stability are important to the evolution of an organism and its competitive success in nature.

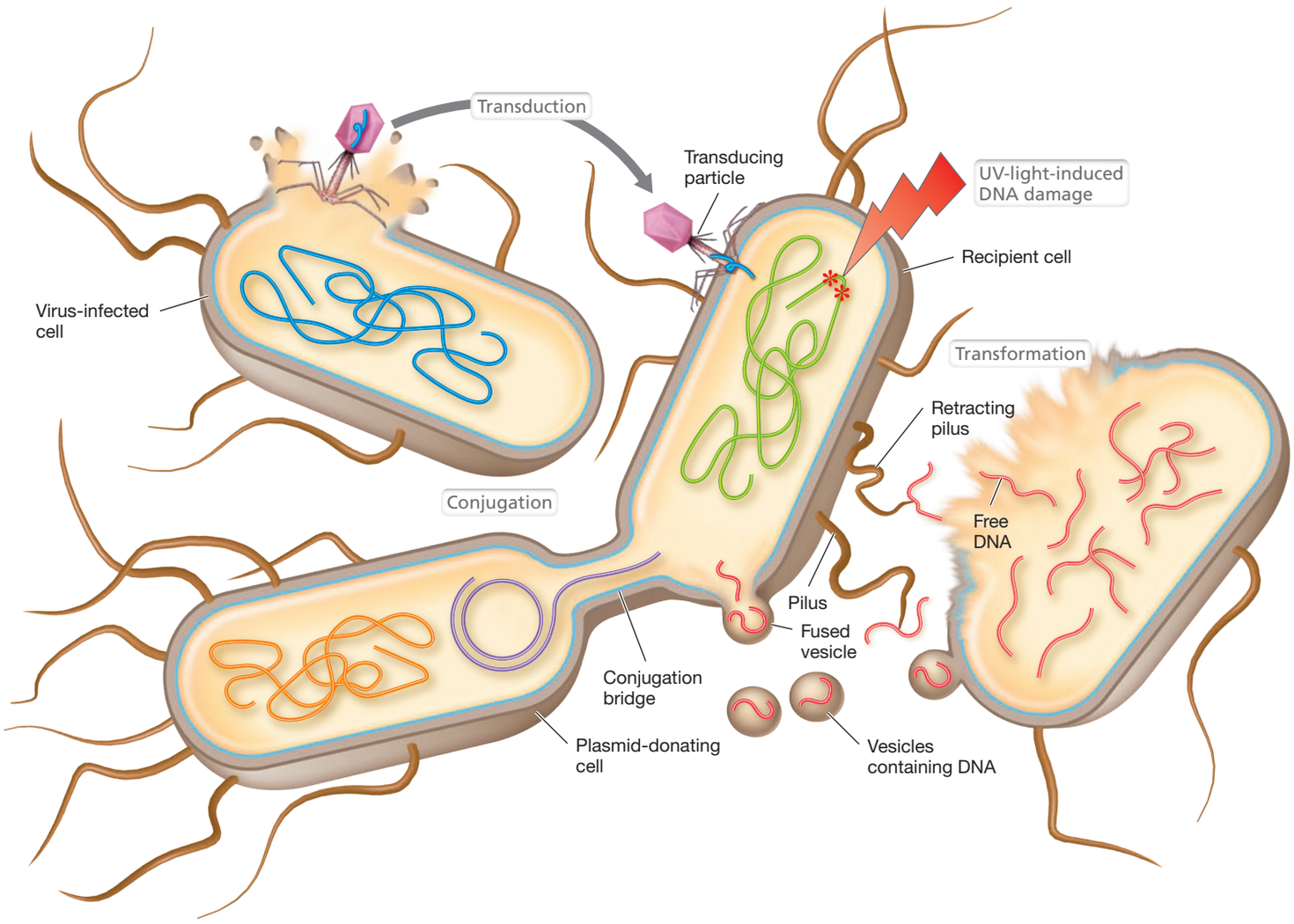


Figure 9.1 Overview of bacterial and archaeal genetics. DNA mutations and mechanisms of DNA transfer contribute to the genetic diversity of *Bacteria and Archaea*. Transduction, transformation, and conjugation are the three known ways by which prokaryotic cells can exchange genes.

I • Mutation

Changes in the human world can be good, bad, or indifferent, and the same is true of the microbial world. Changes in the genetic blueprint of a microbe may fuel its evolutionary progress, accelerate its death, or have no detectable effect on its biology.

All organisms contain a specific sequence of nucleotides in their genome, their genetic blueprint. A **mutation** is a *heritable* change in the base sequence of that genome, that is, a change that is passed from the mother cell to progeny cells. Mutations can lead to changes in the properties of an organism; some mutations are beneficial, some are detrimental, but most are neutral and have no effect. Although the rate of spontaneous mutation is low (Section 9.3), the impressive characteristic of exponential growth in *Bacteria* and *Archaea* ensures that mutations accumulate in a population surprisingly fast. Moreover, whereas a single mutation typically brings about only a small change in a cell, horizontal gene transfer often generates much larger changes. Taken together, mutation and genetic exchange fuel the evolutionary process.

We begin by considering the molecular mechanism of mutation and the properties of mutant microorganisms.

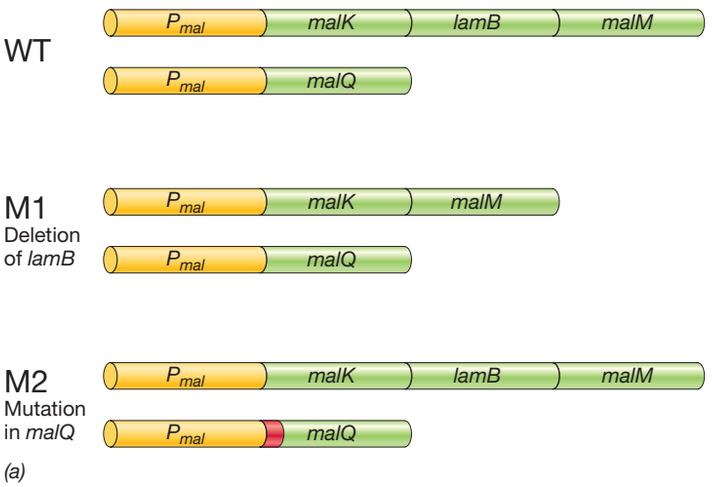
9.1 Mutations and Mutants

In order to appreciate the impact that genome changes can have on a cell (or a virus), we start with a brief genetics primer. The genomes of cells consist of double-stranded DNA. In viruses, by contrast, the genome may consist of double- or single-stranded DNA or RNA, depending on the virus (Chapters 5 and 11). By convention, a strain of an organism or a virus isolated from nature is called the **wild-type strain** and therefore contains the wild-type genome. A cell or virus derived from the wild type whose genome carries a change in nucleotide sequence is called a **mutant**. A mutant by definition differs from the wild-type strain in its **genotype**, the nucleotide sequence of its genome (**Figure 9.2*a***). In addition, the observable properties of a mutant—its **phenotype**—may also be altered relative to its parent (Figure 9.2*b*). This altered phenotype is called a *mutant phenotype*.

The term "wild type" may be used to refer to an entire organism or just to the status of a particular gene that is under investigation. Mutant derivatives can be obtained either directly from a wild-type strain or from another strain—referred to as a *parental strain*—previously derived from the wild type; for example, another mutant. Figure 9.2*b* shows a plate of MacConkey agar (a culture medium that contains a pH indicator that turns red if sugar is fermented) containing the sugar maltose. This medium shows the phenotypic difference between wild-type *Escherichia coli* and mutant derivatives in the maltose utilization pathway.

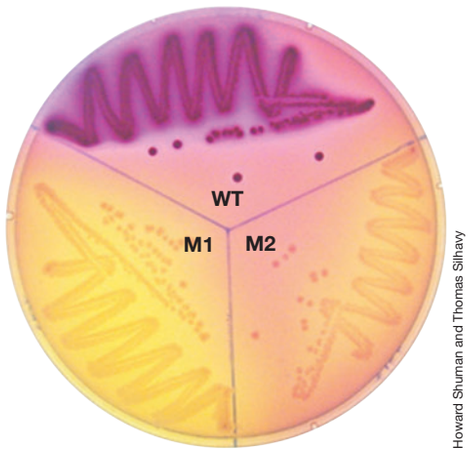
Depending on the mutation, a mutant strain may or may not differ in phenotype from its parent. By convention in bacterial genetics, the *genotype* of an organism is designated by three lowercase letters followed by a capital letter (all in italics) indicating a particular gene. For example, the *hisC* gene of *E. coli* encodes a protein called HisC that functions in biosynthesis of the amino acid histidine (◀ Figure 6.27). Mutations in the *hisC* gene would be designated as *hisC1*, *hisC2*, and so on, the numbers referring to the order of isolation of the mutant strains. Each *hisC* mutation would be different, and each *hisC* mutation might affect the HisC protein in different ways.

The *phenotype* of an organism is designated by a capital letter followed by two lowercase letters, with either a plus or a minus superscript to indicate the presence or absence of that property. For example, a His^+ strain of *E. coli* is one that is capable of making its own histidine, whereas a His^- strain is not. The His^- strain would therefore require a histidine supplement for growth. A mutation in the *hisC* gene leads to a His^- phenotype if it eliminates the function of the HisC protein. Likewise, the *lamB* and *malQ* mutations in Figure 9.2 lead to a Mal^- phenotype, as the mutants can no longer use maltose as an energy source.



(a)

WT	Wild type
M1	Mutant 1
M2	Mutant 2



(b)

Howard Shuman and Thomas Silhavy

Figure 9.2 Wild-type versus mutant phenotype. *(a)* Genotype maps of *Escherichia coli* wild-type and maltose utilization mutants; *P* is the promoter region and *mal* and *lam* regions are structural genes. *(b)* Growth of wild-type *E. coli* and mutants on a plate of MacConkey agar, a differential medium. The medium contains maltose as the carbon source and a pH indicator that turns red if maltose is fermented. While the wild-type strain ferments maltose, mutant strains M1 and M2 are unable to ferment maltose due to a deletion of the *lamB* gene and a point mutation in the *malQ* gene, respectively.

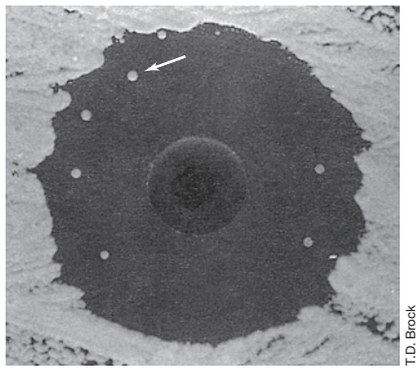
Isolation of Mutants: Screening versus Selection

Virtually any characteristic of an organism can be changed by mutation. Some mutations are *selectable*, conferring some type of advantage on organisms possessing them, whereas others are nonselectable, even though they may lead to a very clear change in the phenotype of an organism. A selectable mutation confers a clear advantage on the mutant strain under certain environmental conditions, so the progeny of the mutant cell are able to grow and replace the parent. A good example of a selectable mutation is drug resistance: An antibiotic-resistant mutant can grow in the presence of an antibiotic that inhibits or kills the parent (**Figure 9.3*a***) and is thus selected under these conditions. It is relatively easy to detect and isolate selectable mutants by choosing the appropriate environmental conditions. **Selection** is therefore an extremely powerful genetic tool, allowing the isolation of a single mutant from a population containing millions or even billions of parental cells.

Mastering Microbiology
Art Activity: Figure 9.4 Screening for nutritional auxotrophs

Some examples of important *nonselectable* mutations are secondary metabolite production loss in an antibiotic-producing organism, loss of attachment ability in a biofilm-forming organism, and loss of color in a pigmented organism (Figure 9.3*b*, *c*). All three types of mutants usually have neither an advantage nor a disadvantage over their parent cells when grown in the laboratory, although antibiotic production (▶ Section 16.12), biofilm formation (◀ Sections 4.9 and 8.10, and ▶ Section 20.4), and pigmentation (for example, of phototrophic organisms) may have a selective advantage in nature. We can detect nonselectable mutations only by examining large numbers of colonies and looking for the "different" ones, an often laborious process called **screening**.

As illustrated in Figure 9.2 (see also Figure 9.35), a thorough understanding of microbial physiology is the key to designing successful genetic screens for nonselectable mutations. In Figure 9.2*b* a genetic screen for Mal$^-$ mutants utilizes a culture medium containing a pH indicator that turns red if maltose is fermented (MacConkey agar). Because the Mal$^-$ mutants are unable to utilize maltose, they are unpigmented compared to the red wild-type strain. To identify mutants in biofilm formation, individual colonies can be grown in liquid medium in a microtiter plate and a stain can be used to track whether cells can form a biofilm and adhere to the microtiter plate (see Figure 9.35). If possible, selection is almost always the preferred strategy over screening for obtaining mutants in a genetic experiment because selective conditions typically place such severe restraints on the population that mutants are easily detectable. Examples of common classes of mutants and the means by which they are detected are listed in **Table 9.1**.



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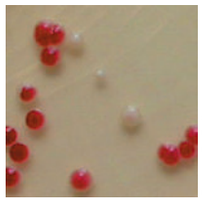

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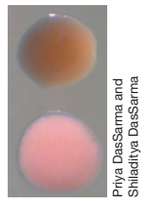


(c)

Figure 9.3 Selectable and nonselectable mutations. *(a)* Development of antibiotic-resistant mutants, a type of easily selectable mutation, within the inhibition zone of an antibiotic assay disk. *(b)* Nonselectable mutations. UV-radiation-induced nonpigmented mutants of *Serratia marcescens*. The wild type has a dark red pigment. The white or colorless mutants make no pigment. *(c)* Colonies of mutants of a species of *Halobacterium*, a member of the *Archaea*. The lower pink wild-type colony contains intracellular gas vesicle nanoparticles that regulate buoyancy and also scatter light, resulting in a milky appearance. The upper red-orange colony is a mutant lacking gas vesicles and is translucent as a result.

Isolation of Nutritional Auxotrophs

Although screening is more tedious than selection, useful methods have been developed for screening large numbers of colonies for certain types of mutations. For instance, nutritionally defective mutants can be detected by the technique of *replica plating* (**Figure 9.4**). A colony from a master plate can be transferred onto an agar plate lacking the nutrient by using a sterile loop, toothpick, or even a robotic arm. Parental colonies will grow normally, whereas those of the mutant will not. Thus, the inability of a colony to grow on a medium lacking the nutrient signals that it is a mutant. The colony on the master plate corresponding to the vacant spot on the replica plate can then be picked, purified, and characterized.

A mutant strain with an additional nutritional requirement above that of the wild type or parental strain from which it was derived is called an **auxotroph** (Table 9.1), and the strain from which an auxotroph originates is called a *prototroph*. For instance, mutants of *E. coli* with His$^-$ and Mal$^-$ (Figure 9.2) phenotypes are histidine and maltose auxotrophs, respectively, while the parental His$^+$ and Mal$^+$ strains from which the auxotrophs were derived are the prototrophs of such strains. As described earlier, many different mutations can lead to a strain showing a His$^-$ or Mal$^-$ phenotype, and thus an initial step in characterizing the genetics of a metabolic pathway (such as histidine biosynthesis and maltose catabolism) would be the isolation of several His$^-$ or Mal$^-$ strains followed by their comparative genetic analyses (Figure 9.2). This comparative analysis process, called *complementation*, is discussed in Section 9.5.

Check Your Understanding

- Distinguish between a mutation and a mutant.
- Distinguish between screening and selection.
- How does an auxotroph differ from a prototroph?

TABLE 9.1 Some examples of mutants

Phenotype	Nature of change	Detection of mutant
Auxotroph	Loss of enzyme in biosynthetic pathway	Inability to grow on medium lacking the nutrient
Temperature-sensitive	Alteration of an essential protein so it is more heat-sensitive	Inability to grow at a high temperature that normally supports growth
Cold-sensitive	Alteration of an essential protein so it is inactivated at low temperature	Inability to grow at a low temperature that normally supports growth
Drug-resistant	Detoxification of drug or alteration of drug target or permeability to drug	Growth on medium containing a normally inhibitory concentration of the drug
Rough colony	Loss or change in lipopolysaccharide layer	Granular, irregular colonies instead of smooth, glistening colonies
Nonencapsulated	Loss or modification of surface capsule	Small, rough colonies instead of larger, smooth colonies
Nonmotile	Loss of flagella or nonfunctional flagella	Compact instead of flat, spreading colonies; lack of motility by microscopy
Pigmentless	Loss of enzyme in biosynthetic pathway leading to loss of one or more pigments	Presence of different color or lack of color
Sugar fermentation	Loss of enzyme in degradative pathway	Lack of color change on agar containing sugar and a pH indicator
Virus-resistant	Loss of virus receptor	Growth in presence of large amounts of virus

9.2 Molecular Basis of Mutation

Mutations can be either spontaneous or induced events. **Spontaneous mutations** are those that occur without external intervention, and most result from occasional errors in the pairing of bases by DNA polymerase during DNA replication (Chapter 6). **Induced mutations**, by contrast, are those caused by environmental agents and include mutations made deliberately by humans. Induced mutations can result from exposure to natural radiation (cosmic rays and so on) that alters the structure of bases in the DNA, or from a variety of chemicals that chemically modify DNA (Section 9.4).

Mutations that change only one base pair are called **point mutations** and occur when a single base-pair *substitution* occurs in

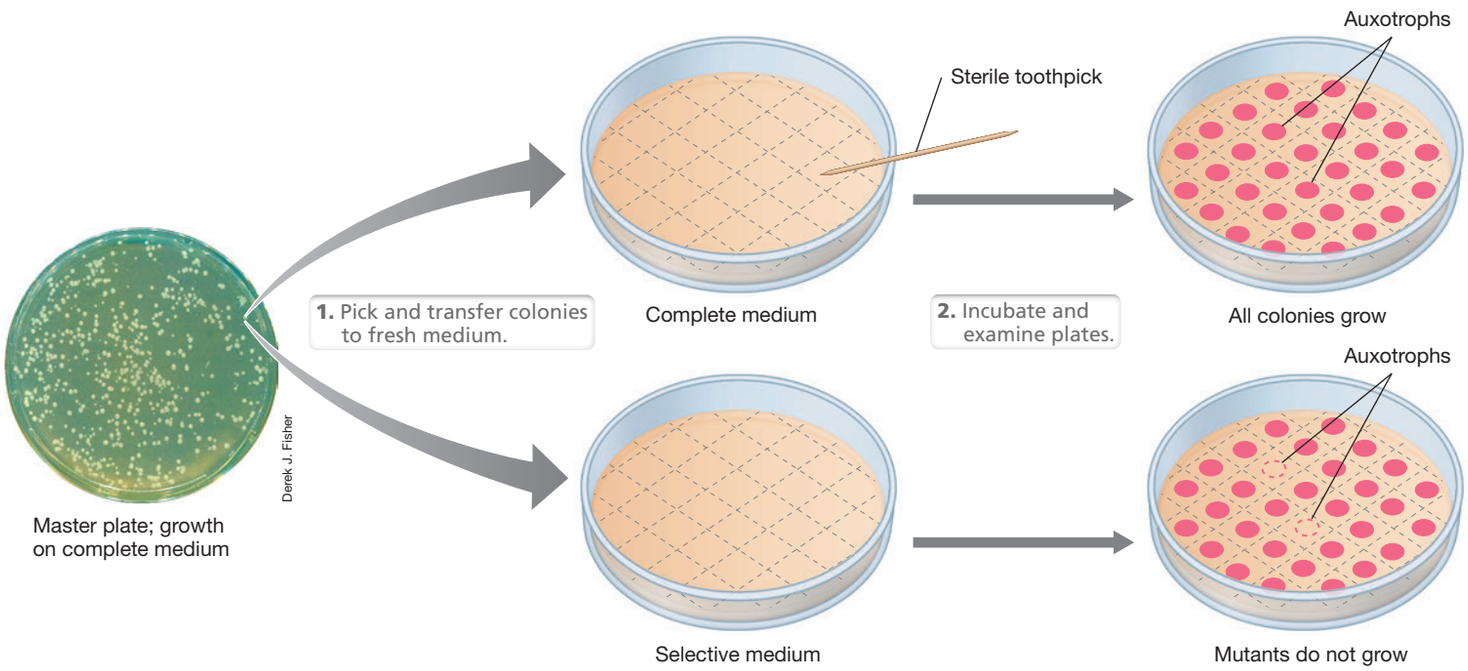


Figure 9.4 Screening for nutritional auxotrophs. The replica-plating method can be used for the detection of nutritional mutants. Colonies from the master plate are transferred using a sterile toothpick to a gridded plate containing different media for selection. The colonies not appearing on the selective medium are labeled as auxotrophs. The selective medium lacked one nutrient (the amino acid leucine) present in the master plate. Therefore, the colonies on the complete medium plate that are not represented on the selective medium plate are leucine auxotrophs (Leu^-).

the DNA. Many point mutations do not actually cause any phenotypic change (although the organism's genotype has changed). However, as for all mutations, any phenotypic change that results from a point mutation depends on exactly where in the genome the mutation occurs and the nature of the nucleotide change, as we will see.

Base-Pair Substitutions: Missense, Nonsense, and Silent Mutations

If a point mutation is within the region of a gene that encodes a polypeptide, any change in the phenotype of the cell is most likely the result of a change in the amino acid sequence of that polypeptide. The error in the DNA is transcribed into mRNA, and the erroneous mRNA in turn is translated to yield a polypeptide. In interpreting the results of a mutation, we must first recall that the genetic code is *degenerate* (◀ Section 6.9 and Table 6.4). Consequently, not all mutations in the base sequence encoding a polypeptide will change the polypeptide sequence. This is illustrated in **Figure 9.5**, which shows several possible results when the DNA that encodes a single tyrosine codon in a polypeptide is mutated. First, a change in the RNA from UAC to UA*U* would have no apparent effect because UAU is also a tyrosine codon. Although they do not affect the sequence of the encoded polypeptide, such changes in the DNA are considered one type of **silent mutation**, that is, a mutation that does not affect the phenotype of the cell. Note that silent mutations in coding regions are almost always in the *third base* of the codon (arginine and leucine can also have silent mutations in the first position) because of genetic code degeneracy.

Changes in the first or second base of the codon more often lead to significant changes in the amino acid sequence of the polypeptide. For instance, a single base change from *U*AC to *A*AC (Figure 9.5) results in an amino acid change within the polypeptide from tyrosine to asparagine at a specific site. This is called a **missense mutation** because the informational "sense" (the precise sequence of amino acids) in the polypeptide has changed. If the change is at a critical location in the polypeptide chain, the protein could be inactive, have reduced activity, or have modified activity. However, not all missense mutations lead to nonfunctional proteins. The outcome depends on where the substitution lies in the polypeptide chain and on how it affects protein folding and activity. While mutations in the active site of an enzyme are more likely to destroy catalytic activity than mutations in other regions of the protein, some active site mutations can alter an enzyme's substrate specificity. This type of change can result in a mutant enzyme with differing activity from the wild-type enzyme, and such events have contributed to the spectacular metabolic diversity we see in the microbial world (Chapters 3 and 14).

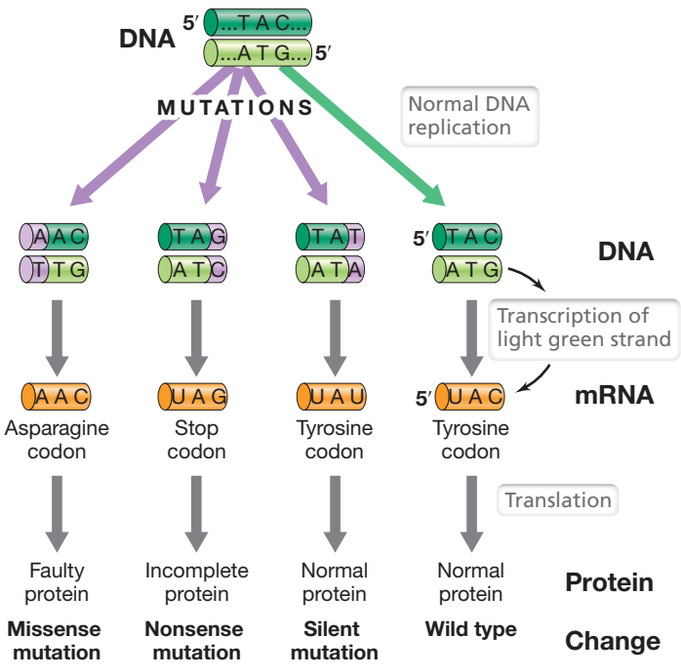


Figure 9.5 Possible effects of base-pair substitution in a gene encoding a protein. Three different protein products are possible from changes in the DNA for a single codon. The possible phenotypes are wild type and strains harboring missense, nonsense, or silent mutations.

Another possible outcome of a base-pair substitution is the formation of a stop (nonsense) codon in the DNA. This results in premature termination of translation, leading to an incomplete polypeptide (Figure 9.5). Mutations of this type are called **nonsense mutations** because the change is from a sense (coding) codon to a nonsense (stop) codon (◀ Table 6.4). Unless the nonsense mutation is very near the end of the gene, the product is incompletely made; such truncated proteins are either inactive or, at the very least, lack normal activity.

Other terms are occasionally used in microbial genetics to describe the precise type of base substitution in a point mutation. **Transitions** are mutations in which one purine base (A or G) is substituted for another purine, or one pyrimidine base (C or T) is substituted for another pyrimidine. **Transversions** are point mutations in which a purine base is substituted for a pyrimidine base, or vice versa.

Frameshifts and Other Insertions or Deletions

Because the genetic code is read from one end of the nucleic acid in consecutive blocks of three bases (codons), any deletion or insertion of a single base pair results in a shift in the reading frame. These **frameshift mutations** often have serious consequences. Single base insertions or deletions change the primary sequence of the encoded polypeptide, typically in a major way (**Figure 9.6**). Such microinsertions or microdeletions can result from DNA polymerase replication errors. Insertion or deletion of two base pairs also causes a frameshift. However, insertion or deletion of three base pairs does not cause a frameshift but does add or remove a codon; this results in the addition or deletion of a single amino acid in the polypeptide sequence. Although an amino acid addition or deletion may well be deleterious to protein function, it is usually not as serious a problem as a frameshift, which scrambles the entire polypeptide sequence downstream of the mutation.

Insertions or deletions can also result in the gain or loss of hundreds or even thousands of base pairs. Such changes inevitably result in complete loss of gene function. Some deletions are so large that they may include several genes. If any of the deleted genes are essential, the mutation will be lethal. Such deletions cannot be restored through further mutations, but only through genetic recombination (Sections 9.5–9.11). Large insertions and deletions may arise as a

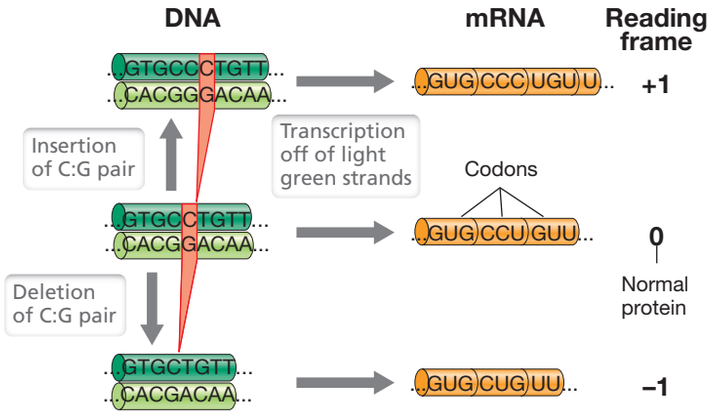


Figure 9.6 Shifts in the reading frame of mRNA caused by insertions or deletions. The reading frame in mRNA is established by properly positioning the message on the ribosome. The mRNA is read beginning at the 5′ end (toward the left in the figure) and proceeds by units of three bases (codons). The normal reading frame is referred to as the 0 frame, that missing a base the −1 frame, and that with an extra base the +1 frame.

result of errors during genetic recombination. In addition, many large insertion mutations are due to the insertion of specific DNA sequences called *transposable elements* (Section 9.11).

We will discuss the effects of mutations on the evolution of bacterial genomes in Chapter 13.

Check Your Understanding

- Do missense mutations occur in genes encoding tRNA? Why or why not?
- Why do frameshift mutations generally have more serious consequences than missense mutations?

9.3 Reversions and Mutation Rates

The rates at which the different kinds of mutations occur vary widely. Some mutations occur so rarely that they are almost impossible to detect, whereas others occur so frequently that they present difficulties for an experimenter trying to maintain a genetically stable stock culture. Sometimes a second mutation can reverse the effect of an initial mutation. Furthermore, all organisms possess a variety of systems for DNA repair. Consequently, the observed mutation rate depends not only on the frequency of DNA changes but also on the efficiency of DNA repair.

Reversions (Back Mutations) and Suppressors

Point mutations are typically reversible, a process known as **reversion**. A *revertant* is therefore a strain in which the original phenotype that was changed by mutation is restored by a second mutation. Revertants can be of two types, same site or second site. In *same-site* revertants, the mutation that restores activity is at the same site as the original mutation. If the back mutation is not only at the same site but also restores the original sequence, it is called a *true* revertant.

In *second-site* revertants, the mutation is at a different site in the DNA. Second-site mutations can restore a wild-type phenotype if they function as *suppressor mutations*—mutations that compensate for the effect of the original mutation. Several classes of suppressor mutations are known. These include (1) a mutation somewhere else in the same gene that restores enzyme function, such as a second frameshift mutation near the first that restores the original reading frame (Figure 9.6); (2) a mutation in another gene that restores the function of the original mutated gene; and (3) a mutation in another gene that results in the production of an enzyme that can replace the nonfunctional one.

Suppressors can be best illustrated by mutations in tRNAs. Nonsense mutations can be suppressed by changing the anticodon sequence of a tRNA molecule so that it now recognizes a stop codon (**Figure 9.7**). Such an altered tRNA is called a *suppressor tRNA* and will insert the amino acid it carries at the stop codon that it now reads. Suppressor tRNA mutations exist in all three domains of life, and their activity has been observed in both *Bacteria* and *Archaea* by introducing reporter genes (◀ Section 8.1) containing nonsense mutations. During these experiments, complete translation and activity of the corresponding reporter protein was observed despite the presence of a stop codon within the coding region of the reporter.

Suppressor tRNA mutations would be lethal unless a cell has more than one tRNA gene for a particular codon. One tRNA gene may then be mutated to produce a suppressor, while the other gene's product performs the original function. Most cells have multiple genes for

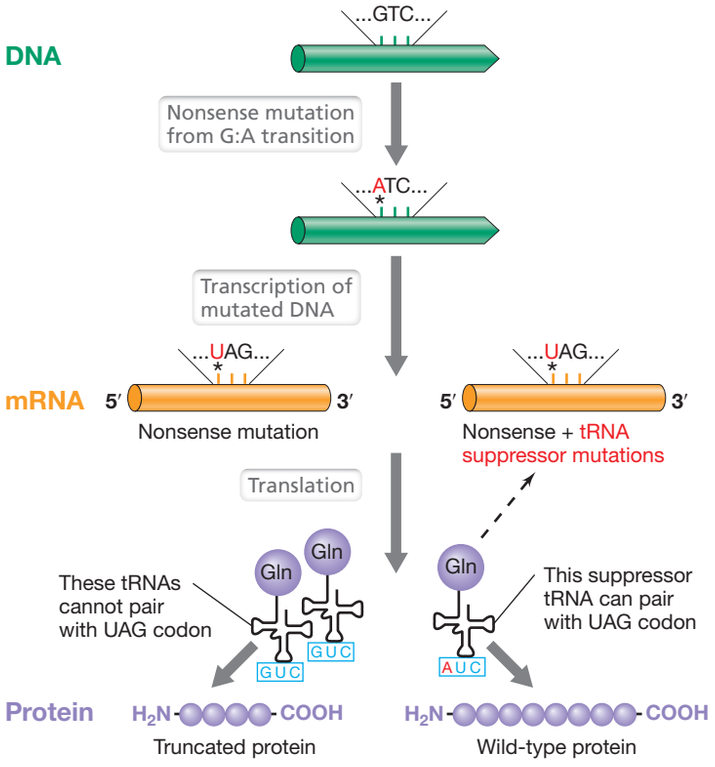


Figure 9.7 Suppression of nonsense mutations. Introduction of a nonsense mutation in a gene encoding a protein results in the incorporation of a stop codon (indicated by the *) in the corresponding mRNA. This single mutation leads to the production of a truncated polypeptide. The mutation is suppressed if a second mutation occurs in the anticodon of a tRNA, a tRNA charged with glutamine in this example, which allows the mutated tRNA or suppressor tRNA to bind to the nonsense codon.

tRNAs, and so suppressor mutations are reasonably common, at least in microorganisms. Sometimes the amino acid inserted by the suppressor tRNA is identical to the original amino acid and the protein is fully active. In other cases, however, a different amino acid is inserted and a protein that is only partially active may be produced.

Mutation Rates

For most microorganisms, errors in DNA replication occur at a frequency of 10^{-6} to 10^{-7} per thousand bases during a single round of replication. A typical gene has about 1000 base pairs. Therefore, the frequency of a mutation *in a given gene* is also in the range of 10^{-6} to 10^{-7} per round of replication. For instance, in a bacterial culture having 10^8 cells/ml, there are likely to be a number of different mutants for any given gene in each milliliter of culture. Eukaryotes with very large genomes tend to have replication error rates about 10-fold lower than typical bacteria, whereas DNA viruses, especially those with very small genomes, may have error rates 100-fold to 1000-fold higher than those of cellular organisms. RNA viruses have even higher error rates due to less effective polymerase proofreading (◀ Section 6.4) and the lack of RNA repair mechanisms.

Single base errors during DNA replication are more likely to lead to missense mutations than to nonsense mutations because most single base substitutions yield codons that encode other amino acids (◀ Table 6.4). The next most frequent type of codon change caused by a single base change leads to a silent mutation. This is because for the most part alternate codons for a given amino acid differ from each other by a single base change in the "silent" third position. A given codon can be changed to any of 27 other codons by a single base substitution, and on average, about two of these will be silent mutations, one a nonsense mutation, and the rest missense mutations.

Unless a mutation can be selected for, its experimental detection is difficult, and much of the skill of the microbial geneticist requires increasing the efficiency of mutation detection. This can be done most effectively by increasing the pool of mutations. As we see in the next section, it is possible to greatly increase the mutation rate by treatment with mutagenic agents. In addition, the mutation rate may change under certain circumstances, such as when cells are placed under high-stress conditions.

Check Your Understanding

- Why are suppressor tRNA mutations not lethal?
- Which class of mutation, missense or nonsense, is more common, and why?

9.4 Mutagenesis

The spontaneous rate of mutation is very low, but a variety of chemical, physical, and biological agents can increase the mutation rate and are therefore said to induce mutations. These agents are called **mutagens**. Because *Bacteria* and *Archaea* are often exposed to mutagens, not only intentionally in the laboratory but also accidentally in their environment, we discuss some of the major categories of mutagens and their activities here.

Chemical Mutagens and Radiation

An overview of some of the major chemical mutagens and their modes of action is given in Table 9.2. Several classes of chemical mutagens exist. The *nucleotide base analogs* are molecules that resemble the purine and pyrimidine bases of DNA in structure but display faulty base-pairing properties (Figure 9.8). If a base analog is incorporated into DNA in place of the natural base, the DNA may replicate normally most of the time. However, DNA replication errors occur at higher frequencies at these sites due to incorrect base pairing. The result is the incorporation of a mismatched base into the new strand of DNA and thus introduction of a mutation. During subsequent segregation of this strand in cell division, the mutation is revealed.

TABLE 9.2 Chemical and physical mutagens and their modes of action

Agent	Action	Result
Base analogs		
5-Bromouracil	Incorporated like T; occasional faulty pairing with G	AT → GC and occasionally GC → AT
2-Aminopurine	Incorporated like A; faulty pairing with C	AT → GC and occasionally GC → AT
Chemicals that react with DNA		
Nitrous acid (HNO_2)	Deaminates A and C	AT → GC and GC → AT
Hydroxylamine (NH_2OH)	Reacts with C	GC → AT
Alkylating agents		
Monofunctional (for example, ethyl methanesulfonate)	Puts methyl on G; faulty pairing with T	GC → AT
Bifunctional (for example, mitomycin, nitrogen mustards, nitrosoguanidine)	Cross-links DNA strands; faulty region excised by DNase	Both point mutations and deletions
Intercalating agents		
Acridines, ethidium bromide	Inserts between two base pairs	Microinsertions and microdeletions
Radiation		
Ultraviolet (UV)	Pyrimidine dimer formation	Repair may lead to error or deletion
Ionizing radiation (for example, X-rays)	Free-radical attack on DNA, breaking chain	Repair may lead to error or deletion

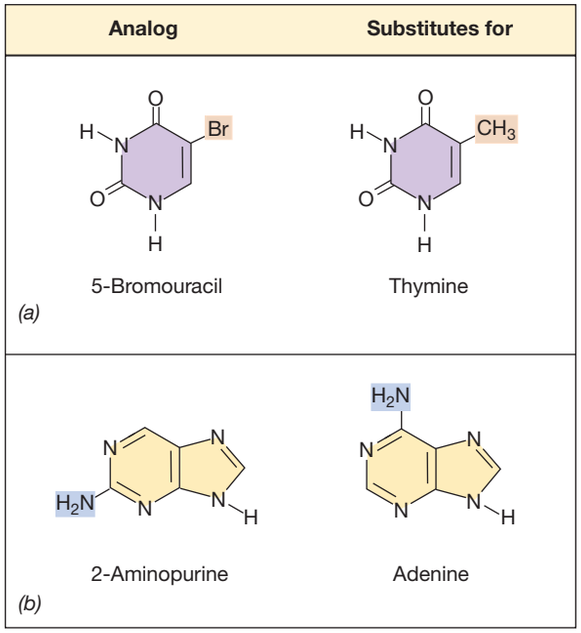


Figure 9.8 Nucleotide base analogs. Structure of two common nucleotide base analogs used to induce mutations compared with the normal nucleic acid bases for which they substitute. *(a)* 5-Bromouracil can base-pair with guanine, causing AT to GC substitutions. *(b)* 2-Aminopurine can base-pair with cytosine, causing AT to GC substitutions.

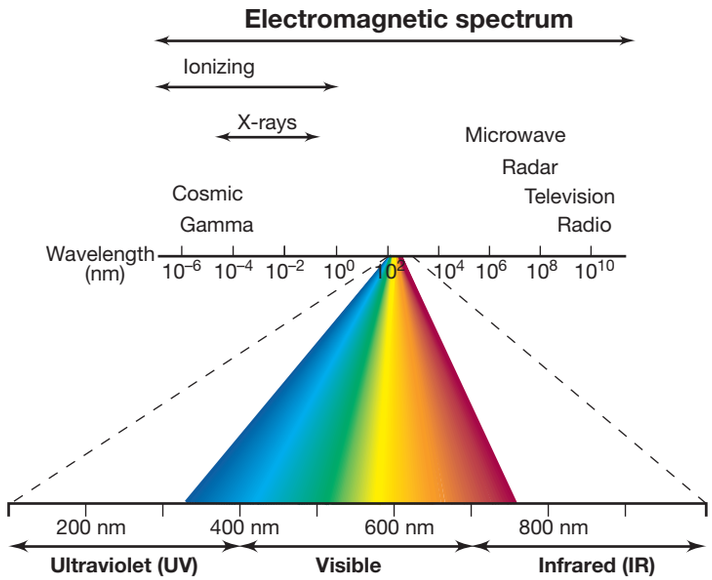


Figure 9.9 Wavelengths of radiation. Ultraviolet radiation consists of wavelengths just shorter than visible light. For any electromagnetic radiation, the shorter the wavelength, the higher the energy. DNA absorbs strongly at 260 nm.

Other chemical mutagens induce *chemical modifications* in one base or another, resulting in faulty base pairing or related changes (Table 9.2). For example, alkylating agents (chemicals that react with amino, carboxyl, and hydroxyl groups by substituting them with alkyl groups) such as nitrosoguanidine are powerful mutagens and generally induce mutations at higher frequency than base analogs. Unlike base analogs, which have an effect only when incorporated during DNA replication, alkylating agents can introduce changes even in nonreplicating DNA. Both base analogs and alkylating agents tend to induce base-pair substitutions (Section 9.2).

Another group of chemical mutagens, the acridines, are planar molecules that function as *intercalating agents*. These mutagens become inserted between two DNA base pairs and push them apart. Then, during replication, this abnormal conformation can trigger single base insertions or deletions. Thus, acridines typically induce frameshift rather than point mutations (Section 9.2). Ethidium bromide, which is commonly used to detect DNA in gel electrophoresis, is also an intercalating agent and therefore a mutagen.

Nonionizing and *ionizing* radiation are two forms of electromagnetic radiation that are highly mutagenic (**Figure 9.9**). Ultraviolet (UV) radiation is widely used to generate mutations because the purine and pyrimidine bases of nucleic acids absorb UV radiation strongly (the absorption maximum for DNA and RNA is at 260 nm). The primary mutagenic effect is the production of *pyrimidine dimers*, in which two adjacent pyrimidine bases (cytosine or thymine) on the same strand of DNA become covalently bonded to one another. This either greatly impedes DNA polymerase activity or greatly increases the probability of DNA polymerase misreading the sequence at this point. Thus the killing of cells by UV radiation is due primarily to its effect on DNA. Conversely, ionizing radiation is more powerful than UV radiation and includes short-wavelength radiation such as X-rays, cosmic rays, and gamma rays (Figure 9.9). These rays cause water and other substances to ionize, resulting in the formation of free radicals such as the hydroxyl radical (OH·, ◀ Section 4.16) that can damage macromolecules in the cell, including DNA. This causes double-stranded and single-stranded breaks that may lead to rearrangements or large deletions.

DNA Repair and the SOS System

By definition, a mutation is a *heritable* change in the genetic material. Therefore, if damaged DNA can be corrected before the cell divides, no mutation will occur. While cells have a variety of different DNA repair processes to correct mistakes (◀ Section 6.4) or repair damage, some are error-prone and the repair process itself introduces the mutation. Some types of DNA damage, especially large-scale damage from highly mutagenic chemicals or large doses of radiation, may cause lesions that interfere with replication. If such lesions are not removed before replication occurs, DNA replication will stall and lethal breaks in the chromosome will result.

In *Bacteria*, stalled replication or major DNA damage activates the **SOS repair system**. The SOS system initiates a number of DNA repair processes, some of which are error-free. However, the SOS system also allows DNA repair to occur without a template, that is, with random incorporation of nucleotide precursors (deoxyribonucleotide triphosphates [dNTPs]). As might be expected, this results in many errors and hence many mutations. However, mutations induced by the SOS repair system are better than the alternative (death of the cell), as mutations can often be corrected while chromosome breaks usually cannot.

In *Escherichia coli* the SOS repair system controls the transcription of approximately 40 genes located throughout the chromosome that

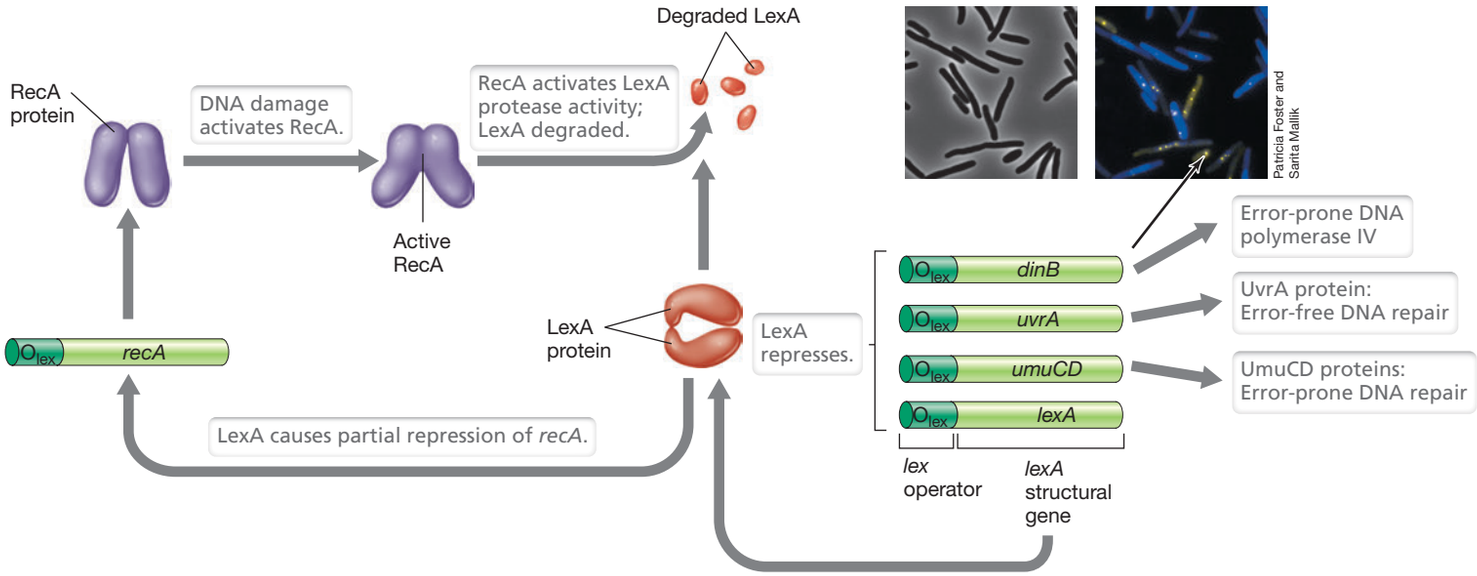


Figure 9.10 SOS response to DNA damage. DNA damage activates RecA protein, which in turn activates the protease activity of LexA, resulting in self-cleavage. LexA normally represses the activities of RecA, the genes *uvrA* and *umuCD* that encode DNA repair functions (the UmuCD proteins are part of DNA polymerase V), and *dinB*, which encodes DNA polymerase IV. However, repression is not complete. Some RecA protein is produced even in the presence of LexA protein. When LexA is inactivated, DNA repair genes are highly transcribed. Inset photos: Both photos show DNA polymerase IV localization to the nucleoid during the SOS response in *Escherichia coli*. Cells containing a fluorescently tagged DNA polymerase IV (DinB) were treated with an antibiotic to induce DNA damage. Left: phase-contrast micrograph. Right: fluorescence micrograph showing cells stained with DAPI (blue) and DNA polymerase IV (yellow, in the nucleoid region). Expression of *dinB* requires not only the loss of LexA repression but also the protein RpoS, an RNA polymerase sigma factor whose synthesis is triggered by various stress responses.

participate in DNA damage tolerance and DNA repair. Not only does the SOS system form a regulon, but the *general stress response* or *RpoS regulon* also plays a role in the repair system (◀ Sections 7.3 and 7.9). In DNA damage tolerance, DNA lesions remain in the DNA, but are bypassed by specialized DNA polymerases that can move past DNA damage—a process called *translesion synthesis*. Even if no template is available to allow insertion of the correct bases, it is less dangerous to cell survival in the long run to fill the gap than to let it remain. Consequently, translesion synthesis generates many errors. In *E. coli*, in which the process of mutagenesis has been studied in great detail, the two error-prone repair polymerases are DNA polymerase V, an enzyme encoded by the *umuCD* genes, and DNA polymerase IV, encoded by *dinB* (**Figure 9.10**). Both are induced as part of the SOS repair system.

The master regulators of the SOS system are the proteins LexA and RecA. LexA is a repressor that normally prevents expression of the SOS system. The RecA protein, which normally functions in genetic recombination (Section 9.5), is activated by the presence of DNA damage, in particular by the single-stranded DNA that results when replication stalls. The activated form of RecA then stimulates LexA to inactivate itself by self-cleavage. This leads to derepression of the SOS system and the coordinate expression of proteins that participate in DNA repair. Because some of the DNA repair mechanisms of the SOS system—such as DNA polymerases IV and V—are inherently error-prone, many mutations arise. However, once the original DNA damage has been repaired, the SOS regulon is repressed and further mutagenesis ceases.

Besides controlling some DNA repair systems and error-prone polymerases, the SOS system also regulates processes that contribute to *horizontal gene transfer*, which we discuss next. Thus, uptake and recombination of foreign DNA can also be used by *Bacteria* and *Archaea* to fix chromosomal breaks. As we shall see in Part II, not only does this form of DNA repair help with cell survival, but it also can increase cell diversity.

Check Your Understanding

- How do mutagens cause mutations?
- What is meant by "error-prone" DNA repair?

II • Gene Transfer in *Bacteria*

Lateral gene flow within and between species of *Bacteria* is highly dynamic and facilitated by at least three different mechanisms. Such genetic exchange is distinct from traditional mother-to-daughter inheritance and allows cells to rapidly acquire new characteristics and increase their competitive fitness.

Comparative genomic analyses of closely related microbes that exhibit different phenotypes have revealed distinct genome differences. Often these idiosyncratic differences result from *horizontal gene transfer*, the movement of genes between cells that are not direct descendants of one another (▶ Section 13.9). Horizontal gene transfer allows cells to quickly acquire new characteristics and fuels metabolic diversity.

Three mechanisms of genetic exchange are known in bacteria: (1) *transformation*, in which free DNA released from one cell is taken up by another (Section 9.6); (2) *transduction*, in which DNA transfer is mediated by a virus (Section 9.7); and (3) *conjugation*, in which DNA transfer requires cell-to-cell contact and a conjugative plasmid in the donor cell (Sections 9.8 and 9.9). While these processes were presented in Figure 9.1, they are compared and contrasted in **Figure 9.11**. It should be noted that DNA transfer typically *occurs in only one direction*, from donor to recipient.

Before discussing the mechanisms of transfer, we consider the fate of transferred DNA. Regardless of how it was transferred, DNA that enters the cell by horizontal gene transfer faces three possible fates: (1) It may be degraded by the recipient cell's restriction enzymes or other DNA destruction systems (Section 9.12); (2) it may replicate by itself (but only if it possesses its own origin of replication, such as a plasmid or phage genome); or (3) it may recombine with the recipient cell's chromosome.

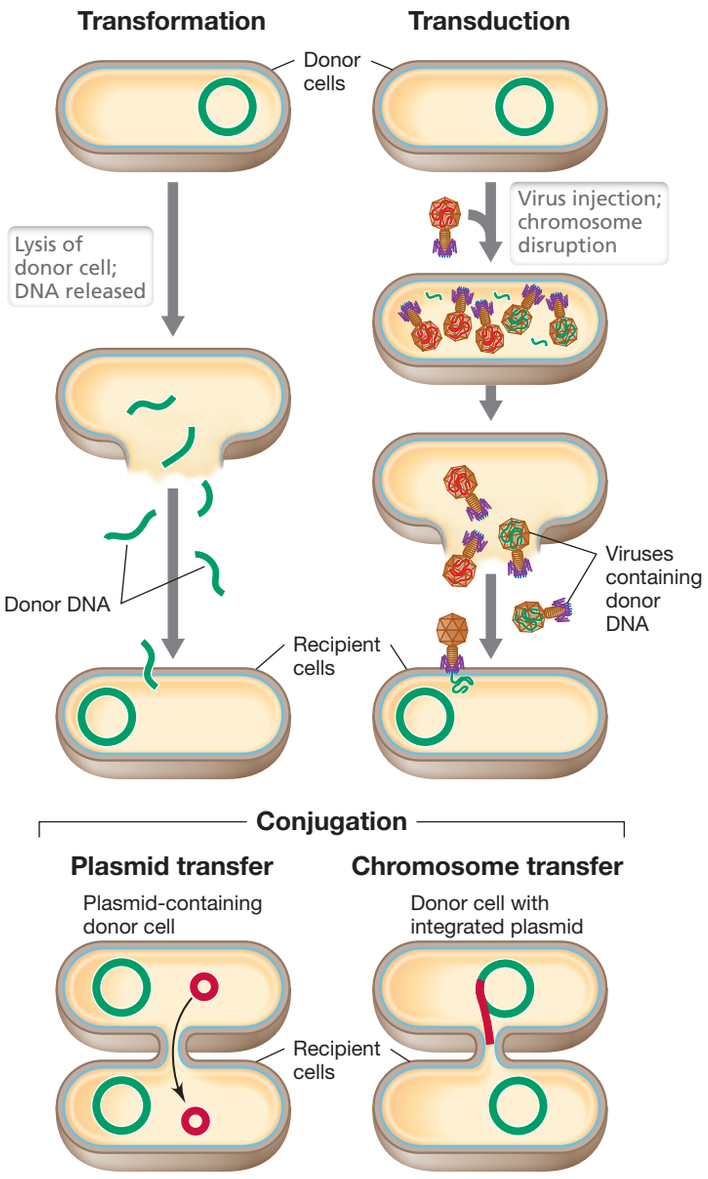


Figure 9.11 Processes by which DNA is transferred from donor to recipient bacterial cell. Just the initial steps in transfer are shown. Note that conjugation requires cell–cell contact, whereas transduction and transformation do not.

9.5 Genetic Recombination

Recombination is the physical exchange of DNA between *genetic elements* (structures that carry genetic information). Here we focus on *homologous* recombination, a process that results in genetic exchange between homologous DNA sequences from two different sources. Homologous DNA sequences are those that have nearly the same sequence; therefore, bases can pair over an extended length of the two DNA molecules to facilitate exchange. This type of recombination is often essential to a cell's ability to retain DNA following genetic exchange and is behind the well-known phenomenon of "crossing over" in the genetics of eukaryotes.

Molecular Events in Homologous Recombination

The RecA protein, previously mentioned in regard to the SOS repair system (Section 9.4 and Figure 9.10), is the key to homologous recombination. RecA is essential in nearly every homologous recombination pathway. RecA-like proteins have been identified in all *Bacteria* examined, as well as in the *Archaea* and most *Eukarya*.

A molecular mechanism for homologous recombination between two DNA molecules is shown in **Figure 9.12**. An enzyme that cuts DNA in the middle of a strand, called an *endonuclease*, begins the process by nicking one strand of the donor DNA molecule. This nicked strand is separated from the other strand by proteins with helicase activity; the resulting single-stranded segment binds single-strand binding protein (◀ Section 6.3) and then RecA. This results in a complex that promotes base pairing with the complementary sequence in the recipient DNA molecule. Base pairing, in turn, displaces the other strand of the recipient DNA molecule (Figure 9.12) and is appropriately called *strand invasion*.

The base pairing of one strand from each of the two DNA molecules over long stretches generates recombination intermediates containing long **heteroduplex** regions, where each strand has originated from a different chromosome. After DNA strands at the branch or crossover regions are joined, the linked molecules are then resolved (separated) by enzymes that cut and rejoin the previously unbroken strands of both original DNA molecules. Depending on the orientation of the junction during resolution, two types of products—referred to as "patches" or "splices"—are formed that differ in the conformation of the heteroduplex regions remaining after resolution (Figure 9.12).

Effect of Homologous Recombination on Genotype

For homologous recombination to generate new genotypes, the two homologous sequences must be related but genetically distinct. This is obviously the case in a diploid eukaryotic cell, which has two sets of chromosomes, one from each parent. However, in bacteria, genetically distinct but homologous DNA molecules are brought together in different ways. Genetic recombination in bacteria occurs after fragments of homologous DNA from a donor chromosome are transferred to a recipient cell by transformation, transduction, or conjugation. It is only after the transfer event, when the DNA fragment from the donor is in the recipient cell, that homologous recombination occurs.

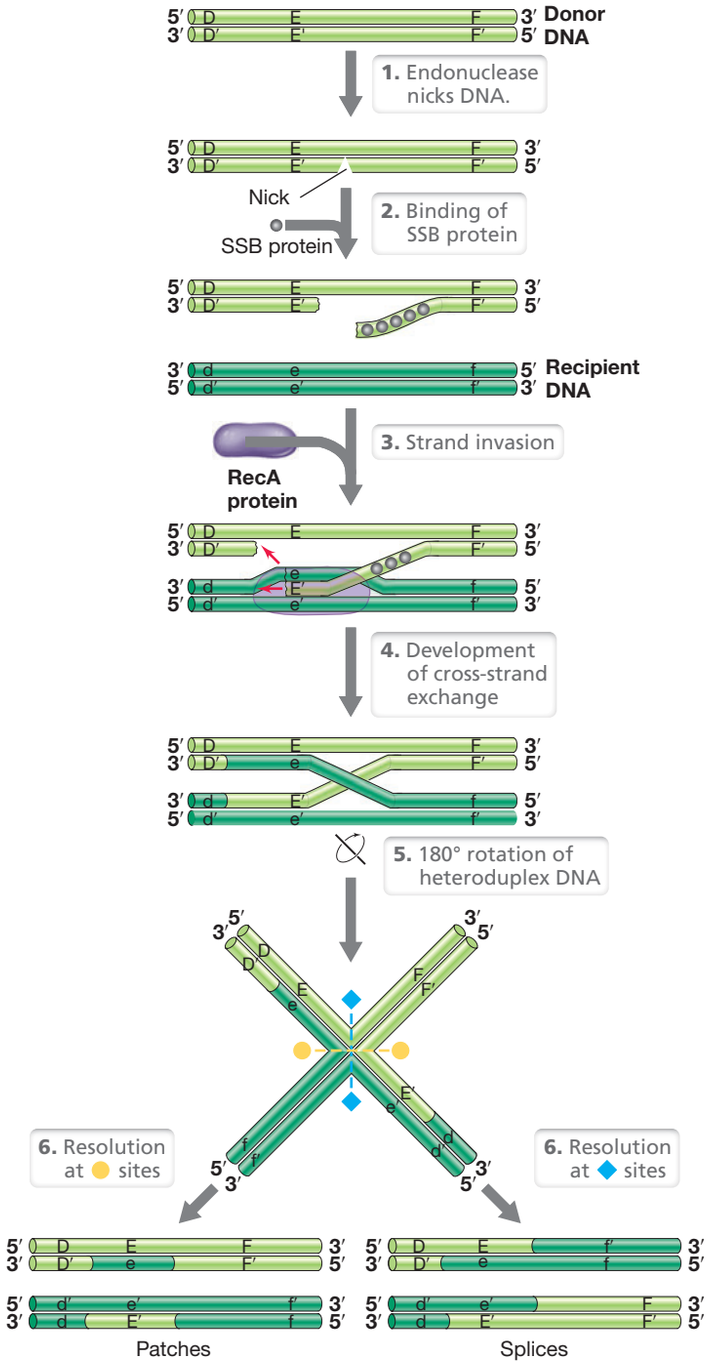


Figure 9.12 Homologous recombination. Homologous DNA molecules pair and exchange DNA segments. The mechanism requires breakage and reunion of paired segments, as indicated by red arrows. Two of the participating proteins, single-strand binding (SSB) protein and the RecA protein, are shown (other participating proteins are not shown). Resolution occurs by cutting and rejoining the cross-linked DNA molecules. Note that there are two possible outcomes, patches or splices, depending on where strands are cut during the resolution process.

For physical exchange of DNA segments to be detected, the cells resulting from recombination must be phenotypically different from both parents (**Figure 9.13**). Genetic crosses in bacteria usually depend on using recipient strains that lack some selectable character that the recombinants will gain. For instance, the recipient may be

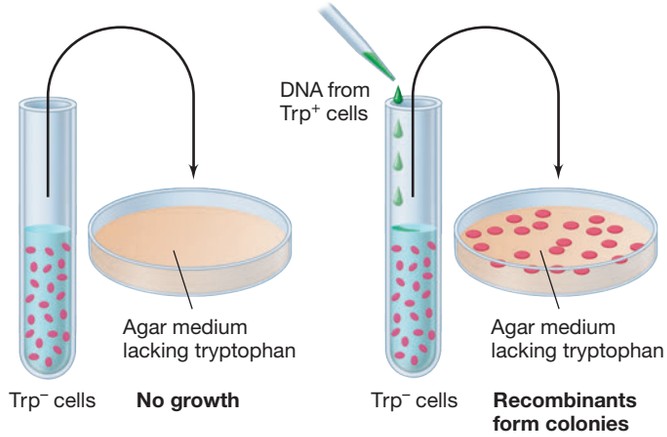


Figure 9.13 Using a selective medium to detect rare genetic recombinants. On the selective medium, only the rare recombinants form colonies even though a very large population of bacteria is plated. Procedures such as this, which offer high resolution for genetic analyses, can ordinarily be used only with microorganisms. The type of genetic exchange being illustrated is transformation, but a similar outcome could result from any of the other forms of horizontal gene transfer (Figure 9.1).

unable to grow on a particular medium or may exhibit a specific phenotype due to a point mutation, while the genetic recombinants can grow on the particular medium or exhibit a phenotype different from the recipient (Figures 9.2 and 9.13). Various kinds of selectable markers, such as drug resistance and nutritional requirements, were discussed in Section 9.1. The exceedingly great sensitivity of the selection process allows even a few recombinant cells to be detected in a large population of nonrecombinant cells, and thus selection is an important tool for the microbial geneticist.

Complementation

In all three methods of bacterial gene transfer, only a portion of the donor chromosome enters the recipient cell and thus transfer is just the first step; unless recombination takes place with the recipient chromosome, the donor DNA will be lost because it cannot replicate independently in the recipient. Nonetheless, it is possible to stably maintain a state of partial diploidy for use in bacterial genetic analysis. A bacterial strain that carries *two copies* of any particular chromosomal segment is known as a partial diploid, or *merodiploid*. In general, one copy is present on the chromosome itself and the second copy on another genetic element, such as a plasmid or a bacteriophage.

Consequently, if the chromosomal copy of a gene is defective due to a mutation, it is possible to supply a functional (wild-type) copy of the gene on a plasmid or bacteriophage. For example, if one of the genes for biosynthesis of the amino acid tryptophan has a mutation resulting in a nonfunctional enzyme, this will yield a Trp^- phenotype (the mutant strain will be a tryptophan auxotroph and must be supplied with tryptophan for growth). However, if a copy of the wild-type gene is introduced into the same cell on a plasmid or viral genome, this gene will encode the necessary protein and, assuming the gene is transcribed and translated, will restore the wild-type phenotype. This process is called *complementation* because the wild-type gene is said to *complement* the mutation, in this case converting the Trp^- cell into Trp^+ (Figure 9.13).

Check Your Understanding

- Which protein, found in virtually all cells, facilitates the pairing required for homologous recombination?
- Explain the fate of transferred chromosomal DNA if recombination does not occur in the recipient cell.
- What is a merodiploid, and what is genetic complementation?

9.6 Transformation

Transformation is a genetic transfer process by which *free DNA* is incorporated into a recipient cell and brings about genetic change. When would a cell encounter free DNA? Because the DNA in bacterial and archaeal cells is present as a large single molecule, chromosomal DNA pours out during cell lysis. Cells that are naturally transformable, which include certain species of both gram-negative and gram-positive *Bacteria* and also some species of *Archaea* (Section 9.10), can take up pieces of this free DNA from their lysed neighbors (Figures 9.1 and 9.11).

Because of their extreme length, bacterial chromosomes break easily. For example, the *Bacillus subtilis* chromosome would be 1700 μm long if linearized. However, even gentle DNA extraction methods result in the *B. subtilis* 4.2-megabase-pair chromosome being broken into fragments of about 10 kilobase pairs each. Because an average gene contains about 1000 nucleotides, each of the fragments of *B. subtilis* DNA contains about ten genes. This is a typical transformable size. A single cell typically incorporates only one or at most a few DNA fragments, so only a small proportion of the genes of one cell can be transferred to another in a single transformation event.

Competence in Transformation

How does a cell take up DNA? Recall from our Chapter 2 discussion that cell membranes are selective barriers. DNA does not freely cross this barrier; thus a cell that is able to take up DNA and be transformed is said to be *competent*. **Figure 9.14** illustrates *Vibrio cholerae*, the causative agent of cholera and a model gram-negative bacterium

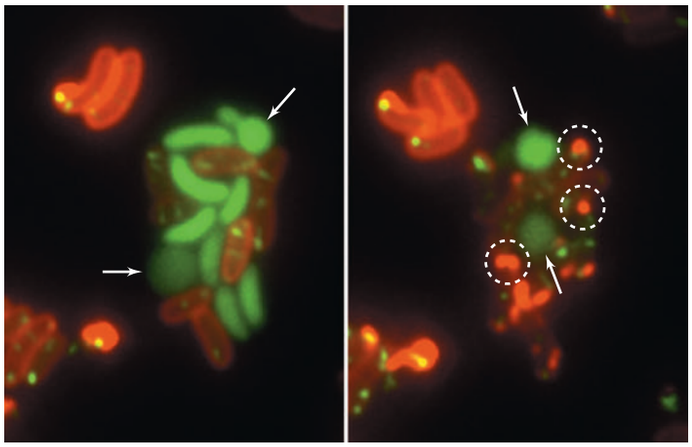


Figure 9.14 ***Vibrio cholerae*** **killing neighboring prey cells and scavenging DNA.** Time-course images of *V. cholerae* type VI secretion system proteins labeled with a green fluorescent protein (GFP) and corresponding DNA uptake proteins labeled with a red fluorescent protein. *V. cholerae* cells expressing both protein types appear red with green "crossbows" inside, while competing cells lacking a type VI secretion system appear as solid green cells. At time 0, white arrows indicate prey cells that have been killed. These cells circularize upon death. After 30 minutes, DNA uptake proteins localize for effective uptake of DNA from killed cells (circled). Images modified from Borgeaud, S., Metzger, L.C., Scrignari, T., and Blokesch, M. 2015. *Science 347*: 63–67.

for natural competence, taking up DNA. In its natural marine environment, *V. cholerae* aggressively competes for nutrients by using its type VI secretion system to inject toxin molecules into neighboring cells (◀ Section 6.13). These toxin molecules ultimately kill competitor cells. Not only do the *V. cholerae* cells obtain nutrients from lysed neighboring cells, but they are also able to scavenge the DNA released from their victims (Figure 9.14).

The capacity to take up free DNA is genetically determined. In *Bacteria* such as *Vibrio*, *Neisseria*, *Acinetobacter*, *Thermus*, *Streptococcus*, and *Bacillus*, competence is directly linked to pili (**Figure 9.15**). Pili

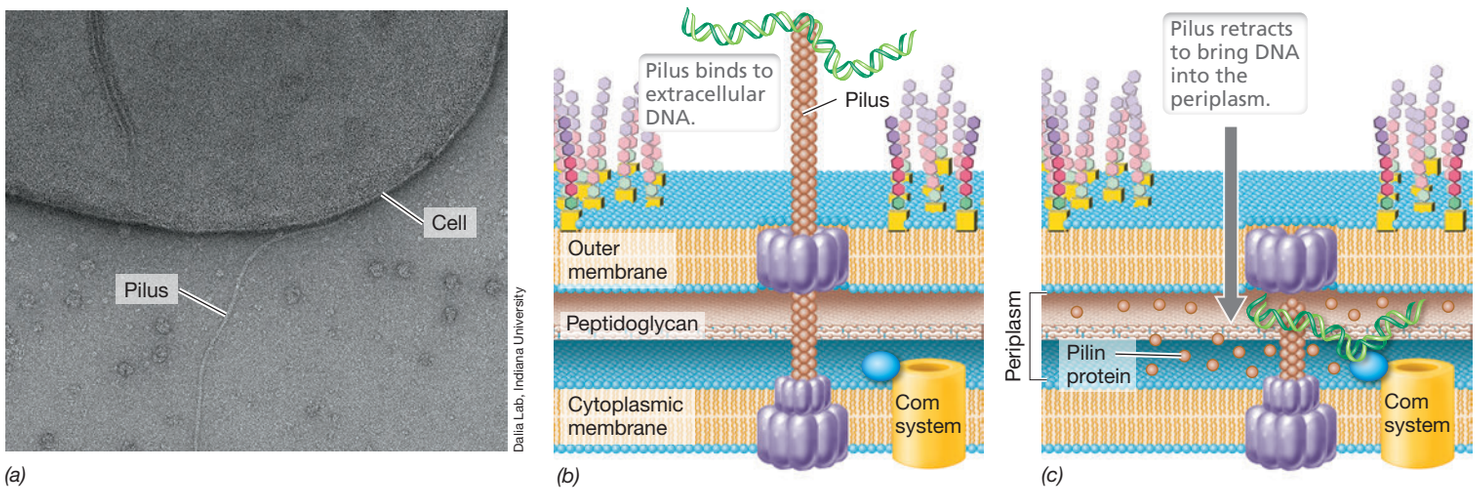


Figure 9.15 ***Vibrio*** **pilus and DNA uptake.** *(a)* Transmission electron micrograph showing *Vibrio cholerae* type IV pilus. *(b)* The pilus binds extracellular DNA, and *(c)* pilus retraction brings extracellular DNA through the outer membrane to the competence (Com) system associated with the cytoplasmic membrane. See the photomicrograph of a *V. cholerae* cell using its pilus to retrieve DNA on page 297.

are versatile multiprotein structures that not only play roles in twitching, gliding motility, biofilm formation, and pathogenesis (◀ Sections 2.6 and 2.10), but are also key to binding and taking up DNA in naturally competent cells. In gram-negative *Bacteria*, pili cross the hydrophobic outer membrane through a specific channel, and protein subunits within the pilus recognize and bind to extracellular DNA (Figure 9.15*b*). After binding to DNA, *V. cholerae* pili pull the DNA through the outer membrane into the periplasm through a process called *pilus retraction* (see MicrobiologyNow in the chapter opener). This retraction of the pilus is the result of depolymerization of individual pilin proteins that make up the pilus structure (Figure 9.15*c*). In gram-positive cells, pili or homologs of type II secretion systems (◀ Section 6.13) are used to bind DNA and bring it through the thick cell wall layer. Once in the periplasm of gram-negative cells or through the cell wall in gram-positive cells, the DNA binds to proteins within the *competence system* associated with the cytoplasmic membrane. These competence-specific proteins include a membrane-associated DNA-binding protein and various nucleases (**Figure 9.16**).

As noted earlier, the sizes of the transforming fragments are much smaller than that of the whole genome, and the fragments are further degraded during the uptake process by competence proteins (Figure 9.16). As these fragments are taken up into the cytoplasm, they are often converted into single-stranded pieces of DNA, with the complementary strand being degraded. After entry into the cytoplasm, a competence-specific protein binds the single-stranded donor DNA. This protects the DNA from nuclease attack until it reaches the recipient's chromosome, where the RecA protein takes over. If the transforming DNA shares sequence homology with that of the chromosome, then it is integrated into the genome of the recipient by recombination (Figures 9.12 and 9.16).

The preceding applies only to small pieces of *linear* DNA. Many naturally transformable *Bacteria* are transformed only poorly by plasmid DNA because the plasmid must remain double-stranded and circular in order to replicate. The success rate of natural plasmid DNA transformation can be enhanced through delivery from a donor to a recipient cell through extracellular vesicles (Figure 9.1), which we will consider in more detail in *Archaea* in Section 9.10.

Regulation of Competence

Competence in most naturally transformable bacteria is regulated. One pathway of natural competence in *Bacillus subtilis*—an easily transformed species—is regulated by quorum sensing, a regulatory system that responds to cell density (◀ Section 7.7). Cells produce and excrete a small peptide during growth, and the accumulation of this peptide to high concentrations induces the cells to become competent. But not all cells in a population become competent. In *Bacillus*, roughly 20% of the cells become competent and stay that way for several hours. By contrast, in *Streptococcus*, 100% of the cells can become competent, but only for a brief period during the growth cycle.

Multiple layers of regulation control natural competence in other bacteria. *Vibrio cholerae* is naturally found in marine and freshwater environments associated with crustacean exoskeletons, which are composed of chitin. Competence in *V. cholerae* is controlled not only by quorum sensing but also by chitin sensing and catabolite repression (◀ Section 7.8). *V. cholerae* can catabolize chitin (a polymer of *N*-acetylglucosamine and an abundant nutrient in the marine environment), and as cells aggregate on a chitin surface, they are in close proximity to one another and more likely to successfully exchange DNA (**Figure 9.17**).

High-efficiency, natural transformation is rare among *Bacteria*. For example, *Acinetobacter, Bacillus, Streptococcus, Haemophilus, Neisseria,* and *Thermus* are naturally competent and easy to transform. This natural competence provides a nutritional advantage, as free DNA is rich in carbon, nitrogen, and phosphorus. By contrast, many *Bacteria* are poorly transformed, if at all, under natural conditions. For example, *Escherichia coli* and many other gram-negative bacteria fall into this category. However, if cells of *E. coli* are treated with high concentrations of Ca^{2+} and then chilled, they become adequately competent. Cells treated in this manner take up double-stranded

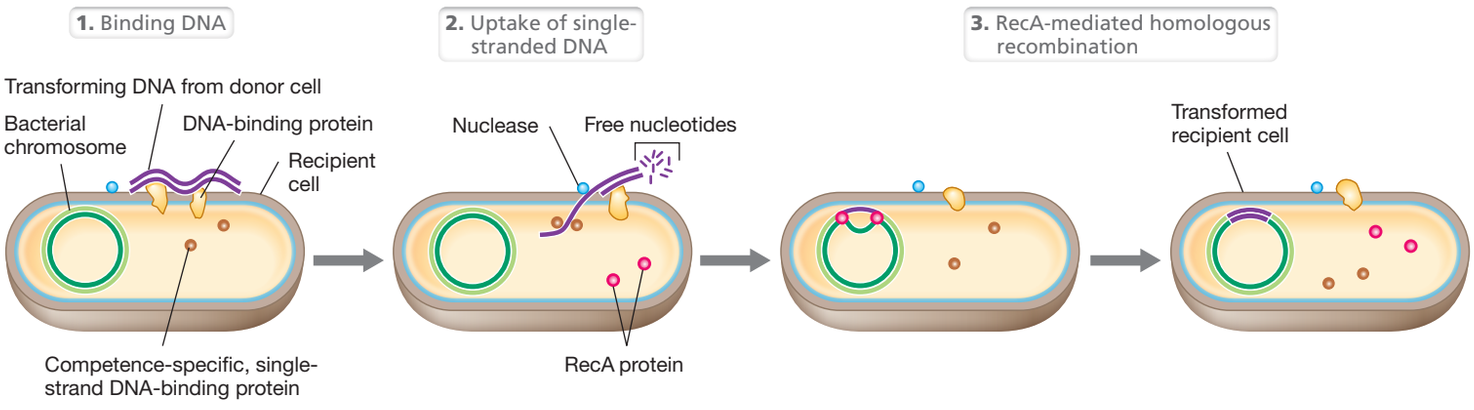


Figure 9.16 General mechanism of transformation. To begin, double-stranded DNA is bound by a membrane-bound DNA-binding protein. Passage of one of the two strands of DNA into the cell is followed by nuclease activity that degrades the complementary strand. Once the single strand of DNA in the cell is bound by specific proteins, recombination with homologous regions of the bacterial chromosome is mediated by the RecA protein with the final product being a transformed cell.

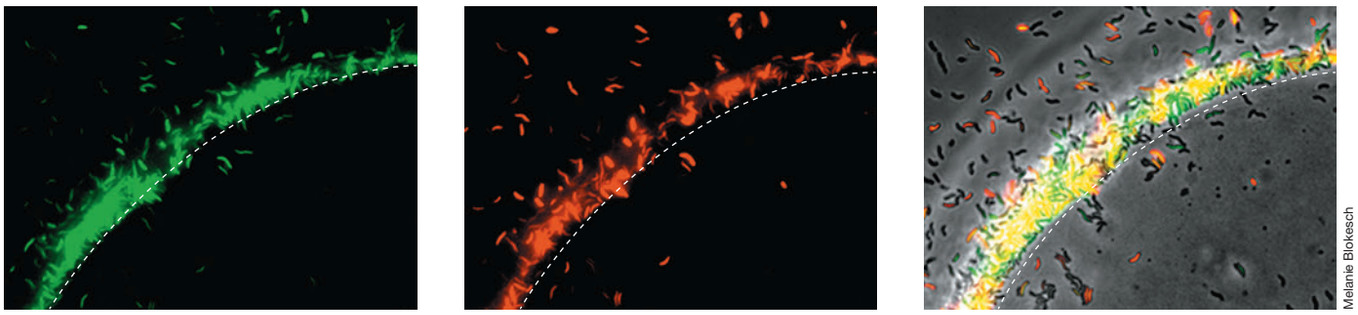


Figure 9.17 Regulation of natural competence in *Vibrio cholerae*. *V. cholerae* cells with fluorescent reporter genes linked to the promoters of competence genes were grown in the presence of chitin beads. White dashed line indicates edge of bead surface. *(a)* Cells with the *pilA* (pilus protein) promoter linked to a green fluorescent protein (GFP). *(b)* Cells with the *comEA* (DNA uptake proteins) promoter linked to a red fluorescent protein. *(c)* Merged image of parts *a* and *b* illustrating expression of competence genes in cells associated with the chitin bead. Cells of *V. cholerae* are about 0.5 μm wide and 1.5 μm long. Adapted from Lo Scrudato, M., and M. Blokesch. 2012. *PLoS Genetics 8*(6): e1002778. See Figure 9.14 for a different view of transformation in *V. cholerae*.

DNA, and therefore transformation of *E. coli* by plasmid DNA can be relatively efficient. This is important because getting DNA into *E. coli*—the workhorse of genetic engineering—is critical for biotechnology, as we will see in Chapter 12.

Electroporation is a physical technique that is used to get DNA into organisms that are difficult or impossible to transform, especially cells that contain thick cell walls. In electroporation, cells are mixed with DNA and then exposed to brief, high-voltage electrical pulses. This makes the cell envelope permeable and allows entry of the DNA. Electroporation works for getting free DNA into most types of cells, including *E. coli*, most other *Bacteria*, some species of *Archaea*, and even yeast and certain plant cells.

Check Your Understanding

- During transformation a cell usually incorporates only one or a few fragments of DNA. Explain.
- In genetic transformation, what is meant by the word competence?
- During natural competence, what type of cellular structure is used to bind DNA and take it to the cytoplasmic membrane?

9.7 Transduction

In **transduction**, a bacterial virus (bacteriophage, Chapter 5) transfers DNA from one cell to another. Viruses can transfer host genes in two ways. In the first, called *generalized transduction*, DNA derived from virtually any portion of the host genome is packaged inside the mature virion in place of the virus genome. In the second, called *specialized transduction*, DNA from a specific region of the host chromosome is integrated directly into the virus genome—usually replacing some of the virus genes. This occurs only with certain temperate viruses such as phage lambda (◀ Section 5.6).

In generalized transduction, the bacterial donor genes cannot replicate independently and are not part of a viral genome. Thus, unless the donor genes recombine with the recipient bacterial chromosome, they will be lost. In specialized transduction, homologous recombination may also occur. However, since the donor bacterial DNA is actually a part of a temperate phage genome, it may be integrated into the host chromosome during lysogeny (◀ Section 5.6). This can lead to an event called phage conversion (see later).

Transduction occurs in a variety of *Bacteria*, including the genera *Desulfovibrio*, *Escherichia*, *Pseudomonas*, *Rhodococcus*, *Rhodobacter*, *Salmonella*, *Staphylococcus*, and *Xanthobacter*, as well as *Methanothermobacter thermautotrophicus*, a species of *Archaea*. Not all phages can transduce and not all bacteria are transducible. However, considering the enormous genetic diversity of viruses (Chapter 11) and the fact that bacteriophages are estimated to outnumber prokaryotic cells in nature by 10-fold, transduction likely plays an important role in gene transfer in the environment for many species of *Bacteria* and *Archaea*. Some examples of genes transferred by transducing bacteriophages include multiple-antibiotic-resistance genes among strains of *Salmonella enterica* (*typhimurium*), Shiga-like toxin genes in *Escherichia coli*, virulence factors in *Vibrio cholerae*, and genes encoding photosynthetic proteins in cyanobacteria (▶ Section 20.13).

Generalized Transduction

In generalized transduction, virtually any gene on the donor chromosome can be transferred to the recipient, forming a *transductant*. Generalized transduction was first discovered and extensively studied in the bacterium *S. enterica* with phage P22 and has also been studied with phage P1 in *E. coli*. The mechanism of transduction is shown in **Figure 9.18**. When a bacterial cell is infected with a transducing phage, the lytic cycle may occur. However, during lytic infection, the enzymes responsible for packaging viral DNA into the bacteriophage sometimes package host DNA accidentally. The result is called a *transducing particle*. These cannot lead to a viral lytic infection because they contain no viral DNA, and are therefore said to be *defective*.

Mastering Microbiology
Art Activity: Figure 9.18 Generalized transduction

Upon lysis of the cell, transducing particles are released along with normal virions that contain the virus genome. When this

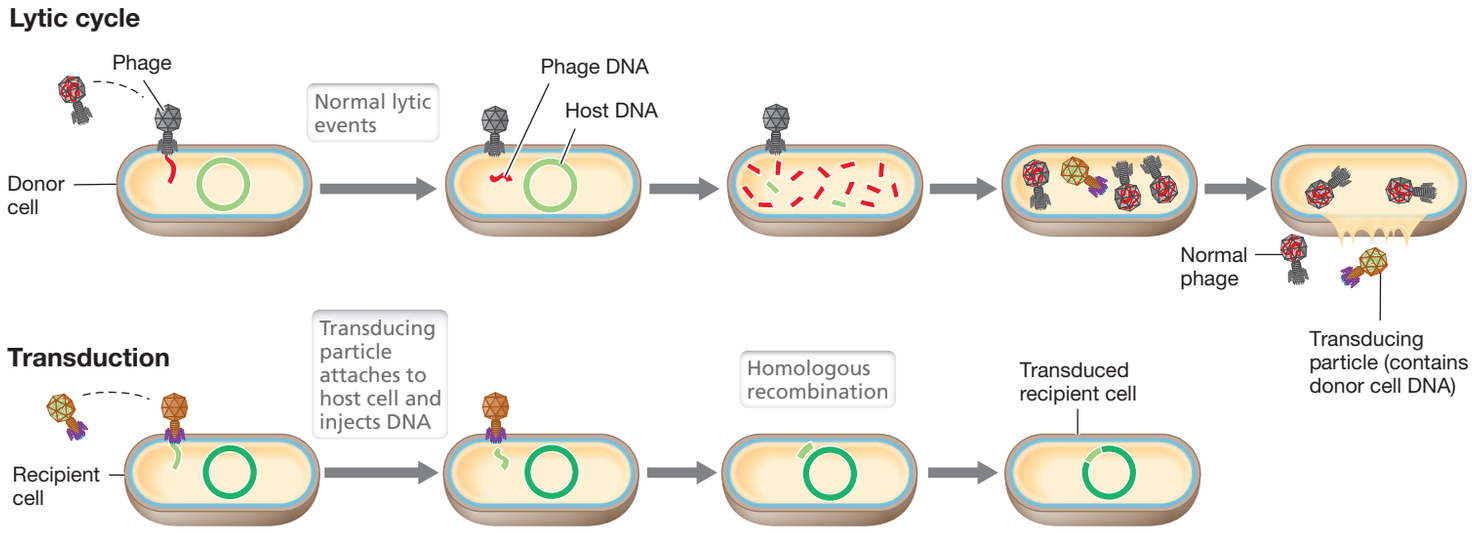


Figure 9.18 Generalized transduction. Note that "normal" virions contain phage genes, whereas a transducing particle contains host genes.

lysate is used to infect a population of recipient cells, most of the cells are infected with a normal (lytic) virus. However, a small proportion of the population receives transducing particles that inject the DNA they packaged from the previous host bacterium. Although this DNA cannot replicate, it can recombine with the DNA (Section 9.5) of the new host (**Figure 9.19**). Because only a small proportion of the particles in the lysate are defective, and each of these contains only a small fragment of donor DNA, the probability of a given transducing particle containing a particular gene is quite low. Typically, only about 1 cell in 10^6 to 10^8 cells is transduced for any given gene.

Lysogeny and Specialized Transduction

Generalized transduction allows the transfer of any gene from one bacterium to another, but at a low frequency. In contrast, specialized transduction allows extremely efficient transfer but is selective and transfers only a small region of the bacterial chromosome. In the first case of specialized transduction to be discovered, genes for galactose catabolism were transduced by the temperate phage lambda of *E. coli*.

Mastering Microbiology
Art Activity: Figure 9.20 Specialized transduction

When lambda lysogenizes a host cell, the phage genome is integrated into the *E. coli* chromosome at a specific site (◀ Section 5.6 and ▶ Section 11.4). This site is next to the cluster of genes that encode the enzymes for galactose utilization. After insertion, viral DNA replication is under control of the bacterial host chromosome. Upon induction, the viral DNA separates from the host DNA by a process that is the reverse of integration (**Figure 9.20**). Usually the lambda DNA is excised precisely, but occasionally the phage genome is excised incorrectly. Some of the adjacent bacterial genes to one side of the prophage (for example, the galactose operon) are excised along with phage DNA. At the same time, some phage genes are left behind (Figure 9.20*b*). This transducing particle can subsequently transfer genes for galactose utilization to a recipient cell. This transfer can only be detected if a galactose-negative (Gal^-) strain is infected with such a transducing particle and Gal^+ transductants are selected.

For a lambda virion to be infectious, there is a limit to the amount of phage DNA that can be replaced with host DNA. Sufficient phage DNA must be retained to encode the phage protein coat and other phage proteins needed for lysis and lysogeny. However, if a helper phage is used together with a defective phage in a *mixed infection*, then far fewer phage-specific genes are needed in the defective phage. Only the *att* (attachment) region, the *cos* site (cohesive ends, for packaging), and the replication origin of the lambda genome (▶ Figure 11.11) are necessary.

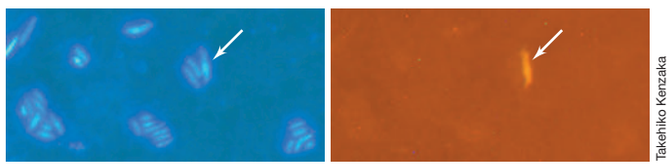


Figure 9.19 Visualization of generalized transduction. *Citrobacter freundii* cells were mixed with P1 bacteriophage carrying the β-lactamase (*bla*) gene for 10 minutes. *(a)* Fluorescence micrograph showing cells by DAPI staining. *(b)* Detection of a single *C. freundii* transductant containing the *bla* gene recombined into the genome as visualized by fluorescence in situ hybridization (FISH, ▶ Section 19.5). Arrows indicate the cell that is transduced.

Phage Conversion

Alteration of the phenotype of a host cell by lysogenization is called *phage conversion*. When a normal (that is, nondefective) temperate phage lysogenizes a cell and becomes a prophage, the cell becomes immune to further infection by the same type of phage. Such immunity may itself be regarded as a change in phenotype. However, other phenotypic changes unrelated to phage immunity are often observed in phage conversion of lysogenized cells due to foreign genes present in the phage genome.

Overall, the genes responsible for the phage conversion are an integral part of the phage genome and hence are automatically

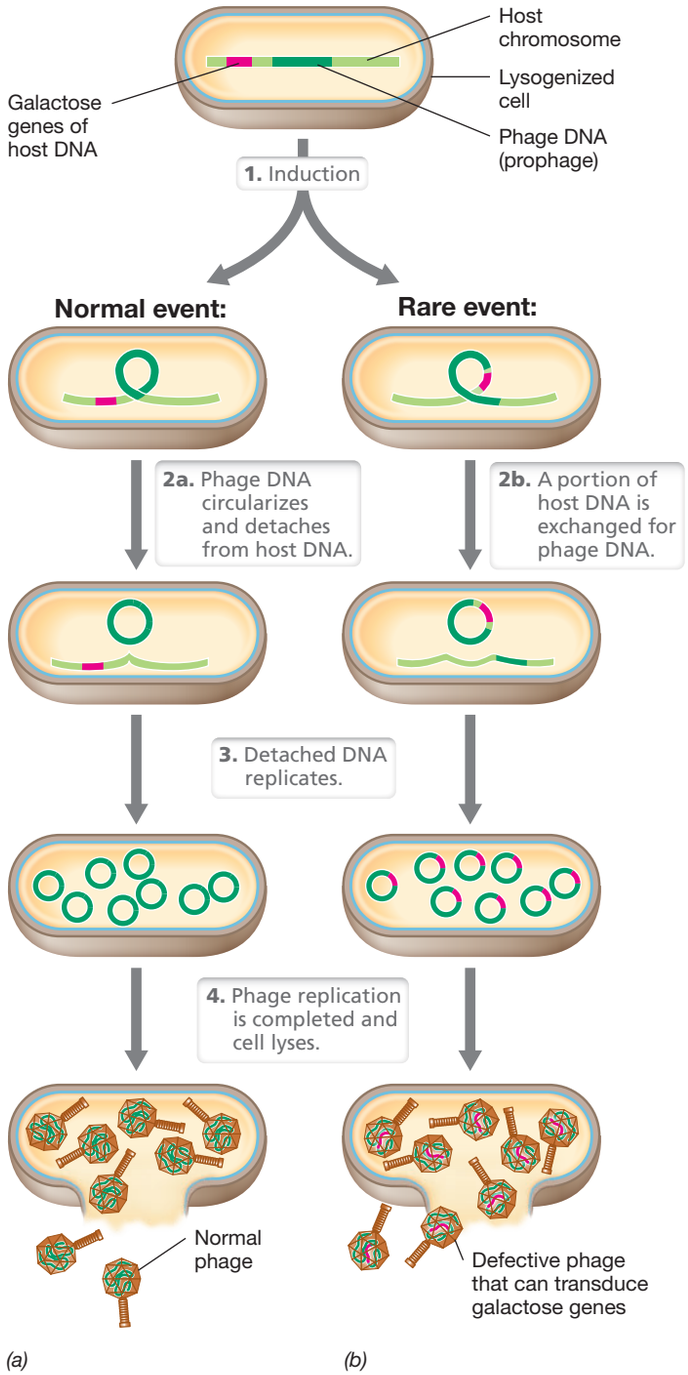


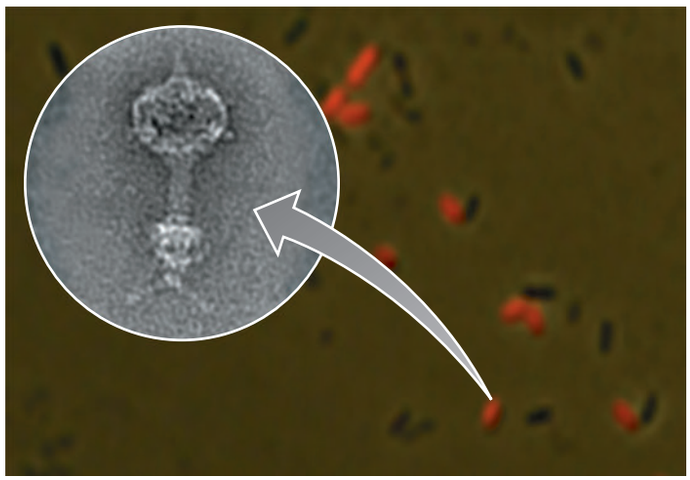
Figure 9.20 **Specialized transduction.** In an *Escherichia coli* cell containing a lambda prophage, *(a)* normal lytic events and *(b)* the production of particles transducing the genes for galactose utilization. Only a short region of the circular host chromosome is shown in the figure.

transferred to the cell upon phage infection and establishment of the lysogenic state. This lysogenic state likely carries strong selective value for the host cell because it confers resistance to infection by viruses of the same type. It has also been found that many bacteria isolated from nature are natural lysogens and that their corresponding prophage encode an abundance of transposons (Section 9.11 and ▶ Section 13.9). Thus it is likely that phage conversion contributes to the formation of new genes that are essential for survival of many host cells in nature.

Cases of phage conversion that promote bacterial virulence have been especially well studied. *Vibrio cholerae*, which causes the disease cholera (▶ Section 33.3), is a fascinating example of how phage conversion can result in a nonpathogenic environmental strain evolving into a pathogen. CTXφ is a lysogenic bacteriophage found integrated into the chromosome of pathogenic *V. cholerae* strains. Besides the normal genes necessary to maintain the lysogenic state, the CTXφ prophage also encodes the cholera toxin that triggers the diarrheal disease. During infection, CTXφ virions specifically bind to the toxin co-regulated pilus (TCP) used by *V. cholerae* to attach to host intestinal cells (▶ Section 33.3). Interestingly, this TCP appears to be encoded by a separate prophage. Strains of *Vibrio* that are not lysogenized by CTXφ or TCP are nonpathogenic, thus highlighting the power of phage conversion in enhancing genetic fitness and pathogen evolution. In later chapters we will see many other human infectious diseases in which phage conversion plays a critical role, including in such distinct diseases as whooping cough and botulism.

Gene Transfer Agents

DNA can also be transferred between prokaryotic cells by defective bacteriophages. These so-called *gene transfer agents* (*GTAs*) are the result of prokaryotic cells hijacking defective viruses and using them specifically for DNA exchange (**Figure 9.21**). GTAs resemble tiny tailed bacteriophages and contain random small pieces of *host* DNA. They are not considered true viruses because they do not contain genes encoding their own production and do not produce characteristic viral plaques. Instead, the genes encoding GTAs lie within the genome of the cell that produces them, while other regions of the genome are packaged within the agents.



A. Westbye, P. Fogg, C. Yip, and J.T. Beatty

Figure 9.21 **Gene transfer agents.** Inset: Transmission electron micrograph of a gene transfer agent (GTA) isolated from *Rhodobacter capsulatus*. Visualization of a subset of *R. capsulatus* cells producing and releasing GTAs during stationary phase using a red fluorescent reporter gene linked to the promoter of an *R. capsulatus* gene essential for GTA production. Note that only a subset of the total population produces GTA.

GTAs have been isolated from a wide range of *Bacteria* including the sulfate-reducing bacterium *Desulfovibrio desulfuricans*, a variety of phototrophic purple bacteria and other *Alphaproteobacteria*, and also from certain methanogenic *Archaea*. GTAs seem to be particularly widely produced by marine *Bacteria*, especially purple bacteria such as *Roseovarius*. GTA synthesis is regulated, and in the purple bacterium *Rhodobacter capsulatus*, GTA synthesis is triggered by the cell's sensing of the stationary growth phase and nutrient fluctuations (Figure 9.21). These conditions lead to only a small subpopulation of *R. capsulatus* cells lysing and releasing the GTAs. However, the same conditions that trigger GTA synthesis in some cells also lead to increased expression of the genes encoding products necessary for the uptake of GTAs. In *R. capsulatus,* stationary phase and nutrient fluctuations lead to the increased expression of genes encoding the capsular polysaccharide (◀ Figure 2.16) receptor that GTAs specifically bind to and natural competence proteins needed for DNA uptake. This suggests that GTAs may have evolved as a mechanism for a subpopulation of cells to sacrifice themselves in order to disperse genes into the environment in a protected form to benefit the rest of their population.

While bacteriophages are considered the most abundant microbes on Earth, the percentage of these that are actually GTAs instead of viruses is unknown, but could be significant. The fact that GTAs are produced by so many different species points to GTAs as major vehicles for gene flow between prokaryotic cells in nature. This could be especially true of open-ocean microbial communities, where constant low nutrient levels might trigger GTA production as a means for cells to scavenge each other's genes in an effort to improve fitness and survival of the overall population.

Check Your Understanding

- How does a transducing particle differ from an infectious bacteriophage?
- What is the major difference between generalized transduction and transformation?
- Why is phage conversion considered beneficial to host cells?

9.8 Conjugation

Conjugation is a form of horizontal gene transfer that requires cell-to-cell contact (mating) and occurs in many species of gram-negative and gram-positive bacteria. Conjugation is a plasmid-encoded mechanism that can mediate DNA transfer between closely related cells or between more distantly related cells (for example, between cells of different genera). Conjugative plasmids use conjugation to transfer copies of the genes that encode themselves and other genes they may contain, such as those that encode antibiotic resistance, to new host cells.

Mastering Microbiology Activities: Microbiology Animation: Conjugation: F Factor

The process of conjugation requires a *donor* cell, which contains the conjugative plasmid, and a *recipient* cell, which does not. In addition, genetic elements that cannot transfer themselves can sometimes be *mobilized* or transferred during conjugation. These other genetic elements can be other plasmids or the host chromosome itself. Indeed, conjugation was discovered because the F plasmid of *Escherichia coli* can mobilize the host chromosome (see Figure 9.27). Transfer mechanisms may differ depending on the participating plasmid, but most plasmids in gram-negative *Bacteria* employ a mechanism similar to that used by the F plasmid.

F Plasmid

The F plasmid (F stands for "fertility") is a circular DNA molecule of 99,159 bp. **Figure 9.22** shows a genetic map of the F plasmid. One region of the plasmid contains genes that regulate DNA replication. It also contains a number of transposable elements (Section 9.11) that allow the plasmid to integrate into the host chromosome. In addition, the F plasmid has a large region of DNA, the *tra* region, containing genes that encode transfer functions. Many genes in the *tra* region participate in mating pair formation, and most of these have to do with the synthesis of the conjugative pilus (◀ Section 2.6) and a type IV secretion system (◀ Section 6.13) to transfer the DNA. Only donor cells produce these pili. Different conjugative plasmids may have slightly different *tra* regions, and the pili may vary somewhat in structure. The F plasmid and its relatives encode F pili.

Pili allow specific pairing to take place between the donor and recipient cells. All conjugation in gram-negative *Bacteria* is thought to depend on cell pairing brought about by pili. The pilus makes specific contact with a receptor on the recipient cell surface and then is retracted by disassembling its subunits. This pulls the two cells

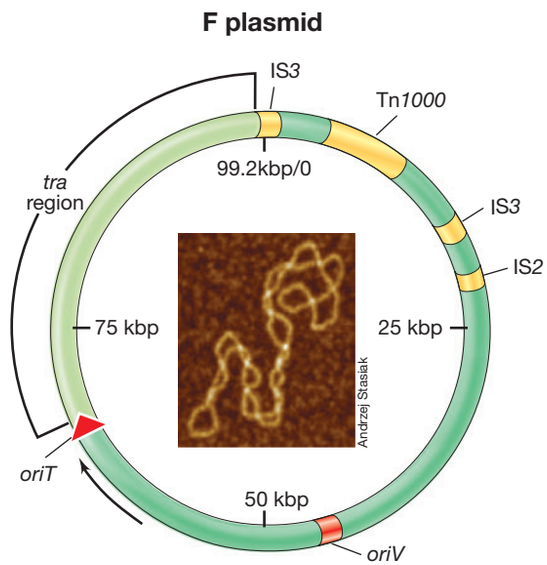


Figure 9.22 Genetic map of the F (fertility) plasmid of *Escherichia coli*. The numbers on the interior mark the size of the plasmid in kilobase pairs (the exact size is 99,159 bp). The region in dark green at the bottom of the map contains genes primarily responsible for the replication and segregation of the F plasmid. The origin of vegetative replication is *oriV*. The light green *tra* region contains the genes needed for conjugative transfer. The origin of transfer during conjugation is *oriT*. The red arrowhead at *oriT* indicates the leading edge and direction of the transfer, and the black arrow is alongside the first part of the plasmid to be transferred behind *oriT*. The *tra* region is transferred last. Insertion sequences are shown in yellow. These may recombine with identical elements on the bacterial chromosome, which leads to integration and the formation of different Hfr strains (Section 9.9). Inset: Atomic force microscopy visualization of supercoiled plasmid DNA. Modified from Witz, G. and Stasiak, A. 2010. *Nucleic Acids Res. 38*: 2119.

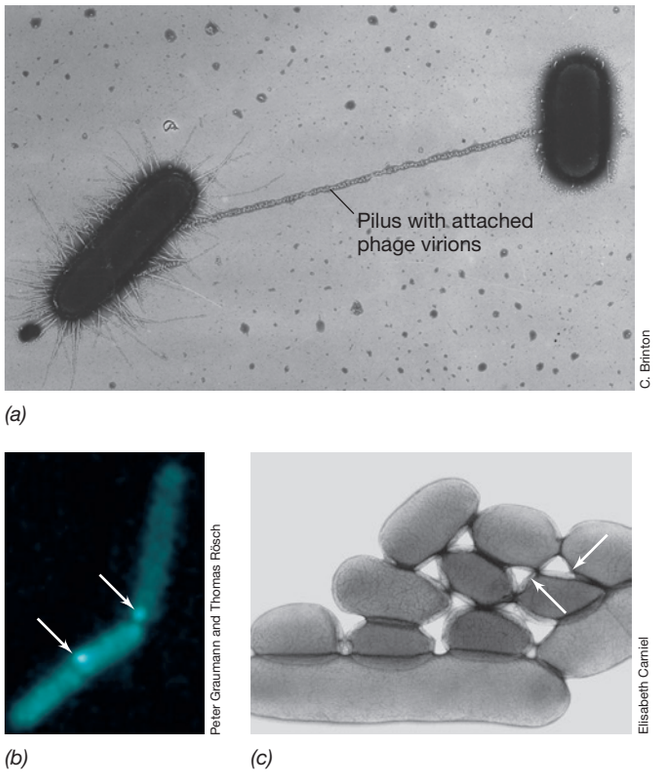


Figure 9.23 Visualization of conjugation. *(a)* Formation of a mating pair. Direct contact between two conjugating *Escherichia coli* cells is first made via a pilus. The cells are then drawn together to form a mating pair by retraction of the pilus, which is achieved by depolymerization. Certain small phages (F-specific bacteriophages) use the conjugative pilus as a receptor and can be seen here attached to the pilus. *(b)* Coupling proteins near the cell membrane (white arrows). These *Bacillus subtilis* cells contain the conjugative plasmid pLS20 encoding the VirD coupling protein linked to a fluorescent reporter gene. *(c)* Conjugation junctions. Negatively stained transmission electron micrograph of conjugation bridges between cells of *Yersinia pseudotuberculosis*. Arrows indicate connection sites. Adapted from Lesic, B., M. Zouine, M. Ducos-Galand, C. Huon, M-L. Rosso, M-C. Prévost, D. Mazel, and E. Carniel. 2012 *PLoS Genet. 8*(3): e1002529.

together (**Figure 9.23*a***). Following this process, donor and recipient cells remain in contact by binding coupling proteins located in the outer membrane of each cell (Figure 9.23*b*). DNA is then transferred from donor to recipient cell through this conjugation junction (Figure 9.23*c*).

Mechanism of DNA Transfer during Conjugation

DNA synthesis is necessary for DNA transfer by conjugation—the donor cell does not lose the genes it transfers but instead makes a copy as the DNA is transferred. In this case, DNA is synthesized not by normal bidirectional replication (◀ Section 6.4) but by **rolling circle replication**, a mechanism also used by some DNA viruses (▶ Section 11.3) and shown in **Figure 9.24**. DNA transfer is triggered by cell-to-cell contact, at which time one strand of the circular plasmid DNA is nicked and transferred to the recipient. The nicking enzyme required to initiate the process, TraI, is encoded by the *tra* operon of the F plasmid. TraI also has helicase activity and thus unwinds the DNA strand to be transferred. As the transfer occurs, DNA synthesis by the rolling circle mechanism replaces the transferred strand in the donor, while a complementary DNA strand is being made in the recipient. Therefore, at the end of the process, both donor and recipient possess complete plasmids. For transfer of the F plasmid, if an F-containing donor cell, which is designated F^+, mates with a recipient cell lacking the plasmid (F^-), the result is two F^+ cells (Figure 9.24).

Transfer of plasmid DNA is efficient and rapid; under favorable conditions virtually every recipient cell that pairs with a donor acquires a plasmid. Transfer of the F plasmid (approximately 100 kbp) takes about 5 min. If plasmid genes can be expressed in the recipient, the recipient itself becomes a donor and can transfer the plasmid to other recipients. In this fashion, conjugative plasmids can spread rapidly among bacterial populations, behaving much like infectious agents. This is of major ecological significance because conjugative plasmids have been found in many *Bacteria* and some *Archaea* (Section 9.10), and a few plasmid-containing cells introduced into a population of potential recipients can convert the entire population into plasmid-bearing (and thus donating) cells in a short time.

Check Your Understanding

- In conjugation, how are donor and recipient cells brought into contact with each other?
- Explain how rolling circle DNA replication allows both donor and recipient to end up with a complete copy of a plasmid transferred by conjugation.

9.9 The Formation of Hfr Strains and Chromosome Mobilization

In addition to plasmid genes, chromosomal genes can be transferred by plasmid-mediated conjugation. As mentioned above, the F plasmid of *Escherichia coli* can, under certain circumstances, *mobilize the chromosome* for transfer during cell-to-cell contact. The F plasmid is actually an *episome*, a plasmid that can integrate into the host chromosome. When the F plasmid is integrated, chromosomal genes can be transferred along with the plasmid. Following genetic recombination between donor and recipient DNA, horizontal transfer of chromosomal genes by this mechanism can be extensive.

Cells possessing a *nonintegrated* F plasmid are called F^+, whereas those with an F plasmid integrated into the chromosome are called **Hfr cells** (for *h*igh *f*requency of *r*ecombination). This term refers to the high rates of genetic recombination between genes on the donor (Hfr) and recipient (F^-) chromosomes. Both F^+ and Hfr cells are donors, but unlike conjugation between an F^+ and an F^-, conjugation between an Hfr donor and an F^- leads to transfer of genes from the host chromosome. This is because the chromosome and plasmid now form a single molecule of DNA. Consequently, when rolling circle replication is initiated by the F plasmid, replication continues on into the chromosome. Thus, the chromosome is also replicated and parts of it get transferred. Hence, integration of a conjugative plasmid provides the mechanism for mobilizing a cell's genome.

Mastering Microbiology
Activities: Microbiology Animation: Conjugation: Hfr Conjugation

Overall, the presence of the F plasmid results in three distinct changes in a cell: (1) the ability to synthesize the F pilus (Figure 9.23*a*), (2) the mobilization of DNA for transfer to another cell, and (3) the

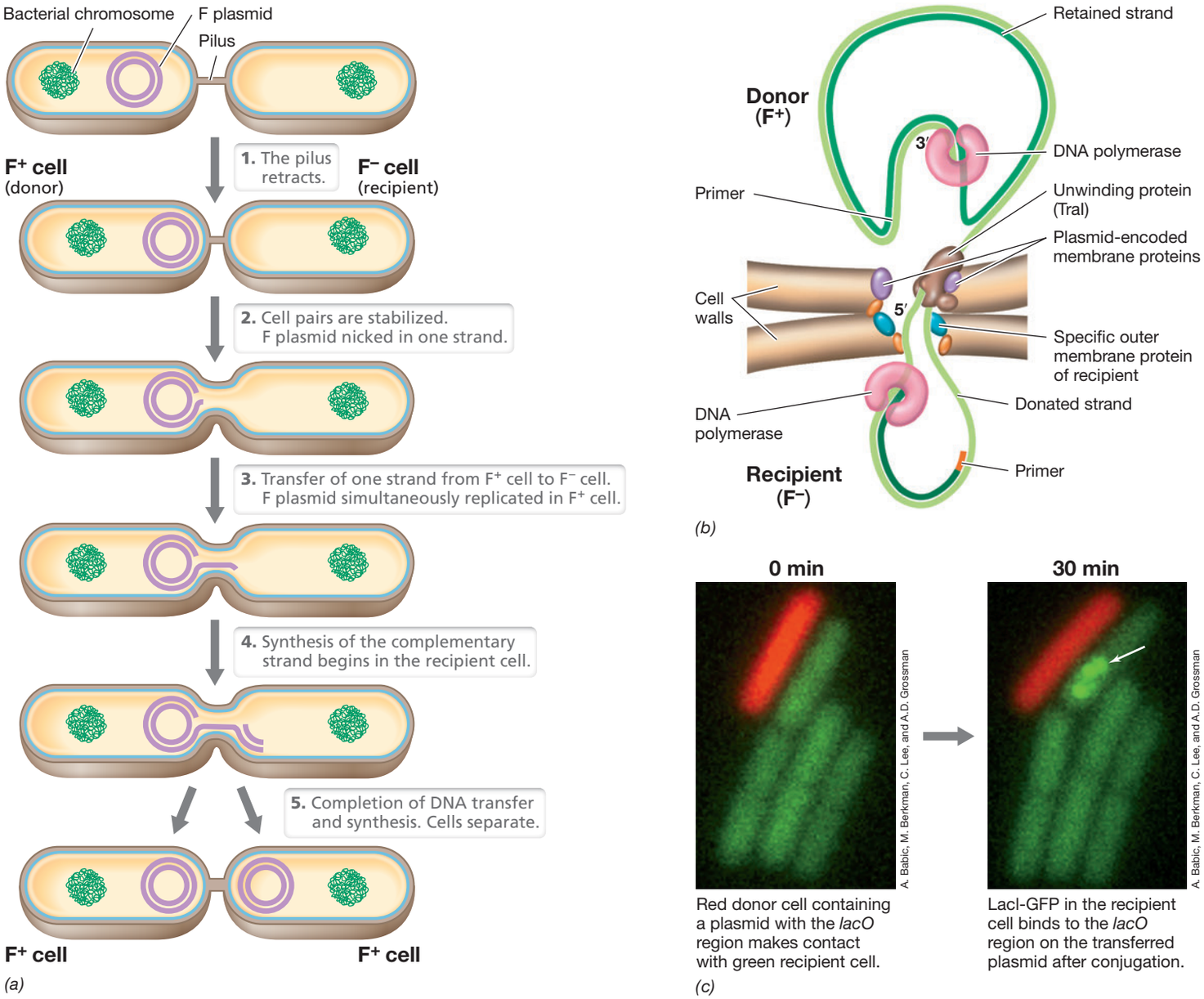


Figure 9.24 **Transfer of plasmid DNA by conjugation.** *(a)* Transfer of the F plasmid converts an F^- recipient cell into an F^+ cell. Note the mechanism of rolling circle replication in steps 4 and 5. *(b)* Details of the replication and transfer process. Note the large number of proteins needed for successful DNA transfer. *(c)* Visualization of DNA transfer by conjugation in *Bacillus subtilis* using fluorescence microscopy. The donor cell constitutively expresses a red fluorescent protein, while the recipient cells fluoresce green due to green fluorescent protein (GFP) fused to LacI. The DNA transferred from the donor contains a *lacO* operator region that binds LacI-GFP. Arrow indicates focal point in the recipient cell where LacI-GFP is bound to the *lacO* region obtained from conjugation.The *lac* operon is discussed in Sections 7.3 and 7.8.

alteration of surface receptors so the cell can no longer be a recipient in conjugation and is therefore unable to take up a second copy of the F plasmid or any genetically related plasmids.

Integration of F Plasmid and Chromosome Mobilization

The F plasmid and the chromosome of *E. coli* both carry several copies of mobile genetic elements called *insertion sequences* (ISs; Section 9.11). These provide regions of sequence homology between chromosomal and F plasmid DNA. Consequently, homologous recombination between an IS on the F plasmid and a corresponding IS on the chromosome results in integration of the F plasmid into the host chromosome (**Figure 9.25**). Once integrated, the plasmid no longer replicates independently, but the *tra* operon still functions normally and the Hfr strain synthesizes pili (Figure 9.23). When a recipient cell is encountered, conjugation is triggered just as in an F^+ cell, and DNA transfer is initiated at the *oriT* (origin of transfer) site. However, because the plasmid is now part of the chromosome, after part of the plasmid DNA is

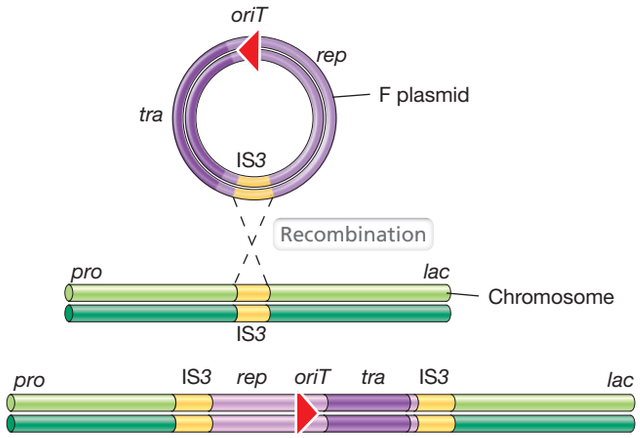


Figure 9.25 The formation of an Hfr strain. Integration of the F plasmid into the chromosome may occur at a variety of specific sites where IS elements are located. The example shown here is an IS*3* located between the chromosomal genes *pro* and *lac*. Some of the genes on the F plasmid are shown. The arrow indicates the origin of transfer, *oriT*, with the arrow as the leading end. Thus, in this Hfr, *pro* would be the first chromosomal gene to be transferred and *lac* would be among the last.

transferred, chromosomal genes begin to be transferred (**Figure 9.26**). As in the case of conjugation with just the F plasmid itself (Figure 9.24), chromosomal transfer also requires DNA replication.

Because the DNA strand usually breaks during transfer, only part of the donor chromosome is typically transferred. Consequently, the recipient does not become Hfr (or F^+) because only part of the integrated F plasmid is transferred (**Figure 9.27**). However, after transfer, the Hfr strain remains genetically Hfr because it retains a copy of the integrated F plasmid. Following recombination, the recipient cell may express a new phenotype due to the incorporation of donor genes, but genetically it remains an F^- cell.

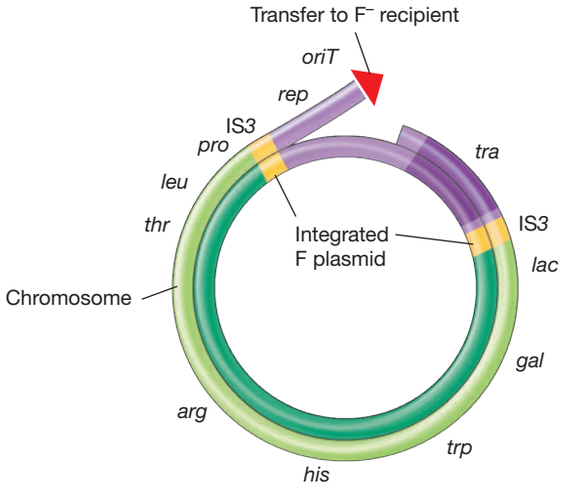


Figure 9.26 Transfer of chromosomal genes by an Hfr strain. The Hfr chromosome breaks at the origin of transfer within the integrated F plasmid. The transfer of DNA to the recipient begins at this point. DNA replicates during transfer as for a free F plasmid (Figure 9.24). This figure is not to scale; the inserted F plasmid is actually less than 3% of the size of the *Escherichia coli* chromosome.

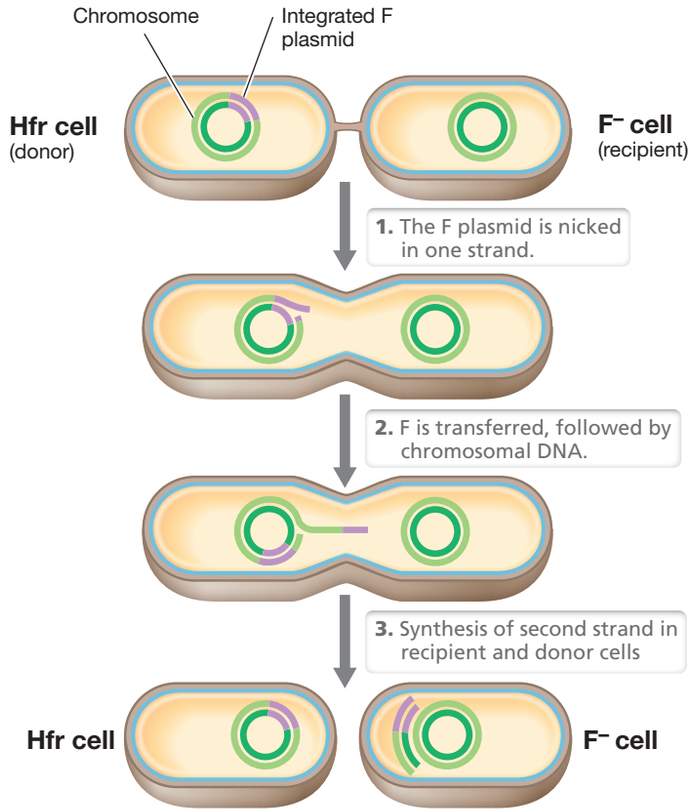


Figure 9.27 Transfer of chromosomal DNA by conjugation. Transfer of the integrated F plasmid from an Hfr strain results in the cotransfer of chromosomal DNA because it is linked to the plasmid. The steps in transfer are similar to those shown in Figure 9.24*a*. However, the recipient remains F^- and receives a linear fragment of donor chromosome attached to part of the F plasmid. For donor DNA to survive, it must be recombined into the recipient chromosome after transfer (not shown).

Because a partial chromosome cannot replicate, for incoming donor DNA to survive, it must recombine with the recipient chromosome. As in transformation and transduction, genetic recombination between donor and recipient genes requires homologous recombination (Figure 9.12) in the recipient cell.

Because several distinct insertion sequences are present on the *E. coli* chromosome, a number of different Hfr strains are possible. A given Hfr strain always donates genes in the same order, beginning at the same position. However, Hfr strains that differ in the chromosomal integration site of the F plasmid transfer their genes in different orders (**Figure 9.28**). At some insertion sites, the F plasmid is integrated with its origin pointing in one direction, whereas at other sites the origin points in the opposite direction. The orientation of the F plasmid determines which chromosomal genes enter the recipient cell first, illustrating how virtually any gene on the chromosome can be mobilized by one Hfr configuration or another (Figure 9.28).

Transfer of Chromosomal Genes to the F Plasmid

Occasionally, integrated F plasmids may be excised from the chromosome. During excision, chromosomal genes may sometimes be

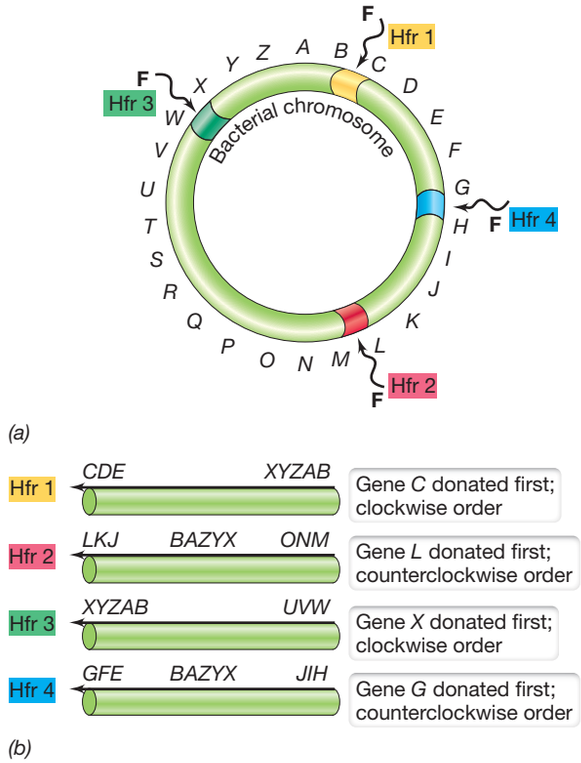


Figure 9.28 Formation of different Hfr strains. Different Hfr strains donate genes from different origins and thus genes are transferred in different orders. *(a)* F plasmids can be inserted into various insertion sequences on the bacterial chromosome, forming different Hfr strains. *(b)* Order of gene transfer for different Hfr strains. See Figures 9.25–9.27 for details of Hfr formation and DNA transfer.

incorporated into the liberated F plasmid. This can happen because both the F plasmid and the chromosome contain multiple identical insertion sequences where recombination can occur (Figure 9.25). F plasmids containing chromosomal genes are called *F′* (F-prime) *plasmids*. When F′ plasmids promote conjugation, they transfer the chromosomal genes they carry at high frequency to the recipients. F′-mediated transfer resembles specialized transduction (Section 9.7) in that only a restricted group of chromosomal genes is transferred by any given F′ plasmid. Transferring a known F′ into a recipient allows one to establish diploids (two copies of each gene) for a limited region of the chromosome. Such partial diploids (merodiploids) are useful for genetic complementation experiments (Section 9.5).

We segue now to the final part of this chapter in which we explore archaeal genetics, movable genetic elements, and a major mechanism prokaryotic cells have evolved to protect their genomic integrity, a mechanism that has been exploited to revolutionize biology.

Check Your Understanding

- In conjugation involving the F plasmid of *Escherichia coli*, how is the host chromosome mobilized?
- Why does an Hfr × F^- mating not yield two Hfr cells?
- At which sites in the chromosome can the F plasmid integrate?

III • Gene Transfer in *Archaea* and Other Genetic Events

Although phylogenetically distinct from *Bacteria*, *Archaea* have evolved similar mechanisms for horizontal gene transfer. Some genetic elements can move about the chromosome, and a host of surveillance systems are present in prokaryotic cells that function to "search and destroy" foreign DNA.

Although studies of the genetics of *Archaea* lag behind genetics research in *Bacteria*, they are showing progress, along with the development of archaeal versions of the tools necessary for detailed genetic analyses. In addition, some other genetic events in *Bacteria* reveal important genetic concepts even though they do not involve horizontal gene flow per se. These events can trigger genomic duplications and rearrangements, which increase genetic diversity (Chapter 13).

9.10 Horizontal Gene Transfer in *Archaea*

Although *Archaea* contain a single circular chromosome (Figure 9.29), as do most *Bacteria* (◀ Section 6.2), and genome analysis clearly shows that horizontal transfer of archaeal DNA occurs in nature, experimental gene transfer systems are not as well developed for *Archaea* as they are for *Bacteria*. Some reasons for this are of a practical nature including the fact that most well-studied *Archaea* are extremophiles, capable of growth only under conditions of high salt or high temperature (Chapter 17). The temperatures necessary to culture some hyperthermophiles, for example, will melt agar, and alternative materials are required to form solid media and obtain colonies.

Another problem is that most known antibiotics do not affect *Archaea*. For example, penicillins do not affect *Archaea* because their cell walls lack peptidoglycan. The choice of selectable markers for use in genetic crosses is therefore often limited. However, novobiocin (a DNA gyrase inhibitor) and mevinolin (an inhibitor of isoprenoid biosynthesis) have been used to inhibit growth of extreme halophiles, and puromycin and neomycin (both protein

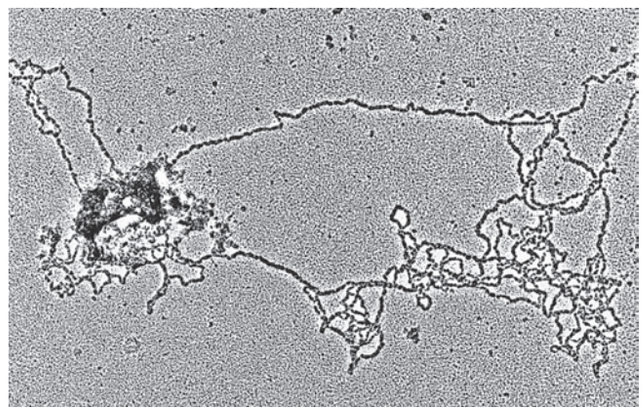


Figure 9.29 An archaeal chromosome, as shown by transmission electron microscopy. The circular chromosome is from the hyperthermophile *Sulfolobus*, a member of the *Archaea*.

synthesis inhibitors) inhibit methanogens. Auxotrophic strains (Section 9.1) of a few *Archaea* have also been isolated for genetic selection purposes.

Examples of Archaeal Genetics

No single species of *Archaea* has become a model organism for archaeal genetics, although more genetic work has been done on select species of extreme halophiles (*Halobacterium*, *Haloferax*, ▶ Section 17.1) than on any other *Archaea*. Various mechanisms of gene transfer have been discovered in select species of *Archaea*, including transformation, transduction, and conjugation. In addition, several plasmids are known and some have been used to construct cloning vectors, allowing genetic analysis through gene cloning and sequencing rather than through traditional genetic means, such as genetic crosses (Section 9.5). Transposon mutagenesis (Section 9.11) has been well developed in certain methanogens, including species of *Methanococcus* and *Methanosarcina*, and other tools such as shuttle vectors and other in vitro methods of genetic analysis (Chapter 12) have been developed for study of the unique biochemistry of the methanogens (▶ Sections 14.15 and 17.2).

Archaeal Competence and Viral Transfer

Transformation occurs in the methanogen *Methanococcus voltae* and the hyperthermophiles *Thermococcus kodakarensis* and *Pyrococcus furiosus*, all of which are naturally competent (Section 9.6). Intraspecies exchange of plasmids occurs in some species of *Thermococcus* and *Halorubrum* through the budding of the cell envelope, a process that results in DNA-containing membrane vesicles as vehicles of genetic exchange. Formation of membrane vesicles occurs in species of all three domains of life, and these structures can carry both protein and nucleic acid. While DNA transfer by membrane vesicles has also been observed in *Bacteria*, the mechanism observed in transferring the pR1SE plasmid by the extreme halophile *Halorubrum lacusprofundi* blurs the line between transformation and transduction. While pR1SE does not encode genes of viral origin, the plasmid does contain genes encoding membrane proteins that are inserted into the host's membrane; these are then used to form regularly shaped membrane vesicles that function to transfer the plasmid between cells (**Figure 9.30**). Thus, pR1SE mimics a virus because the genetic element encodes a portion of a structurally defined particle that transfers the plasmid between *H. lacusprofundi* strains.

Other conditions for transformation work reasonably well in several *Archaea* although the details vary from organism to organism. One approach requires removal of divalent metal ions, which in turn results in the partial disassembly of the glycoprotein cell wall layer that surrounds many archaeal cells (the S-layer, ◀ Section 2.5), and this allows access to transforming DNA. However, *Archaea* with rigid cell walls have proven difficult to transform, although electroporation (Section 9.6) sometimes works. One exception is in *Methanosarcina* species, organisms with a thick polysaccharide cell wall (▶ Section 17.2). In this organism, a high-efficiency transformation system has been developed that employs DNA-loaded lipid preparations (liposomes) that are able to traverse the cell wall and deliver DNA into the cell.

Although viruses that infect *Archaea* are plentiful, transduction is rare. Only one archaeal virus, which infects the thermophilic methanogen *Methanothermobacter thermautotrophicus*, has been shown to transduce host genes. Unfortunately, the low burst size (about six phage virions liberated per cell) makes it impractical to use this system for gene transfer. Gene transfer agents (Section 9.7) have been found in one species of methanogen but do not appear to be widespread in *Archaea*.

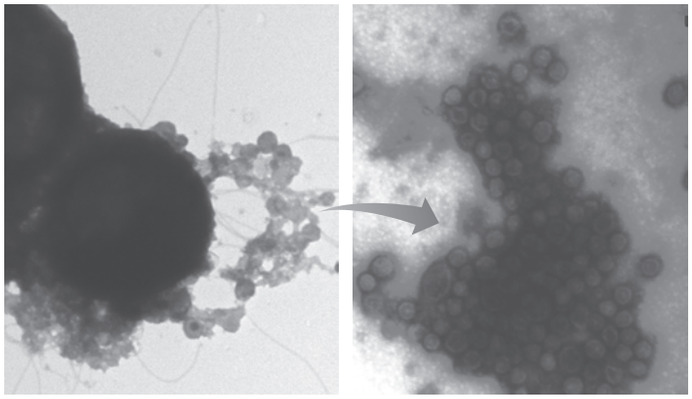


Figure 9.30 Membrane vesicles and plasmid transfer in *Halorubrum lacusprofundi* R1S1. *(a)* Transmission electron micrograph showing an isolated cell with membrane vesicles budding from the cell surface. Filaments around the cell are cell appendages and archaella. *(b)* Membrane vesicles containing plasmid DNA isolated from the supernatant of R1S1 cells. Each vesicle is 80–110 nm in diameter. Images modified from Erdmann, S., Tschitschko, B., Zhong, L., Raftery, M.J., and Cavicchioli, R. 2017. *Nat. Microbiol. 2*: 1446.

Transfer by Cell-to-Cell Contact

Different types of conjugation have been detected in *Archaea*. Some strains of the thermophilic and acidophilic *Sulfolobus solfataricus* (▶ Section 17.10) contain plasmids that promote conjugation between two cells in a manner similar to that seen in *Bacteria*. In this process, cell pairing is independent of pili formation, but as for *Bacteria*, DNA transfer is unidirectional. However, most of the genes encoding conjugation in *S. solfataricus* have little similarity to those in gram-negative *Bacteria*. An exception is a gene similar to the gene *traG* located on the *Escherichia coli* F plasmid (Sections 9.8 and 9.9); the protein product of the archaeal version of this gene participates in stabilizing mating pairs. Thus it is likely that the overall mechanism of conjugation in *Archaea* differs in significant ways from that in *Bacteria*.

Most species of *Sulfolobus* can also exchange DNA without the participation of a fertility plasmid. Such exchange is dependent on cell aggregation, a process that requires pili whose synthesis is triggered by UV radiation. While the exact mechanism of this unusual DNA exchange is unknown, these pili bring cells of the same species together through recognition of S-layer glycosylation patterns present on the cell walls.

Other *Archaea* form specialized structures between cells that allow for genetic exchange, and the formation of these structures is also independent of a fertility plasmid. For example, **Figure 9.31** illustrates the formation of DNA-transferring nanotubes between cells of a *Thermococcus* species. Interestingly, unlike the other means of horizontal gene transfer discussed in this chapter, the *Thermococcus*

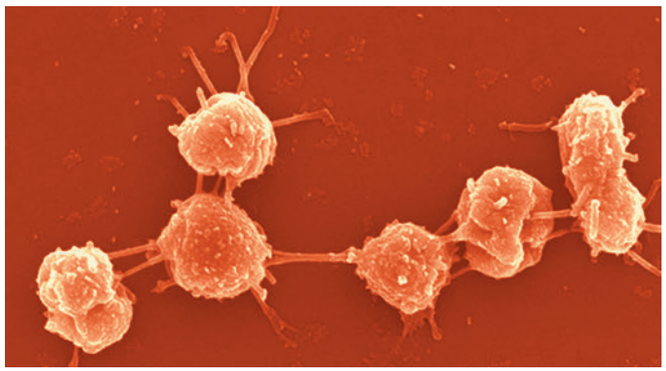


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Figure 9.31 Nanotubes and *Thermococcus*. Scanning electron micrograph showing nanotubes linking cells of a species of *Thermococcus*. A single *Thermococcus* cell is about 1 μm in diameter.

nanotubes allow for *bidirectional* transfer of DNA. Thus, with both cells in an exchange able to function as recipients, the *Thermococcus* nanotubes likely facilitate very dynamic gene flow. Similarly, some halobacteria also form cytoplasmic bridges between mating cells that are used for DNA transfer. Although the nanotube and cytoplasmic bridge systems are not yet routine laboratory tools, they may well prove useful for the future development of more facile archaeal genetic transfer systems.

Check Your Understanding

- Why is it usually more difficult to select recombinants with *Archaea* than with *Bacteria*?
- Why do penicillins not kill species of *Archaea*?

9.11 Mobile DNA: Transposable Elements

As we have seen, molecules of DNA may move from one cell to another, but to a geneticist, the phrase "mobile DNA" has a special meaning. Mobile DNA refers to discrete segments of DNA that move as units from one location to another *within* other DNA molecules.

Although the DNA of certain viruses can be inserted into and excised from the genome of the host cell (◀ Section 5.6 and ▶ Section 11.4), most mobile DNA consists of **transposable elements**. These are stretches of DNA that can move from one site to another. However, transposable elements are always found inserted into another DNA molecule such as a plasmid, a chromosome, or a viral genome. Transposable elements do not possess their own origin of replication. Instead, they are replicated when the host DNA molecule into which they are inserted is replicated.

Mastering Microbiology Activities: Microbiology Animation: Transposons: Insertion Sequences

Transposable elements move by *transposition*, a process that is important in both genome rearrangement and genetic analysis. Transposable elements are extremely abundant and widespread in nature and are present in the genomes of all three domains of life and in many viruses and plasmids, suggesting that the elements offer a selective advantage by accelerating genome rearrangement and gene duplication (▶ Section 13.8).

The two major types of transposable elements in *Bacteria* are *insertion sequences* (ISs) and *transposons*. Both elements have two important features in common: They carry genes encoding *transposase*, the enzyme necessary for transposition, and they have short inverted terminal repeats at their ends that are also needed for transposition (the ends of transposable elements are not free but are continuous with the host DNA molecule into which the transposable element has inserted). **Figure 9.32** shows genetic maps of two well-studied transposable elements: the insertion element IS*2* and the transposon Tn*5*.

Insertion Sequences and Transposons

Insertion sequences (ISs) are the simplest type of transposable element. They are short DNA segments, about 1000 nucleotides long, and typically contain inverted repeats of 10–50 base pairs. Each different IS has a specific number of base pairs in its terminal repeats, and the only protein encoded is the transposase. Several hundred distinct IS elements have been characterized. IS elements are found in the chromosomes and plasmids of both *Bacteria* and *Archaea*, as well as in certain bacteriophages. Individual strains of the same bacterial species vary in the number and location of the IS elements they harbor. For instance, the genome of one strain of *Escherichia coli* has five copies of IS*2* and five copies of IS*3*. Many plasmids, such as the F plasmid, also carry IS elements. Indeed, integration of the F plasmid into the *E. coli* chromosome is facilitated by recombination between identical IS elements on the F plasmid and the chromosome (Section 9.9 and Figure 9.25).

Transposons are larger than IS elements but have the same two essential components: inverted repeats at both ends and a gene encoding the transposase (Figure 9.32*b*). The transposase recognizes the inverted repeats and moves the segment of DNA flanked by them from one site to another. Consequently, any DNA that lies between the two inverted repeats is moved and is, in effect, part of the transposon. Genes included inside transposons vary widely. Some of these genes, such as antibiotic resistance genes, confer important new properties on the organism. Because antibiotic resistance is both important and easy to detect, most well-studied transposons

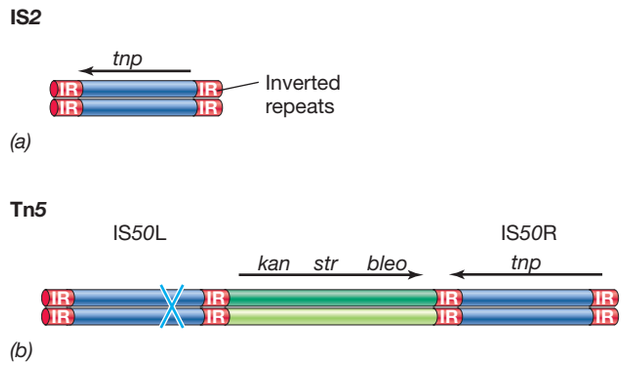

Figure 9.32 Maps of the transposable elements IS*2* and Tn*5*. The arrows above the maps show the direction of transcription of any genes on the elements. The gene encoding the transposase is *tnp*. *(a)* IS*2* is an insertion sequence of 1327 bp with inverted repeats of 41 bp at its ends. *(b)* Tn*5* is a composite transposon of 5.7 kbp containing the insertion sequences IS*50*L and IS*50*R at its left and right ends, respectively. IS*50*L is not capable of independent transposition because there is a nonsense mutation, marked by a blue cross, in its transposase gene. The genes *kan, str,* and *bleo* confer resistance to the antibiotics kanamycin (and neomycin), streptomycin, and bleomycin.

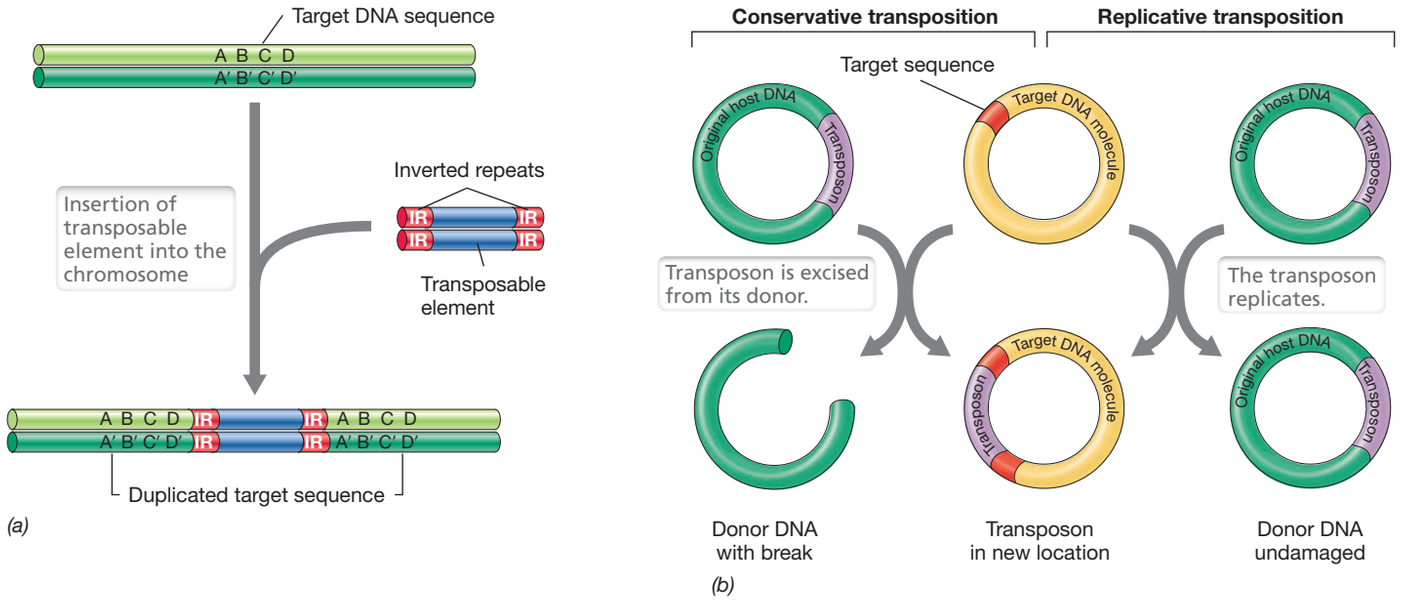

Figure 9.33 Transposition. *(a)* Insertion of a transposable element generates a duplication of the target sequence. Note the presence of inverted repeats (IR) at the ends of the transposable element. *(b)* Two mechanisms of transposition. Donor DNA (carrying the transposon) is shown in green, and recipient DNA carrying the target sequence is shown in yellow. In both conservative and replicative transposition, the transposase inserts the transposon (purple) into the target site (red) on the recipient DNA. During this process, the target site is duplicated. In conservative transposition, the donor DNA is left with a double-stranded break at the previous location of the transposon. In contrast, after replicative transposition, both donor and recipient DNA possess a copy of the transposon.

contain antibiotic resistance genes as selectable markers. Examples include transposon Tn*5*, which encodes resistance to the antibiotics kanamycin (and neomycin), streptomycin, and bleomycin (Figure 9.32*b*), and Tn*10*, which encodes tetracycline resistance.

Because any genes lying between the inverted repeats become part of a transposon, it is possible to get hybrid transposons that display complex behavior. For example, conjugative transposons contain *tra* genes (Figures 9.25 and 9.26) and can therefore move between bacterial species by conjugation as well as transpose from place to place within a bacterial genome.

Mechanisms of Transposition

Both the inverted repeats (located at the ends of transposable elements) and transposase are essential for transposition. The transposase recognizes, cuts, and ligates the DNA during transposition. When a transposable element is inserted into target DNA, a short sequence in the target DNA at the site of integration is duplicated during the insertion process (**Figure 9.33*a***). The duplication arises because single-stranded DNA breaks are made by the transposase. The transposable element is then attached to the single-stranded ends that have been generated. Finally, enzymes of the host cell repair the single-strand portions, which results in the duplication.

Two mechanisms of transposition are known: conservative and replicative (Figure 9.33*b*). In *conservative* transposition, as occurs with the transposon Tn*5* (Figure 9.32*b*), the transposon is excised from one location and reinserted at a second location. The copy number of a conservative transposon therefore remains at one. By contrast, during *replicative* transposition, a new copy of the transposon is produced and inserted at the second location. Thus, after a replicative transposition event, one copy of the transposon remains at the original site, while a second copy is incorporated at the new site.

Utility of Transposon Mutagenesis

When a transposon inserts itself within a gene, the DNA sequence in the gene is altered and a mutation occurs (**Figure 9.34**). Mutations due to transposon insertion do occur naturally. However, laboratory use of transposons has been a powerful genetic tool to create a library of bacterial mutants. To do this, transposons carrying antibiotic resistance genes are used. With conjugation or transformation

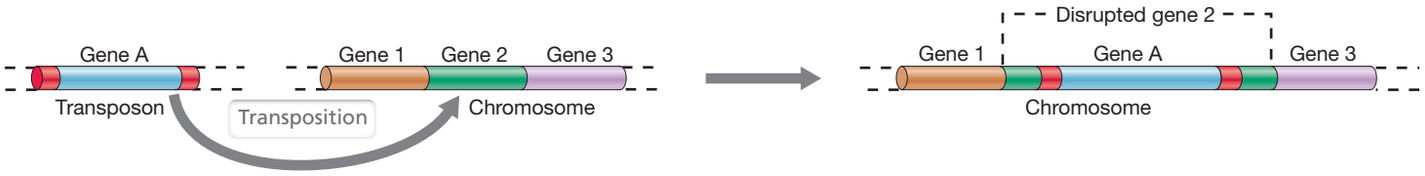

Figure 9.34 Transposon mutagenesis. The transposon moves into the middle of gene 2. Gene 2 is now disrupted by the transposon and is inactivated. Gene A from the transposon is now expressed from the chromosome.

as the transfer mechanism, the transposon is introduced into the target cell on a plasmid that cannot replicate in that particular host. Consequently, antibiotic-resistant colonies will mostly be due to insertion of the transposon into the bacterial genome.

Because bacterial genomes contain relatively little noncoding DNA, most transposon insertions will occur in genes that encode proteins. This technique can be used to determine the function of a novel gene if a screening method is available. For example, if a transposon inserts into a gene encoding a product required for biofilm formation (biofilms are colonies of microbes encased in an adhesive and attached to a surface, ◀ Section 4.9), the transposon mutant will no longer grow in the biofilm mode. Then, further genetic analyses can be performed to reveal which gene the transposon has disrupted. **Figure 9.35** illustrates the use of transposon mutagenesis to study flagella structure and function in a notorious biofilm-producing bacterium, *Pseudomonas aeruginosa* (◀ Section 8.10). In this research, transposon mutagenesis combined with a special staining technique was used to identify the protein FlgK of the flagellar hook (◀ Section 2.9) as a protein required for biofilm formation.

Two transposons widely used for mutagenesis of *E. coli* and related bacteria are Tn*5* (Figure 9.32*b*) and Tn*10*. Many *Bacteria*, a few *Archaea*, and the yeast *Saccharomyces cerevisiae* have all been mutagenized using genetically engineered transposons. In Chapter 10 we will learn about how engineered transposons can be used in metagenomic studies to determine the role of individual genes in a microbe's overall fitness. More recently, transposons have even been used to isolate mutations in animals, including mice.

We finish this chapter with a consideration of genomic stability (in contrast to the genomic alterations we have discussed thus far) and preview the application of this process to biotechnology and synthetic biology.

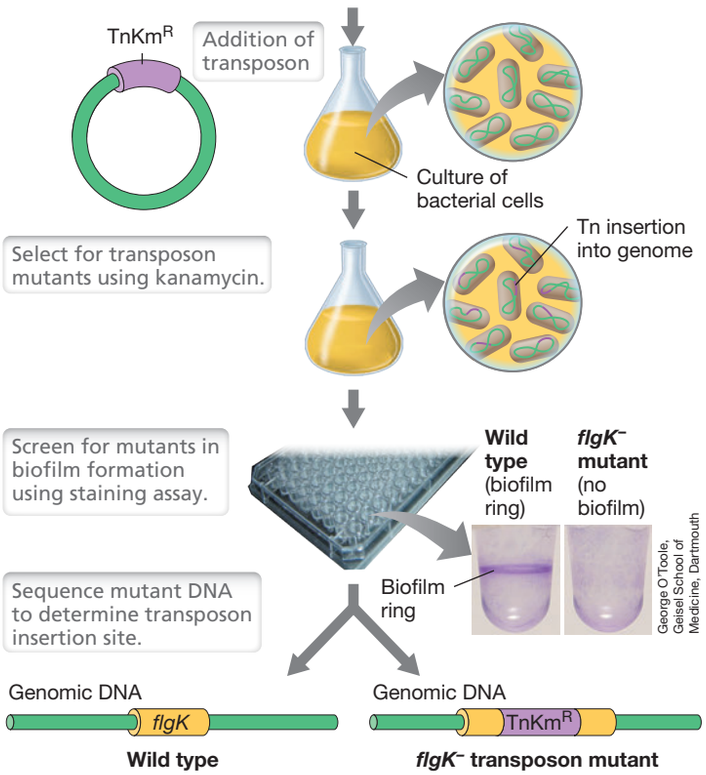

Figure 9.35 Utility of transposon mutagenesis. A transposon (Tn) conferring kanamycin resistance (Km^R) is added to a culture of wild-type *Pseudomonas aeruginosa*. After selection for integration of the transposon using the antibiotic kanamycin, the mutants are screened for biofilm formation in a microtiter plate. Cultures that produce biofilm adhere to the microtiter plate (ring in left tube of inset photo) and the resulting biofilm can be stained with crystal violet. Primers specific to the Tn can be used to determine the Tn insertion site and the identity of the interrupted gene. *flgK* encodes a protein in the flagellar hook.

Check Your Understanding

- Which features do insertion sequences and transposons have in common?
- What is the significance of the terminal inverted repeats of transposons?
- How can transposons be used in bacterial genetics?

9.12 Preserving Genomic Integrity and CRISPR

While DNA transfer is key to the diversity and evolution of *Bacteria* and *Archaea*, cells also have mechanisms to prevent horizontal gene transfer and viral infection from occurring. With viruses outnumbering prokaryotic cells in nature by a factor of 10, barriers against gene transfer are key to preserving genome integrity and ultimately species maintenance. **Figure 9.36** provides an overview of gene transfer barriers that work in concert in both *Bacteria* and *Archaea*. While our focus here is on mechanisms to prevent DNA transfer from viruses, such as modification of viral host cell receptors (◀ Section 5.5 and Figure 9.36), these barriers also target incoming naked and plasmid DNA.

Because barriers to horizontal gene transfer must distinguish invading DNA from self DNA, and protect the latter, they are considered bacterial and archaeal *immune systems*. Thus, processes that are *nonspecific* are considered analogous to *innate immunity* (Chapter 26), whereas mechanisms that target *specific* incoming nucleic acid are considered a form of *adaptive immunity* (Chapter 27). Despite engaging these forms of bacterial and archaeal immunity, the relationship between viruses and their bacterial and archaeal hosts is not static but instead extremely dynamic. That is, although microbial cells possess several weapons to battle viral attack, viruses counter these immune systems with weapons of their own, triggering a microbial "arms race" for cell survival and viral propagation.

Bacterial and Archaeal Innate Immunity

In our discussion of viruses in Section 5.5, we briefly considered restriction endonucleases—proteins produced by both *Bacteria* and *Archaea* that target and destroy foreign DNA—and how these enzymes protect the cell from viral attack. Restriction enzymes also target foreign DNA entering the cell as a result of transformation and conjugation events (Figure 9.36). The cell's DNA is protected from its own restriction endonucleases because the enzymes' recognition sequences are chemically modified (▶ Section 12.2). As we mentioned in Chapter 5, some viruses avoid restriction endonuclease surveillance by modifying their DNA to mimic the chemical

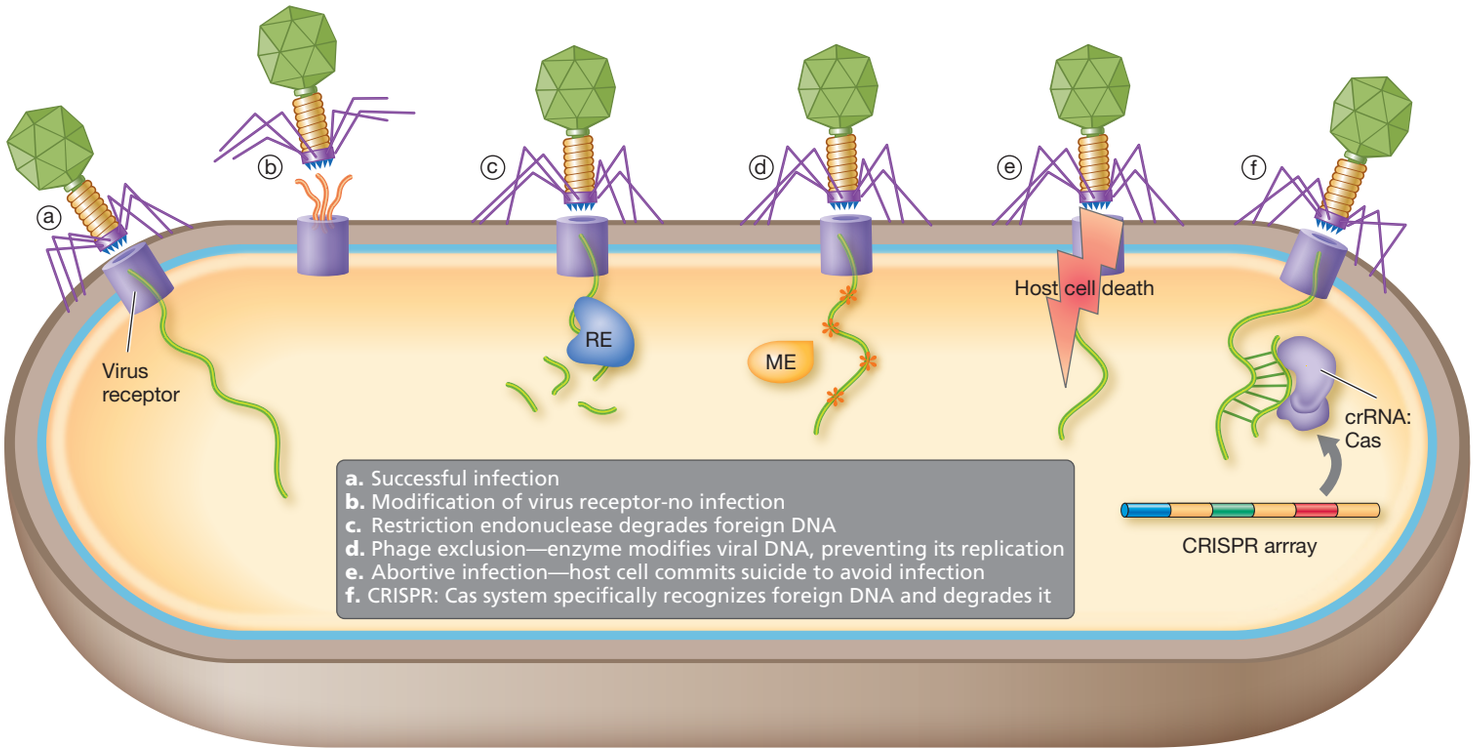

Figure 9.36 Barriers to horizontal gene transfer in *Bacteria* and *Archaea* using phage infection as an example. Some possible outcomes of viral attack: *(a)* Successful viral infection. *(b)* The virus is prevented from recognizing and binding to its host cell; viral DNA cannot enter. *(c)* The foreign DNA is degraded by host cell restriction endonucleases (host cell DNA is not degraded because it is chemically modified at restriction cut sites). *(d)* The foreign DNA is modified by the host cell's replication machinery such that it is not replicated. *(e)* Programmed cell death kills the host and thus prevents successful infection. *(f)* CRISPR activity destroys foreign DNA: crRNAs and Cas proteins detect DNA as previous invading DNA and degrade it through nuclease activity. Abbreviations: RE, restriction endonuclease; ME, modification enzyme; crRNA, CRISPR (*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeat) RNA; Cas (CRISPR-associated) protein.

modification found in the host genome. In response, some bacteria have evolved altered restriction enzyme systems that recognize this viral DNA modification and still degrade the incoming viral DNA, preventing viral infection.

An example of this microbial arms race is that of T4 bacteriophage and its host cell *Escherichia coli*. The adaptation of *E. coli* recognizing modified phage DNA has now selected for T4 bacteriophages that modify their DNA in a different manner—by glycosylating (adding sugars) to specific DNA bases. Then, as one might expect in an expanding arms race, some strains of *E. coli* have evolved restriction systems that recognize glycosylated viral DNA and destroy it. As a countermeasure to this, some T4 strains have evolved a protein that inhibits these modified restriction enzymes. In a constant attempt to stay ahead, other *E. coli* strains have evolved endonucleases that are not inhibited by these bacteriophage proteins, and so it goes, back and forth as both predator and prey fight to survive and propagate in the face of "the enemy."

Recent studies have uncovered yet additional mechanisms for thwarting viral infection, such as the *phage exclusion* and *abortive infection* systems present in both *Bacteria* and *Archaea* (Figure 9.36). Phage exclusion systems are a variant of restriction enzyme systems. Genes of the phage exclusion system encode enzymes that recognize and then modify incoming foreign DNA so that it cannot be replicated; this modification results in replication of only the host cell's DNA and not the modified DNA. In contrast, abortive infection systems trigger the host cell to commit suicide (Figure 9.36). These systems are encoded by toxin–antitoxin systems (◀ Section 8.12) that result in *programmed cell death*. Although the host cell dies during this abortive infection, its death prevents more virions from being produced and released to infect other cells in the population, thereby increasing the survival rate in the host population.

Adaptive Immunity and CRISPR

A major antiviral defense system present in both *Bacteria* and *Archaea* is CRISPR—*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats—which functions to seek out and destroy foreign nucleic acid. While some CRISPR systems can target RNA viruses, our discussion will focus on systems that target foreign DNA. Besides protecting against viral infection, CRISPR also helps the cell maintain the stability and integrity of its genome by destroying certain plasmids and other genes obtained from horizontal transfers.

CRISPR regions are short repeats of *constant* DNA sequence present on the host chromosome that alternate with short *variable* DNA sequences called *spacers* (**Figure 9.37**). Spacers correspond to sequences of viral or other foreign DNA and function as a "memory bank" of past encounters with a virus in a manner reminiscent of how animals produce antibodies and long-lasting memory cells

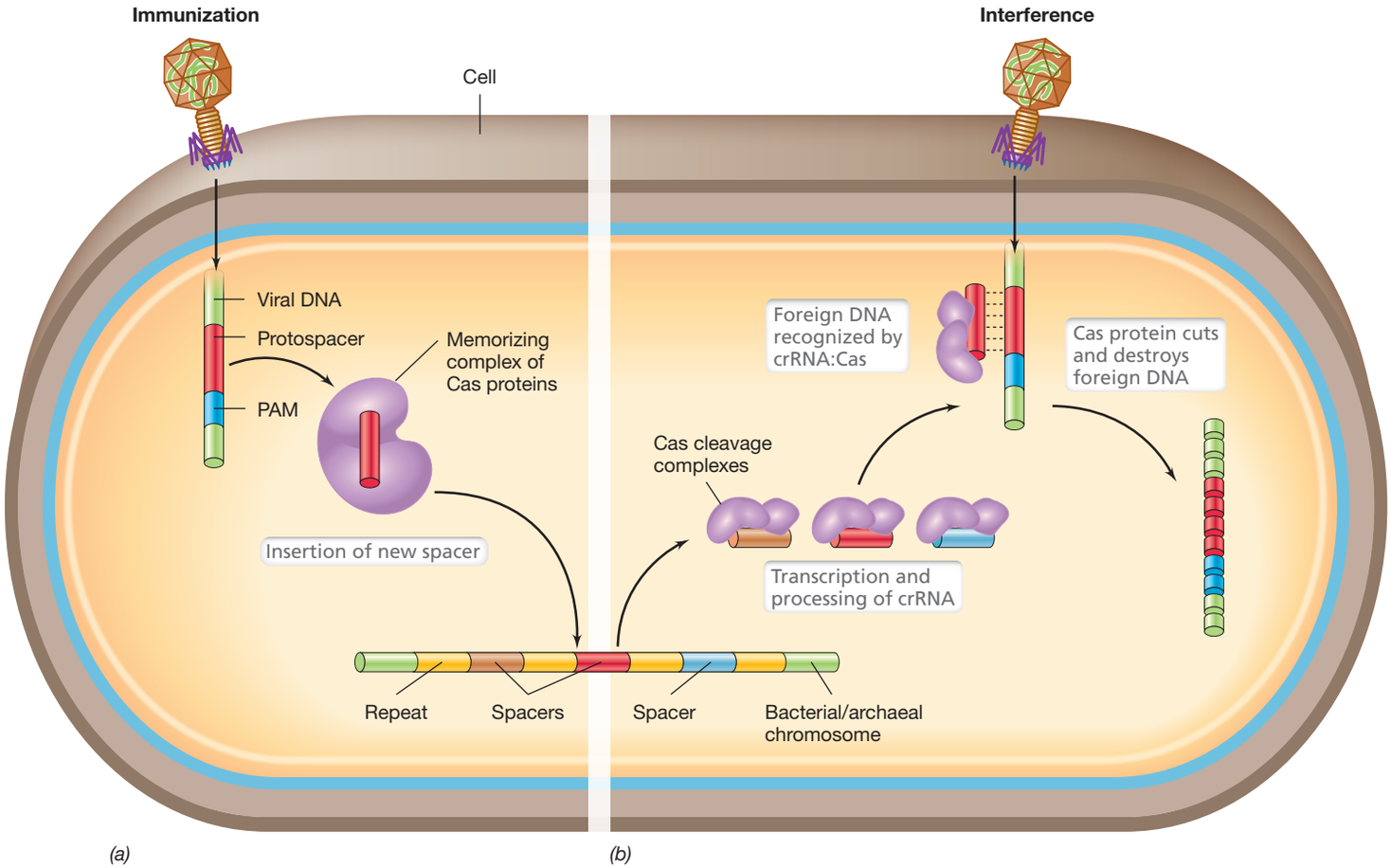

Figure 9.37 CRISPR defense system against viruses and foreign DNA. *(a)* Immunization. Incoming viral DNA is targeted by the memorizing complex of Cas proteins. This complex selects a protospacer region based on protospacer adjacent motif (PAM) sequences located on the viral genome. Once the protospacer is excised from the viral genome, the memorizing complex inserts the protospacer into the CRISPR region of the chromosome, resulting in a unique spacer region. *(b)* Interference. The chromosomal CRISPR region is transcribed and processed by Cas proteins into crRNAs that correspond to the individual spacer regions. Each spacer segment corresponds to previous encounters with incoming foreign nucleic acid. Cas proteins bind to these crRNAs and search for complementary DNA. If a crRNA binds to the DNA of an invading virus, forming a crRNA:DNA duplex, the endonuclease activity of the cleavage complex is triggered and results in the degradation of incoming viral DNA.

against an infecting virus. Because of this specificity, the CRISPR system is analogous to the adaptive immune response of vertebrate animals (Chapter 27).

Besides the spacer regions, another essential component of the CRISPR system is the *Cas* (CRISPR-*a*ssociated) *proteins*. These proteins possess endonuclease activity and both mediate the defense against foreign DNA and incorporate new spacers into the CRISPR region. When a virus attaches to a host cell and injects its DNA, the Cas proteins of a CRISPR region may recognize specific DNA sequences known as *p*rotospacer *a*djacent *m*otifs (PAMs) (Figure 9.37*a*). The Cas protein then cleaves the viral DNA at a locus near this PAM (termed the *protospacer*) and inserts the short DNA region into the CRISPR region of the chromosome, where it becomes a *spacer* (Figure 9.37*a*). The insertion of a spacer into the CRISPR region confers "genetic memory" (referred to as *immunization*) on the cell and sets the stage for later encounters with the same virus.

When an immunized cell encounters the same virus later, the Cas proteins quickly destroy the incoming DNA in an RNA-dependent process. While the genomic CRISPR region does not contain open reading frames, it does have a promoter (◀ Section 6.5). The resulting transcript is considered the *pre-CRISPR RNA* (pre-crRNA) and contains an array of RNA sequences complementary to both the repeat and spacer regions. The Cas proteins then process the transcript into individual spacer RNAs by targeting the repeat regions (Figure 9.37*b*). These crRNAs then associate with Cas proteins within the cleavage complex and begin surveillance for complementary incoming viral DNAs. Any viral DNA:crRNA duplexes formed are cleaved by the endonuclease activity of Cas proteins, and the invading DNA is degraded in a process called *interference* (Figure 9.37*b*). With part of its DNA destroyed in this way, the foreign DNA molecule of an invading virus cannot proceed to replicate and the infection (and threat to the cell) is thwarted.

One of the major conundrums of the CRISPR system is how a host can survive the *initial* viral invasion long enough to become immunized. For the CRISPR system to successfully prevent viral infection, a spacer corresponding to a region of the viral genome must already be present in the CRISPR locus. Immunization probably occurs when an incoming virus has been inactivated by environmental

factors (such as ultraviolet radiation) or when the host's restriction enzyme system cleaves the invading DNA before a successful infection can be initiated.

Distribution of CRISPR Systems and Anti-CRISPR Systems

CRISPR systems are widely distributed in both *Archaea* and *Bacteria*. Approximately 90% of the sequenced genomes of *Archaea* and 70% of those of *Bacteria* possess a CRISPR system. The utility of the system was first demonstrated in the dairy industry where starter cultures used for milk fermentation were susceptible to rampant bacteriophage infection. However, a strain of *Streptococcus thermophilus* was found to be resistant to virulent bacteriophage, and the difference between this *S. thermophilus* strain and those susceptible to viral infection was the spacers within its CRISPR region.

Because of the dynamic nature of the phage–host interaction, viruses have evolved mechanisms to avoid CRISPR surveillance and destruction. These include mutation of the PAM regions recognized by the memorizing complex of Cas proteins and the production of proteins that inhibit activity of the cleavage complex of Cas proteins (Figure 9.37*b*). This illustrates how the CRISPR system—central as it may be to preventing attacks on a cell's genome—does have a drawback in its reliance on recognizing previously encountered DNA sequences that can continue to mutate in their host and then reappear in the future in a form not recognized by CRISPR.

In addition, a *phage*-encoded (in contrast to *cell*-encoded) CRISPR system has been identified in the genome of a bacteriophage that infects *Vibrio cholerae*, the bacterium that causes cholera. The phage-encoded crRNAs target genes in *V. cholerae* that encode a defense system that prevents bacteriophage propagation; when these genes are inactivated, the cell's defense system is inoperative, a rather elaborate example of the "arms race" between bacteria and their viruses.

While CRISPRs are a major weapon *Bacteria* and *Archaea* have evolved for viral surveillance and maintaining genomic integrity, the mechanisms employed by CRISPR/Cas have not been overlooked by the scientific community. In Chapter 12 we discuss how the CRISPR/Cas system has been exploited as a powerful tool for synthetic biology and has opened up the possibility of radically new therapeutic strategies for human medicine (▶ Section 12.13).

Check Your Understanding

- Describe two ways that bacterial and archaeal cells can avoid the entry of foreign DNA. How can viruses overcome these defenses?
- Why is the CRISPR system considered a prokaryotic "adaptive immune system"?
- How do the crRNAs and Cas proteins protect the cell from invading DNA?

CHAPTER REVIEW

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

I • Mutation

9.1 Mutation is a heritable change in the nucleotide sequence of the genome and may lead to a change in phenotype. Selectable mutations are those that give the mutant a growth advantage under certain environmental conditions and are especially useful in genetic research. If selection is not possible, mutants must be identified by screening.

Q Write a one-sentence definition of the term "genotype." Do the same for "phenotype." Does the phenotype of an organism automatically change when a change in genotype occurs? Why or why not? Can phenotype change without a change in genotype? In both cases, give examples to support your answer.

9.2 Mutations, either spontaneous or induced, are in the base sequence of the nucleic acid in a genome. A point mutation is due to a single base-pair change. In a nonsense mutation, the codon becomes a stop codon and an incomplete polypeptide is made. Deletions and insertions cause more dramatic changes in the DNA, including frameshift mutations that often result in complete loss of gene function.

Q What are point mutations? How do silent mutations differ from missense and nonsense mutations?

9.3 Different types of mutations occur at different frequencies. For a typical bacterium, mutation rates of 10^{-6} to 10^{-7} per kilobase pair are generally seen. Although RNA and DNA polymerases introduce sequence errors at about the same rate, RNA genomes typically accumulate mutations at much higher frequencies than DNA genomes.

Q What is a revertant? Explain the two common types of revertants.

9.4 Mutagens are chemical, physical, or biological agents that increase the mutation rate. Mutagens can alter DNA in many different ways. However, alterations in DNA are not mutations unless they are inherited. Some DNA damage can lead to cell death if not repaired, and both error-prone and high-fidelity DNA repair systems exist.

Q Explain the primary mechanisms of each of the three types of mutagens.

II • Gene Transfer in *Bacteria*

9.5 Homologous recombination occurs when closely related DNA sequences from two distinct genetic elements are combined together in a single element. Recombination is an

important evolutionary process, and cells have specific mechanisms for ensuring that recombination takes place.

Q What are heteroduplex regions of DNA, and what process leads to their formation?

9.6 Certain bacteria exhibit competence, a state in which cells are able to take up free DNA released by other bacteria, a process called transformation. Once this DNA has been taken up, typically through the action of pili, incorporation of donor DNA into a recipient cell requires the activity of the single-strand binding protein RecA and several other enzymes. Only competent cells are transformable.

Q Explain why recipient cells do not successfully take up plasmids during natural transformation.

9.7 Transduction is the transfer of host genes from one bacterium to another by a bacterial virus. In generalized transduction, defective virus particles randomly incorporate fragments of the cell's chromosomal DNA, but the transducing efficiency is low. In specialized transduction, the DNA of a temperate virus excises incorrectly and takes adjacent host genes along with it; the transducing efficiency here may be very high.

Q Explain how a generalized transducing particle differs from a specialized transducing particle.

9.8 Conjugation is a mechanism of DNA transfer in *Bacteria* and *Archaea* that requires cell-to-cell contact. Conjugation is controlled by genes carried by certain plasmids (such as the F plasmid) and requires transfer of the plasmid from a donor cell to a recipient cell. Plasmid DNA transfer requires replication using the rolling circle mechanism.

Q What is a conjugative pilus and which cell type, F^- or F^+, would produce this structure?

9.9 The donor cell chromosome can be mobilized for transfer to a recipient cell. This requires an F plasmid to integrate into the chromosome to form the Hfr phenotype. Because transfer of the integrated F plasmid is rarely complete, recipient cells rarely become F^+. F′ plasmids are previously integrated F plasmids that have excised and captured some chromosomal genes.

Q What is a merodiploid, and how does an F′ plasmid yield a merodiploid?

III • Gene Transfer in *Archaea* and Other Genetic Events

9.10 Archaeal research lags behind bacterial research in the development of systems for gene transfer. Many antibiotics are ineffective against *Archaea*, making it difficult to select recombinants effectively, and the unusual growth conditions needed by many *Archaea* also make genetic experimentation challenging. Nevertheless, the genetic transfer systems of *Bacteria*—transformation, transduction, and conjugation—are all known in *Archaea*.

Q Explain one type of conjugation in *Archaea* and how it differs from F-plasmid-mediated conjugation.

9.11 Transposons and insertion sequences are genetic elements that can move from one location on a host DNA molecule to another by transposition. Transposition can be either replicative or conservative. Transposons often carry genes encoding antibiotic resistance and can be used to identify the functions of unknown genes.

Q What are the major differences between insertion sequences and transposons?

9.12 Bacterial and archaeal cells employ various mechanisms to prevent gene transfer and viral infection. However, the fast mutation rate of viruses allows them to adapt to these strategies. The clustered regularly interspaced short palindromic repeat (CRISPR) system is an RNA-based immune system that protects the genome of *Bacteria* and *Archaea* from invading DNA resulting from viral infection or conjugation. If small RNA molecules resulting from the spacer regions of the CRISPR region bind to incoming complementary DNA, Cas proteins destroy the nucleic acid duplex.

Q Explain why incoming DNA recognized by a short RNA molecule expressed from the CRISPR region cannot be completely foreign to the cell.

APPLICATION QUESTIONS

1. A constitutive mutant is a strain that continuously makes a protein that is inducible in the wild type. Describe two ways in which a change in a DNA molecule could lead to the emergence of a constitutive mutant. How could these two types of constitutive mutants be distinguished genetically?
2. Although a large number of mutagenic chemicals are known, none is known that induces mutations in only a single gene (gene-specific mutagenesis). From what you know about mutagens, explain why it is unlikely that a gene-specific chemical mutagen will be found. How then is site-specific mutagenesis accomplished?
3. Why is it difficult in a single experiment to transfer a large number of genes to a recipient cell using transformation or transduction?
4. Transposable elements cause mutations when inserted within a gene. These elements disrupt the continuity of a gene. Introns also disrupt the continuity of a gene, yet the gene is still functional. Explain why the presence of an intron in a gene does not inactivate that gene but insertion of a transposable element does.

CHAPTER GLOSSARY

Auxotroph an organism that has developed an additional nutritional requirement compared with the wild type, often as a result of mutation

Conjugation the transfer of genes from one prokaryotic cell to another by a mechanism requiring cell-to-cell contact

Frameshift mutation a mutation in which insertion or deletion of nucleotides changes the groups of three bases in which the genetic code is read within an mRNA, usually resulting in a faulty product

Genotype the complete genetic makeup of an organism; the complete description of a cell's genetic information

Heteroduplex a DNA double helix composed of single strands from two different DNA molecules

Hfr cell a cell with the F plasmid integrated into the chromosome

Horizontal gene transfer the unidirectional transfer of genes between cells through a process uncoupled from reproduction

Induced mutation a mutation caused by external agents such as mutagenic chemicals or radiation

Insertion sequence (IS) the simplest type of transposable element, which carries only genes that participate in transposition

Missense mutation a mutation in which a single codon is altered so that one amino acid in a protein is replaced with a different amino acid

Mutagen an agent that causes mutation

Mutant an organism whose genome carries a mutation

Mutation a heritable change in the base sequence of the genome of an organism

Nonsense mutation a mutation in which the codon for an amino acid is changed to a stop codon

Phenotype the observable characteristics of an organism

Point mutation a mutation that involves a single base pair

Recombination a resorting or rearrangement of DNA fragments resulting in a new sequence

Reversion an alteration in DNA that reverses the effects of a prior mutation

Rolling circle replication a mechanism, used by some plasmids and viruses, of replicating circular DNA, which starts by nicking and unrolling one strand. For a single-stranded genome, this is preceded by using the still-circular strand as a template for DNA synthesis; for a double-stranded genome, the unrolled strand is used as a template for DNA synthesis

Screening a procedure that permits the identification of organisms by phenotype or genotype, but does not inhibit or enhance the growth of particular phenotypes or genotypes

Selection placing organisms under conditions that favor or inhibit the growth of those with a particular phenotype or genotype

Silent mutation a change in DNA sequence that has no effect on the phenotype

SOS repair system a DNA repair system activated by DNA damage

Spontaneous mutation a mutation that occurs "naturally" without the help of mutagenic chemicals or radiation

Transduction the transfer of host cell genes from one cell to another by a virus

Transformation the transfer of bacterial genes through the uptake of free DNA from the environment

Transition a mutation in which a pyrimidine base is replaced by another pyrimidine or a purine is replaced by another purine

Transposable element a genetic element able to move (transpose) from one site to another on host DNA molecules

Transposon a type of transposable element that carries genes in addition to those required for transposition

Transversion a mutation in which a pyrimidine base is replaced by a purine or vice versa

Wild-type strain a bacterial strain isolated from nature or one used as a parent in a genetics investigation

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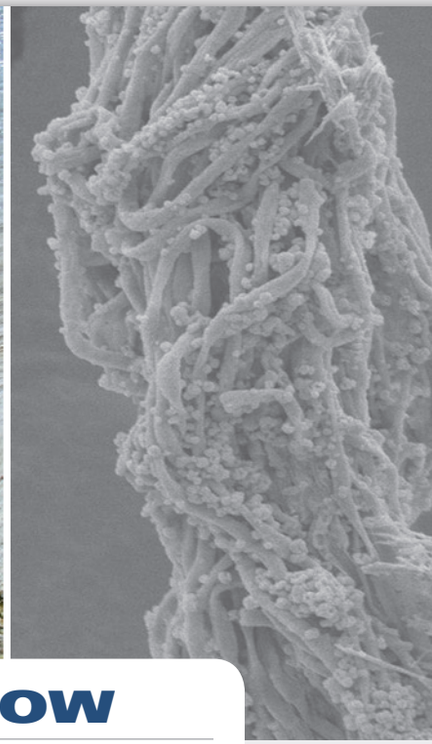


MICROBIOLOGYNOW

Omics Tools Unravel Mysteries of "Fettuccine" Rocks

Mammoth Hot Springs (MHS) in Yellowstone National Park (USA) displays many geochemical traits similar to those on Mars. Because of this, geomicrobiologists study these hot, oxygen-limited and sulfur-rich springs for microbial fossils whose fingerprints of life (biomarkers) could be used to detect life on other planets. The photo on the left shows filamentous microbial mats from MHS that resemble fettuccine pasta as a result of mineral encrustation. Tens of centimeters long, these streamers contain extremophilic microbes that entomb themselves in 5 millimeters of travertine (calcium carbonate) per day, a striking biomarker that can be easily observed. How are these pasta-looking rocks formed?

Using a combination of geochemical measurements, microscopy, and metagenomic, metatranscriptomic, and metaproteomic approaches, scientists have unraveled many of the mysteries surrounding the MHS streamers. These mats are dominated by the thermophilic, chemolithotrophic bacterium *Sulfurihydrogenibium yellowstonense*, which uses CO_2 and reduced sulfur as carbon and energy sources, respectively. Multi-omic data sets indicate that these remarkable cells produce pili as well as extracellular polymeric substances to latch onto one another and form streamers in the fast-moving spring water. Once the cells have formed microbial filaments (see scanning electron micrograph on the right), proteins expressed on the surface of *S. yellowstonense* catalyze the formation of travertine over a billion times faster than it would occur abiotically.

What is the advantage of forming these mineral-encrusted filamentous streamers? By undulating within travertine ridges, *S. yellowstonense* is able to maximize its access to the minimal oxygen and sulfide—key substrates for its energy metabolism—available within MHS waters. Thus, this organism has evolved an ingenious way to optimize chemolithotrophic growth.

Besides revealing fascinating microbiology, this study illustrates the power of omics tools to characterize microbes and their physiology in natural environments. It also highlights the utility of combined omics approaches for detecting biomarkers useful for searching for life beyond Earth.

Source: Dong, Y., et al. 2019. Physiology, metabolism, and fossilization of hot-springs filamentous microbial mats. *Astrobiology* doi:10.1089/ast.2018.1965.

Traditional approaches to studying microbial physiology and biochemistry have focused on the analysis of individual biochemical pathways or molecular responses under specific conditions. While informative, this reductionist approach can only target a specific gene or subset of genes or gene products—RNAs and proteins—and fails to address the dynamic nature of microorganisms and how a network of biological molecules controls their behavior in a coordinated fashion. By contrast, "*omics*" is a broad discipline that integrates different methodologies to characterize and quantify large pools of biological molecules. Through the power of combining various omics, a detailed picture of an organism's response to its environment can be generated. Because the ability to store and analyze massive amounts of biological information by computer is essential to omics, the understanding of entire biological systems is evolving in parallel with computing power and storage and retrieval capabilities.

I • Genomics

Buried within a genome sequence lies the heart of a cell's biology, and with the sequencing technology available today, microbiology—indeed all of biology—is moving ahead faster than ever as genomes unveil their secrets at a breathtaking pace.

The foundation of omics biology lies in nucleic acid and protein *sequences*, characteristics ultimately controlled by the cell's genome. The **genome** is an organism's entire complement of genetic information, including genes that encode proteins, RNAs, and regulatory sequences, as well as any noncoding DNA that may be present. The genome sequence of an organism not only reveals its genes, but also yields important clues to how the organism functions. While new omics are coined with regularity, this chapter focuses on the major omic themes of biological molecules—*genomics*, *transcriptomics*, *proteomics*, and *metabolomics*—and describes how these various pieces of the puzzle are integrated to yield important information about the biology of a single organism, or even an entire microbial community in the case of *metagenomics*, the comprehensive analysis of specific genes or genomes in an environment (Figure 10.1).

The word **genomics** refers to the discipline of mapping, sequencing, analyzing, and comparing genomes. Here we review how genomes are sequenced and some techniques used to analyze these genomes and their gene content.

10.1 Introduction to Genomics

Advances in genomics rely heavily on improvements in molecular technologies and computing power. The automation of DNA sequencing and the development of powerful computational tools for DNA and protein sequence analysis have reduced the cost and increased the speed at which genomes are analyzed. Thus the number of sequenced genomes has grown rapidly, with the major genomics bottleneck being the digestion of vast amounts of nucleic acid sequence data.

Genomics: Then and Now

The first genomes sequenced were those of small viruses over 40 years ago, and the first bacterial genome sequence was published in 1995. Today, DNA sequences from over 125,000 *Bacteria*, *Archaea*, and viruses, as well as datasets from metagenomic projects (Section 10.7), are available in public databases such as the Genomes *On*line *D*atabase (GOLD; see https://gold.jgi.doe.gov for an up-to-date list). With the goal of using genome sequences to advance systems and ecosystems biology, the United States Department of Energy's *Joint Genome Institute* (JGI) sponsors GOLD. The JGI has also joined the Genomic *E*ncyclopedia of *Bacteria* and *Archaea* (GEBA) in an effort to expand the coverage of genome sequences across the phylogenetic tree of life (◀ Figure 1.41*b*). At the time of this writing, GEBA had added over 1000 new genome sequences to the databases. These genome sequences represent a phylogenetically diverse range of *Bacteria* and *Archaea*. Despite these additions, there is plenty of "space" within the genomic tree of life (◀ Figure 1.41) for new microbial genome sequencing projects. Table 10.1 lists some representative genomes from *Bacteria* and *Archaea*. The genomes of many eukaryotic organisms have also been sequenced, including the haploid human genome, which contains about 3.2 billion bp (~21,000 protein-encoding genes, Figure 10.2). As astonishing as this large number might seem, the human genome is far from the largest genome known. Of all life forms on Earth, certain plants are known to contain the largest genomes (Figure 10.2).

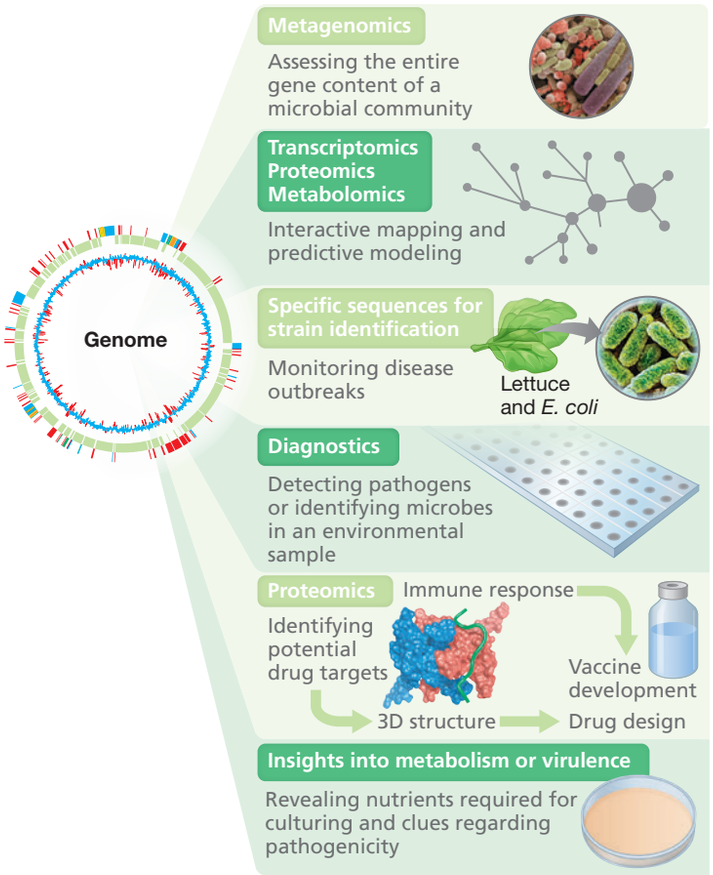


Figure 10.1 Utility of microbial genome sequences. A genome sequence allows for the development of omics approaches and tools for understanding, investigating, and monitoring microorganisms, both in culture and in nature. It can also provide targets for drug and vaccine design.

TABLE 10.1 Genomes of select species of *Bacteria* and *Archaea*[a]

Organism	Lifestyle[b]	Size (bp)	ORFs[c]	Features
Bacteria				
Nasuia deltocephalinicola	E	112,091	137	Degenerate sap-feeding insect endosymbiont
Tremblaya princeps	E	138,931	121	Degenerate mealybug endosymbiont
Hodgkinia cicadicola	E	143,795	169	Degenerate cicada endosymbiont
Mycoplasma genitalium	P	580,070	525	Smallest nonsymbiotic bacterial genome
Rickettsia prowazekii	P	1,111,523	834	Obligate intracellular parasite, causes epidemic typhus
Treponema pallidum	P	1,138,006	1,041	Spirochete, causes syphilis
Methylophilaceae family, strain HTCC2181	FL	1,304,428	1,354	Marine methylotroph, smallest free-living genome
Thermotoga maritima	FL	1,860,725	1,877	Hyperthermophile
Deinococcus radiodurans	FL	3,284,156	2,185	Radiation resistant, multiple chromosomes
Bacillus subtilis	FL	4,214,810	4,100	Gram-positive genetic model
Mycobacterium tuberculosis	P	4,411,529	3,924	Causes tuberculosis
Escherichia coli K-12	FL	4,639,221	4,288	Gram-negative genetic model
Escherichia coli O157:H7	FL	5,594,477	5,361	Enteropathogenic strain of *E. coli*
Bradyrhizobium japonicum	FL	9,105,828	8,317	Nitrogen fixation, nodulates soybeans
Sorangium cellulosum	FL	14,782,125	11,559	Forms multicellular fruiting bodies
Minicystis rosea	FL	16,040,666	14,018	Forms multicellular fruiting bodies
Archaea				
Nanoarchaeum equitans	P	490,885	552	Smallest nonsymbiotic cellular genome
Methanocaldococcus jannaschii	FL	1,664,976	1,738	Methanogen, hyperthermophile
Pyrococcus horikoshii	FL	1,738,505	2,061	Hyperthermophile
Sulfolobus solfataricus	FL	2,992,245	2,977	Hyperthermophile, sulfur chemolithotroph
Haloarcula marismortui	FL	4,274,642	4,242	Extreme halophile, bacteriorhodopsin
Methanosarcina acetivorans	FL	5,751,000	4,252	Acetate using methanogen

[a]Information on prokaryotic genomes can be found at https://gold.jgi.doe.gov.
[b]E, endosymbiont; P, parasite; FL, free-living.
[c]Open reading frames. Genes encoding known proteins are included, as well as ORFs that could encode a protein greater than 100 amino acid residues. Smaller ORFs are not included unless they show similarity to a gene from another organism or unless the codon bias is typical of the organism being studied.

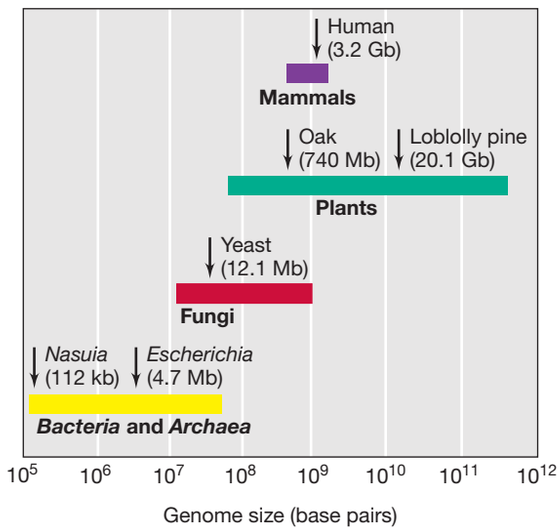
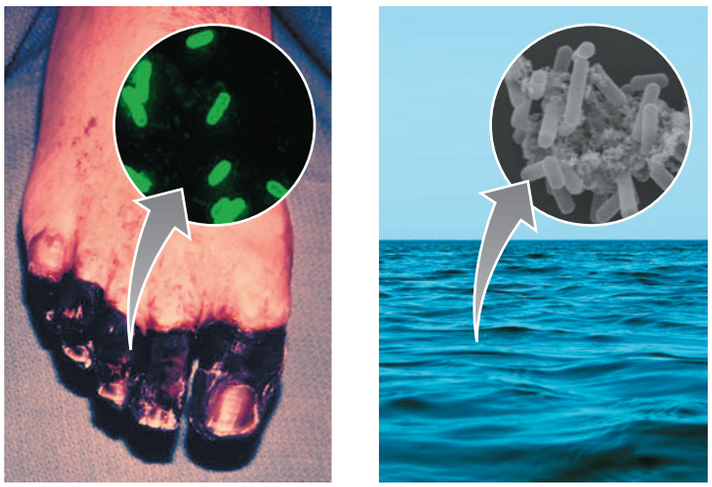


Figure 10.2 Genome sizes of microbial cells and higher organisms. Compare with viral genome sizes in Chapter 11 (▶ Figure 11.1). The flowering plant *Paris japonica* has the largest known genome, some 50 times that of humans.

What Can Genomes Tell Us?

As we will discuss throughout this chapter, modern microbiology thrives on genome sequences; indeed, little in microbiology has been left untouched by genomic sequences. Microbial genome sequencing has discovered everything from genes encoding heat-stable enzymes in microbes that thrive in boiling water (◀ Figure 4.25*b*) to genes that encode virulence factors in the most vicious pathogens. Genome sequencing has also been instrumental in developing microarrays for studying gene expression (Section 10.8), detecting horizontal transfer events (between microbes of different species, genera, and even phyla; Chapters 9 and 13), monitoring and diagnosing disease outbreaks (based on the presence of "signature genes" of different pathogens), discovering CRISPRs (◀ Section 9.12) and other anti-bacteriophage systems (Section 10.5), understanding metabolic pathways, and discerning the growth requirements of microbes that have thus far defied laboratory culture.

The ability to sequence genomes has also been used to solve obscure medical mysteries. An excellent example is the genomics that revealed the causative agent of the "Black Death," which swept through Europe in the middle of the fourteenth century (**Figure 10.3*a***).



(a) (b)

Figure 10.3 Diverse examples of what genomes can tell us. *(a)* Genomics helped solve an ancient medical mystery surrounding plague. The blackened skin on the toes of this modern plague victim originates from hemorrhaging due to systemic infection with the pathogenic bacterium *Yersinia pestis*, shown in inset. *(b)* Genome sequencing was used to assign the marine ammonia-oxidizing archaeon *Nitrosopumilus* to a new phylum of *Archaea*, the *Thaumarchaeota*.

While it was believed that the Black Death was caused by a massive outbreak of bubonic plague, a typically fatal disease caused by the bacterium *Yersinia pestis* (▶ Section 32.7), scientists could not be positive until they recovered and sequenced DNA samples from the teeth and bones of corpses of people known to have died from the Black Death. By comparisons of this ancient DNA with the genome of *Y. pestis*, the mystery behind this devastating medieval disease was unraveled: The Black Death was indeed bubonic plague.

Microbial genomics has also been used to identify new microbial phyla. For example, until recently, only three phyla of *Archaea* were known—*Euryarchaeota*, *Crenarchaeota*, and *Nanoarchaeota* (Chapter 17). Because every cultured species was isolated from an extreme environment, many microbiologists concluded that *Archaea* were mainly extremophiles and that they did not inhabit oceans, lakes, and soil in significant numbers. However, based on environmental 16S rRNA gene sequencing, *Archaea* only marginally affiliated with *Crenarchaeota* were detected in marine and freshwater samples. Who were these organisms, and how were they making a living? Subsequently, *Nitrosopumilus*, the first ammonia-oxidizing (nitrifying) archaeon known, was isolated (Figure 10.3*b*; ▶ Section 14.9). Using the powerful analytical tools of genomics, the genomes of two distinct ammonia-oxidizing *Archaea* were compared with those of all other *Archaea*. This genomic analysis clearly showed that these ammonia-oxidizing *Archaea* belonged in a new phylum, now called the *Thaumarchaeota* (▶ Section 17.5).

The above is just a taste of how genomics has impacted microbiology. Other relevant examples will appear regularly as you make your way through this text. The major message here is twofold: (1) We are clearly living in the era of microbial genomics, and (2) the genomics revolution has spawned a wealth of powerful tools to attack old problems in new ways. Indeed, in the past 40 years or so, microbiology as a science has leapt forward farther and faster than at any time in its history.

Check Your Understanding

- How many protein-encoding genes are in the human genome?
- List three examples of how genomics has led to major new discoveries in microbiology.

10.2 Sequencing and Annotating Genomes

In biology, the term **sequencing** refers to determining the precise order of subunits in a macromolecule. In the case of DNA (or RNA), the sequence is the *order* in which the nucleotides are aligned. DNA sequencing today forms the heart of the omics revolution and its technology is advancing so quickly that new methods appear every year. Yet despite the technological breakthroughs that have catapulted us into the omics age, some of the earliest sequencing methodologies—born of simple yet brilliant basic science—form the foundation of the latest methods today (see next subsection).

After sequencing and assembly of the gene fragments, the next step is *genome annotation*, the conversion of raw sequence data into a list of the genes and other functional sequences present in the genome. The term **bioinformatics** refers to the use of computers to store and analyze the sequences and structures of nucleic acids and proteins. Improved sequencing methods are now generating data almost faster than it can be properly analyzed. Thus, although automated annotation software exists and powerful new versions are being developed at a rapid pace, annotation remains the major "bottleneck" in genomics. Here we focus on the process of genome sequencing, assembly, and annotation.

DNA Sequencing

The first widely used method for sequencing DNA was the *dideoxy method* developed by the British scientist Fred Sanger, who won a Nobel Prize (his second) for this accomplishment. In the Sanger procedure the sequence is determined by making a copy of the original single-stranded DNA in a process similar to the polymerase chain reaction (PCR, ▶ Section 12.1). The secret behind the Sanger method was the addition of a mixture of normal deoxyribonucleotides (dNTPs) and small amounts of the corresponding *dideoxyribonucleotides* (ddNTPs), one for each of the four bases—adenine, guanine, cytosine, and thymine—to the mixture used to make the DNA copy (**Figure 10.4*a***). The dideoxy analog is a specific *chain-terminator*; because it lacks a 3′-hydroxyl, the analog prevents further elongation of the chain after its insertion. Because ddNTPs insert randomly, DNA chains of varying length are produced in the synthesis reaction (Figure 10.4*a*). Sanger sequencing originally used radioactive labels, but automated systems were quickly developed that used a separate fluorescent label for each different ddNTP and detected the DNA products (separated by passing through a sizing column) with a laser (Figure 10.4*a*).

Mastering Microbiology
Art Activity: Figure 10.4*a* DNA sequencing

Because the original Sanger method was dependent on primers binding to a known sequence and was limited to around 800 nucleotides per reaction, entire chromosomes or large DNA molecules could not be sequenced in a single reaction. Instead large DNA molecules had to be cut into smaller fragments and cloned into vectors for sequencing. This led to the development of new sequencing technologies, which appear now with such regularity that the term "next-generation sequencing" is commonly used to describe the

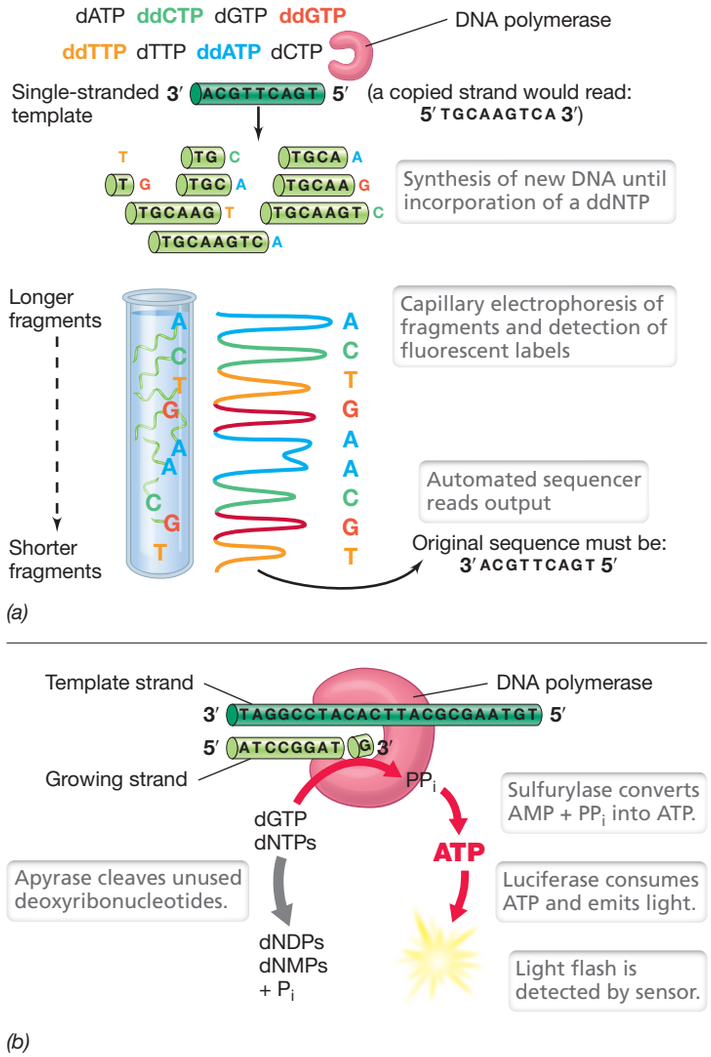


Figure 10.4 **DNA sequencing.** *(a)* Sanger sequencing. When a polymerase incorporates a ddNTP during synthesis, the chain of DNA is terminated. The identity of the terminal ddNTP can be determined by capillary electrophoresis and fluorescence detection. *(b)* Pyrosequencing. Whenever a new dNTP is inserted into the growing strand of DNA (red arrows), pyrophosphate (PP_i) is released and is used to make ATP from AMP by the enzyme sulfurylase. The ATP is consumed by the enzyme luciferase, which releases light. Unused dNTPs are degraded by the enzyme apyrase (gray arrow).

latest and greatest in nucleic acid sequencing. For example, *pyrosequencing*, a *second-generation sequencing method* still widely used today, is based on the Sanger method and employs the light-emitting enzyme luciferase to detect incorporation of dNTPs by emitting a pulse of light (Figure 10.4*b*). It should be noted that despite the advent of new sequencing technologies, the Sanger method is still routinely used to sequence plasmid constructs and PCR products. **Table 10.2** summarizes modern sequencing methods and illustrates how the cost of sequencing 1 megabase (Mbp, million base pairs) of DNA dropped over 100,000-fold in 15 years.

Mastering Microbiology
Art Activity: Figure 10.4*b* Pyrosequencing

Genome Assembly and Annotation

Regardless of which sequencing system is used—even hand-held systems are available today (see Explore the Microbial World, DNA Sequencing in the Palm of Your Hand, on page 336)—the sequences obtained must be *assembled* before they can be analyzed. Genome assembly consists of putting the fragments in the correct order to reconstruct the chromosome and eliminating any overlaps that may appear. Then, for assembled genomic sequences to be useful, they must be *annotated* in order to identify genes and other functional regions. Many of the tasks surrounding genome assembly and annotation are highly computational. For genome assembly, a computer examines many short DNA fragments that have been sequenced and deduces their order by detecting all of the instances where two fragments of DNA possess overlapping sequence (**Figure 10.5**). These overlaps are used to merge sequencing reads into *contigs*, or contiguous consensus sequences. Individual contigs with overlapping ends are then aligned to form scaffolds (contigs as well as gaps) that are ultimately used to generate a map representing the complete genome.

From the genome map, the annotation process can begin. Because the genomes of *Bacteria* and *Archaea* possess very few intervening sequences (introns, ◀ Section 6.6), their genomes essentially consist of a series of **open reading frames (ORFs)** separated by short regulatory regions and transcriptional terminators. A *functional ORF* is one that actually encodes a protein (◀ Section 6.9) and can be identified from a computer search of the sequence (**Figure 10.6**). Although any given cellular gene is always transcribed from one DNA strand, a gene can actually be located on either strand and thus computer inspection of both strands of DNA is required.

Finding and Identifying ORFs

The first step in finding an ORF is to locate *start* and *stop* codons in the sequence (◀ Section 6.9 and Table 6.4). However, in-frame start and stop codons appear randomly with reasonable frequency;

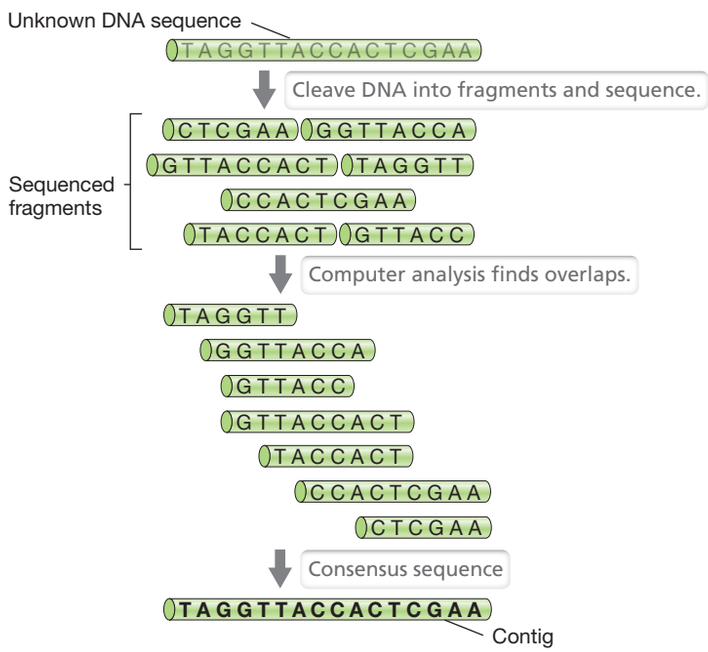


Figure 10.5 **Computer assembly of DNA sequence.** Most DNA sequencing methods generate vast numbers of short sequences (30 to several hundred bases) that must be assembled. The computer searches for overlaps in the short sequences and then arranges them to form contigs, or a consensus sequence.

TABLE 10.2 DNA sequencing methods

Generation	Method	Features
First generation	Sanger dideoxy method (radioactivity or fluorescence; DNA amplification)	Read length: 700–900 bases Used for the Human Genome Project
Second generation	454 Pyrosequencing (fluorescence; DNA amplification; massively parallel) Illumina/Solexa method (fluorescence; DNA amplification; massively parallel) SOLiD method (fluorescence; DNA amplification; massively parallel) Ion torrent (electronic—pH; DNA amplification)	Read length: 400–700 bases Used to sequence genome of James Watson (completed 2007) Read length: 50–150 bases Giant panda genome (2009; Beijing Genome Institute); Denisovan genome (2010) Read length 50–100 bases Read length: 200–400 bases Used to sequence genome of Intel cofounder Gordon Moore
Third generation	Pacific Biosciences SMRT (fluorescence; single molecule; zero mode waveguide) Oxford nanopore (electronic—current; single molecule; real time)	Read length: 2500–3000 bases Read length: 9000 bases Portable MinION unit is approximately the size of a flash drive

thus, further clues are needed. In *Bacteria*, translation begins at start codons located immediately downstream of a ribosome-binding sequence (RBS or Shine–Dalgarno site) on the mRNA (◀ Section 6.9). Thus, locating potential ribosome-binding sequences in addition to start and stop codons helps decide both whether an ORF is functional and which start codon is actually used. In addition, an ORF is more likely to be functional if its sequence is similar to those of ORFs in the genomes of other organisms (regardless of whether they encode known proteins) or if the ORF includes a sequence known to encode a protein functional domain. This is because proteins with similar functions in different cells tend to share a common evolutionary origin and thus share sequence and structural features (▶ Section 13.8). A computer can search for sequence similarities in major databases such as GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) using *BLAST* (*B*asic *L*ocal *A*lignment *S*earch *T*ool), an algorithm that can compare a nucleic acid or protein sequence with all other such sequences in the database.

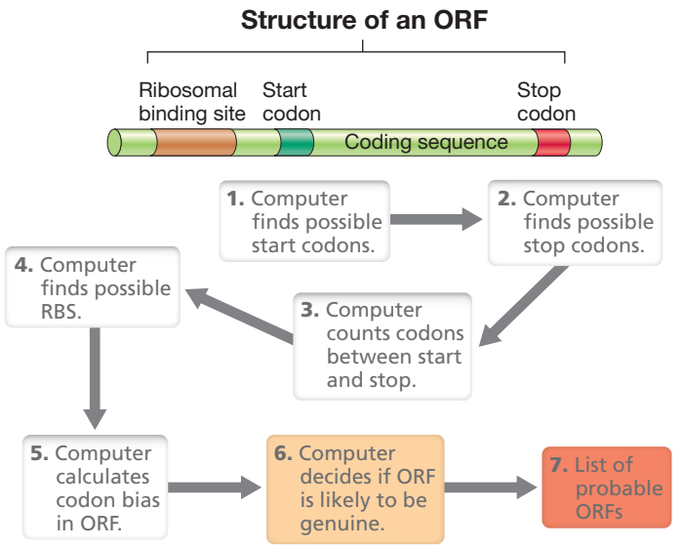


Figure 10.6 Computer identification of possible ORFs. The computer scans the DNA sequence looking first for start and stop codons. It then counts the number of codons in each uninterrupted reading frame and rejects those that are too short. The probability of a genuine ORF is made stronger if a likely ribosomal binding site (RBS) is found the correct distance in front of the reading frame. Codon bias calculations are used to test whether an ORF complies with the codon usage of the organism being examined.

Other issues must be considered in a genome annotation as well. For example, more than one codon exists for many of the 20 common amino acids (◀ Table 6.4), and some codons are used more frequently than others. The latter is known as **codon bias** (codon usage) and differs greatly between organisms. For example, **Table 10.3** shows the different usage of the six arginine codons in *Escherichia coli* compared to their usage in humans and fruit flies. If the codon bias in a given ORF differs greatly from the consensus for the organism containing it, that ORF may be nonfunctional or may be functional but obtained by horizontal gene transfer (▶ Section 13.9).

Genomic Analyses: The Final Tally

No genome sequence project ends with 100% of the genome identified. In fact, this is one of the most exciting and challenging findings of genomic analyses: Many genes in microbes almost certainly encode proteins whose function(s) remain unknown. Although there are differences among organisms, in most genomes the percentage of genes whose role can be clearly identified is approximately 70% of the total number of ORFs detected. Uncharacterized (or unknown) ORFs are said to encode *hypothetical proteins*, proteins that probably exist although their function is unknown. These ORFs have uninterrupted reading frames of reasonable length and the necessary start and stop codons and ribosome-binding site (Figure 10.6); however, the proteins they encode lack sufficient amino acid sequence homology with any known protein to be unambiguously identified. Some gene annotations can only assign a gene to a protein family or to a general function (such as "transport protein") without being more specific. Many of the unidentified genes in *E. coli* are thought to encode proteins that play a role in some unidentified regulatory process or are proteins

TABLE 10.3 Examples of codon bias

Arginine codon[a]	Usage of each arginine codon (%)		
	Escherichia coli	Fruit fly	Human
AGA	1	10	22
AGG	1	6	23
CGA	4	8	10
CGC	39	49	22
CGG	4	9	14
CGU	49	18	9

[a]Arginine has six codons; see Table 6.4.

required only for special nutritional or environmental conditions. A few may also function as "backups" of key enzymes. Later in this chapter we will discuss methods, which are only possible because of the omics revolution, to help identify the function of these hypotheticals (Sections 10.5 and 10.7).

In addition to protein-encoding genes, some genes encode RNA molecules that are not translated. Such genes therefore lack start codons and may well have multiple stop codons within the gene. Some noncoding RNAs, such as tRNAs and rRNAs, are easy to detect because they are well characterized and are highly conserved. However, many noncoding regulatory RNA molecules (◀ Section 7.12) are conserved only in their three-dimensional structure, with little sequence homology. Thus transcriptomics, specifically RNA-Seq (Section 10.8), has become instrumental in identifying these noncoding genes.

With this general background in nucleic acid sequencing and the coding features of genomes, we move on to compare the nature of genomes in various microbial groups. We begin with the *Bacteria* and *Archaea* where thousands of genome sequences are available for comparative analyses.

Check Your Understanding

- What key molecules are essential for Sanger sequencing?
- What is an open reading frame (ORF)? What is a hypothetical protein?
- What is the major problem in identifying genes encoding non-translated RNA?

10.3 Genome Size and Gene Content in *Bacteria* and *Archaea*

Once a genome has been assembled, *comparative genomics* using databases such as MicrobesOnline (http://www.microbesonline.org)—which contains nearly 4000 microbial genome sequences—can be used to probe its biological secrets. By using comparative genomics, it has been determined that genomes of *Bacteria* and *Archaea* show a strong correlation between genome size and open reading frame (ORF) content (**Figure 10.7*a***). Regardless of the organism, each megabase pair of DNA in a prokaryotic cell encodes about 1000 ORFs, and as the size of these genomes increases, the gene number also increases proportionally. This contrasts markedly with the genomes of eukaryotes, in which noncoding DNA (introns, ◀ Section 6.6) may constitute a large fraction of the genome, especially in organisms with large genomes (Figure 10.2).

While the sizes of bacterial genomes vary 100-fold, from the insect symbiont *Tremblaya princeps* with 121 protein-encoding genes to the soil-dwelling *Minicystis rosea* with over 14,000 genes, archaeal genomes only vary in size 10-fold (Figure 10.7*b*). This archaeal range encompasses the ectosymbiont *Nanoarchaeum equitans* with 552 protein-encoding genes to the methanogen *Methanosarcina acetivorans* with 4252 genes (Figure 10.2 and Table 10.1). Whereas about 1300 genes have been the predicted minimum for the number necessary for a cell to have a *free-living* existence, recent environmental

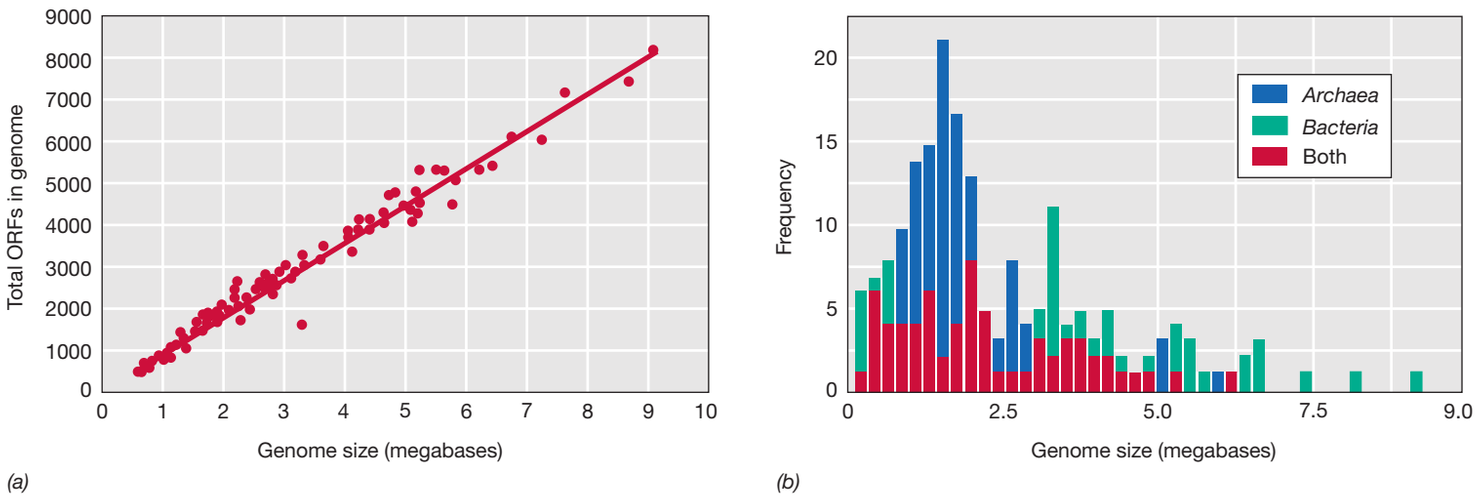
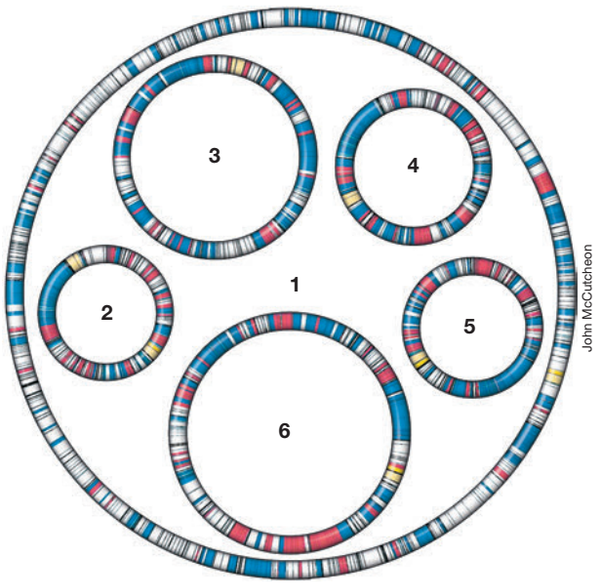


Figure 10.7 Bacterial and archaeal genome size and content. *(a)* Correlation between genome size and ORF content in prokaryotic cells. Analyses of 115 completed genomes from species of both *Bacteria* and *Archaea*. Data from Konstantinidis, K.T., and Tiedje, J.M. 2004. *Proc. Natl. Acad. Sci. (USA) 101*: 3160. *(b)* Distribution of genome sizes among a representative sample of *Archaea* (blue) and *Bacteria* (green). Genomes were chosen based on even distribution across the two phylogenetic domains. Shared frequency of occurrence data points are represented in red. Data adapted from Kellner, S., Spang, A., Offre, P., Szöllosi, G.J., Petijean, C., and Williams, T.A. 2018. *Emerging Top. Life Sci.* doi: 10.1042/ETLS20180021.

metagenomic (◀ Figure 1.1 and Section 10.7) datasets have called this earlier estimate into question; individual genomes have been assembled from various environments that contain only 600 to 800 genes (◀ Chapter 1 Explore the Microbial World, "Tiny Cells"). Representative organisms for these genomes are not yet in culture to determine if they are free-living bacteria, but if they are, the genetic requirements for a free-living cell are far lower than originally suspected.

Small Genomes

The smallest cellular genomes belong to bacteria that are parasitic or endosymbiotic (cells that live inside other cells), with the insect symbionts *Tremblaya* (in mealybugs) and *Hodgkinia* (in cicadas) possessing some of the smallest genomes (around 140 kbp, Table 10.1 and **Figure 10.8**). The absolute smallest genome discovered thus far is that of *Nasuia deltocephalicola*, a sap-feeding insect symbiont whose genome is only 112 kbp. Because of their reduced genome size, such symbionts are totally dependent on their insect host cells for survival and nutrients. In turn, the symbionts provide the insect with essential amino acids and other nutrients that the insect cannot synthesize.



1. ***Mycoplasma genitalium*** (*Mollicutes*)
   580.1 kbp
   GC: 31.7%
2. ***Tremblaya*** (*Betaproteobacteria*)
   138.9 kbp
   GC: 58.8%
3. ***Zinderia*** (*Betaproteobacteria*)
   208.5 kbp
   GC: 13.5%
4. ***Carsonella*** (*Gammaproteobacteria*)
   159.6 kbp
   GC: 16.6%
5. ***Hodgkinia*** (*Alphaproteobacteria*)
   143.7 kbp
   GC: 58.4%
6. ***Sulcia*** (*Bacteroidetes*)
   245.5 kbp
   GC: 22.4%

Figure 10.8 Symbiont genomes. Five insect symbiont genomes are shown drawn to scale inside the circle representing the genome of a *Mycoplasma*. Blue: genes encoding genetic information processing; red: genes encoding amino acid and vitamin biosyntheses; yellow: rRNA genes; white: other genes; gaps indicate noncoding DNA. Kbp, kilobase pairs. GC indicates percentage of nucleotides that are guanine or cytosine.

With genomes of around 500 kbp, *Mycoplasma* (*Bacteria*) and *Nanoarchaeum equitans* (*Archaea*) have the smallest genomes among parasitic prokaryotic cells (Table 10.1). *N. equitans* is a hyperthermophile and a parasite of another hyperthermophile, the archaeon *Ignicoccus* (▶ Section 17.11). *N. equitans* lacks virtually all genes that encode metabolic proteins and presumably depends on its host for most catabolic as well as anabolic functions. While some pathogens such as *Mycobacterium tuberculosis* have quite large genomes (4.4 Mbp), the genomes of most human pathogens, such as *Mycoplasma*, *Chlamydia*, and *Rickettsia*, are smaller than the largest known viral genome, that of *Pandoravirus* (2.5 Mbp, ▶ Section 11.1).

Using *Mycoplasma*, which has around 525 genes, as a starting point, it has been estimated that around 250–300 genes are the minimum number possible for a viable cell. These estimates rely partly on comparisons with other small genomes. In addition, systematic mutagenesis has been performed to identify essential genes. For example, experiments with *Escherichia coli* and *Bacillus subtilis*, both of which have about 4000 genes, indicate that approximately 300–400 genes are essential depending on the growth conditions. In fact, synthetic biologists have been able to create a minimal *Mycoplasma* bacterium with 473 genes (▶ Section 12.12). While this is only 52 genes fewer than that of the *Mycoplasma genitalium* genome (Table 10.1), the designer microbe has a doubling time of 3 hours versus the weeks of the original *M. genitalium*. Thus the trimmed-down genome benefits the synthetic bacterium in the laboratory.

Large Genomes

Some *Bacteria* have genomes that are as large as those of some eukaryotic microbes. In fact, because eukaryotes tend to have significant amounts of noncoding DNA and bacteria do not (Section 10.4), some bacterial genomes actually have more genes than microbial eukaryotes, despite having less DNA. For example, the genome of *Bradyrhizobium japonicum*, a bacterium that forms nitrogen-fixing root nodules on leguminous plants such as soybeans (▶ Section 23.4), has 9.1 Mbp of DNA and 8300 ORFs, whereas the genome of the baker's yeast *Saccharomyces cerevisiae*, a eukaryote, has 12.1 Mbp of DNA and only 5400 ORFs (see Tables 10.1 and 10.5).

The second largest bacterial genome known is that of *Sorangium cellulosum*, a species of the gliding myxobacteria (▶ Section 15.16; little has been reported on the larger *M. rosea* genome, Table 10.1). With just under 14.8 Mbp on a single circular chromosome, *S. cellulosum* has more DNA than several eukaryotes including yeast and the pathogenic protozoans *Cryptosporidium* and *Giardia* (see Table 10.5). The *S. cellulosum* genome is composed of roughly 10.5% noncoding DNA and 11,559 protein-encoding genes, making it over three times larger than the genome of *E. coli*. Interestingly, the *S. cellulosum* genome encodes 508 kinases (enzymes that phosphorylate other proteins to regulate their activity), which is over three times that of any other genome including those of eukaryotes. This suggests that the lifestyle of *S. cellulosum* is highly diverse and that its ecological success requires extensive regulation. In contrast to *Bacteria*, the largest genomes found in species of *Archaea* thus far are only about 5 Mbp (Table 10.1).

DNA Sequencing in the Palm of Your Hand

DNA sequencing technologies are revolutionizing microbiology at a remarkable pace. Innovations in next-generation sequencing have even tackled the difficult issues of cost and portability. The world's first mobile nucleic acid sequencer—the MinION—is a palm-sized device that possesses 2000 tiny pore-containing proteins called nanopores. As single strands of nucleic acid travel through the nanopores, individual nucleotides are identified in real time based on changes in electrical current (**Figure 1**). These current changes are relayed to a computer through a USB connection, which also powers the MinION. This miniature but mighty machine can display nucleic acid sequences from critical field samples in real time on a computer screen.

Besides its portability, the methodology behind the nanopore technology yields extremely long sequencing reads compared to other next-generation sequencing methods (Table 10.2). This feature is being exploited to quickly sequence complete microbial genomes as well as to "close" those genomes that have been difficult because of their troublesome nucleotide repeats. The identification of nucleotides based on changes in electrical current as they travel through a nanopore also allows for modified nucleotides, such as those with additional methyl groups, to be detected. This feature is of interest to microbiologists who study gene regulation, as the methylation state of gene promoters can control gene expression; such is the case with genes that encode mobile gene elements such transposases (◀ Section 9.11) that play critical roles in horizontal gene transfer and genome evolution (Chapter 13).

The utility of the MinION's portability was clearly on display during the 2014–2015 Ebola virus hemorrhagic fever outbreak in West Africa. Scientists traveled to Guinea with three MinIONs in their luggage, a feat in itself as most DNA sequencers are too large and delicate to travel in baggage. Once in Guinea, scientists were able to survey the spread of different Ebola virus strains by analyzing unique nucleotide sequences present in each strain's genome. In as little as 48 hours after sample collection, Ebola virus genomes from 14 patients were determined using MinION sequencing.[1] **Figure 2** shows a researcher loading a patient's sample onto a MinION set up in a mobile field laboratory (inset).

Because the Ebola genome mutates rapidly, the astonishing turnaround time provided by the MinION allowed epidemiologists to track geographical movements of different strains of the virus. This real-time analysis indicated that two major viral strains were the cause of Ebola persistence and that cross-border transmission between Sierra Leone and Guinea severely prolonged the outbreak.[2] Traditional sequencing methods would not have supported such surveillance, as it requires weeks to obtain results after shipment of samples to remote laboratories.

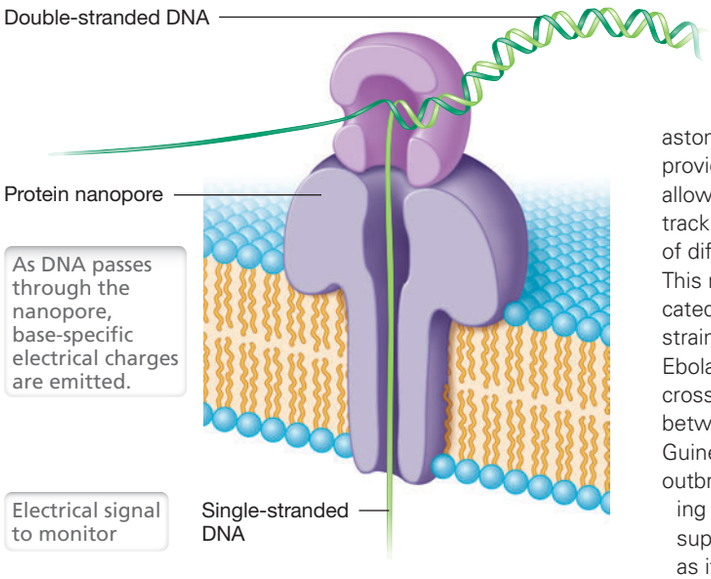


Figure 1 Nanopore-MinION sequencing. A DNA double helix is converted to a single strand for passage through a membrane-bound pore. As the DNA transits the nanopore, it causes changes in electric current that are base-specific and thus reveal the DNA sequence.

Figure 2 Use of the MinION in the field during the 2015 Ebola outbreak in West Africa. Credits: © EMLab/Tommy Trenchard 2016.

While biologists have envisioned numerous uses for the MinION, it has already been tested by NASA on the International Space Station. During this test, the MinION's ability to sequence the genomes of *Escherichia coli*, bacteriophage lambda, and mouse DNA was not hampered by the microgravity of outer space.[3] In addition, the MinION has proven durable and dependable even in such remote and extreme locations as Antarctica,[4] opening up the possibility that the large sequencing requirements of modern-day microbial ecology may be fulfilled, at least in part, right in the field at the time of sample collection. Moreover, developers are currently attempting to modify the portable sequencer to operate from a smartphone instead of a computer. And, because of its size, relatively low cost, and ease of use, the next frontier for the MinION will undoubtedly be the classroom.

[1]Quick, J., et al. 2016. Real-time, portable genome sequencing for Ebola surveillance. *Nature 530*: 228.
[2]Ibid.
[3]Castoro-Wallace, S.L., et al. 2017. Nanopore DNA sequencing and genome assembly on the International Space Station. *Sci. Rep. 7*: 18022.
[4]Johnson, S.S., et al. 2017. Real-time DNA sequencing in the Antarctic Dry Valleys using the Oxford nanopore sequencer. *J. Biomol. Tech. 28*(1): 2.

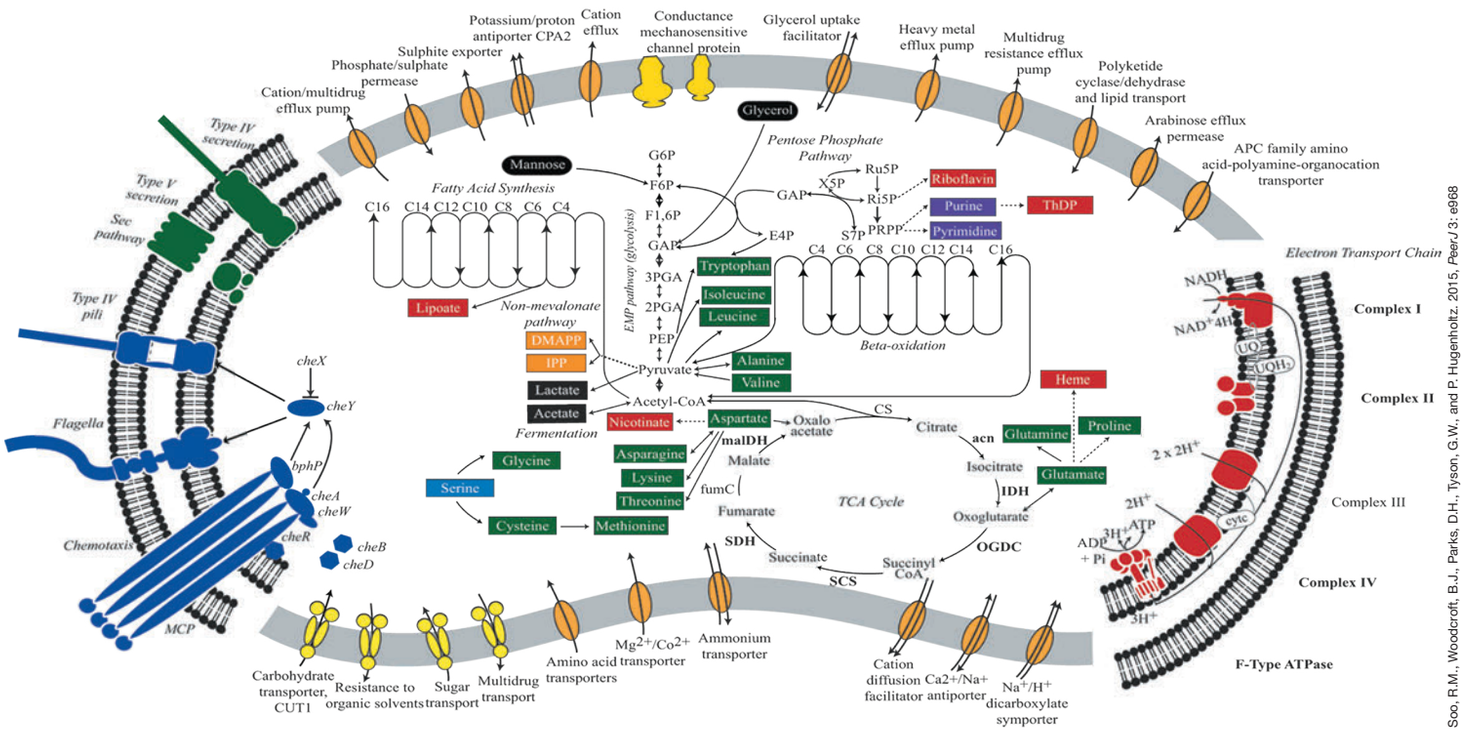
Gene Content of Bacterial Genomes

The complement of genes in a particular organism reveals its capabilities. Conversely, genomes are molded by adaptation to particular lifestyles. Comparative analyses are useful when searching for genes that encode enzymes that probably exist because of the lifestyle of an organism, and in some cases these searches yield big surprises. For example, *Vampirovibrio chlorellavorus* is a predatory bacterium that attacks its host, the green alga *Chlorella*, by surface attachment and ultimate ingestion of its cellular contents (thus the terms "vámpír" from Hungarian, meaning "blood sucker," and "vorus" from Latin, meaning "to devour," in the organism's name). Isolates of *V. chlorellavorus* existed only as 36-year-old freeze-dried samples that had not been successfully revived. However, by using advanced sequencing techniques, the genome of *V. chlorellavorus* was recovered, and surprisingly, genomic analyses indicated that *V. chlorellavorus* falls within the phylum *Cyanobacteria* (▶ Section 15.3) even though it lacks genes for photosynthesis.

Figure 10.9, reprinted from a scientific journal, is included here to give you an idea of why a microbe's genome should be sequenced and the amazing amount of information that can be gleaned from annotation, though the details are beyond the scope of this chapter. The figure summarizes some of the metabolic pathways and transport systems of *V. chlorellavorus* deduced from analysis of its genome. These include an electron transport chain for microaerobic growth (◀ Section 4.16), the ability to ferment (◀ Sections 3.6 and 3.7), chemotaxis abilities (◀ Section 2.11), and the synthesis of 15 of the 20 essential amino acids (◀ Figure 6.27). Comparative genomics also indicated that *V. chlorellavorus* used a conjugative type IV secretion system (◀ Section 6.13) to attack its prey, the first discovery of this strategy in a predatory bacterium.

A functional analysis of genes and their activities in several bacteria is given in **Table 10.4**. Thus far, a distinct pattern of gene distribution in *Bacteria* has emerged. Metabolic genes are typically the most abundant class in bacterial genomes, although genes for protein synthesis overtake metabolic genes on a percentage basis as genome size decreases (Table 10.4 and **Figure 10.10**). Although many genes can be dispensed with, genes that encode the protein-synthesizing apparatus cannot. Thus, the *smaller* the genome, the greater the percentage of genes that encode translational processes. Conversely, the *larger* the genome, the more genes there are for transcriptional regulation and signal transduction (Chapter 7).

Analyses of gene categories have also been done for several *Archaea*. On average, *Archaea* devote a higher percentage of their genomes to energy and coenzyme production than do *Bacteria* (this result is undoubtedly skewed a bit due to the large number of novel coenzymes produced by methanogenic *Archaea*, ▶ Section 14.15 and Figure 14.36). On the other hand, *Archaea* appear to contain fewer genes for carbohydrate metabolism and membrane functions



Soo, R.M., Woodcroft, B.J., Parks, D.H., Tyson, G.W., and P. Hugenholtz. 2015, *PeerJ* 3: e968

Figure 10.9 Functional and metabolic predictions for *Vampirovibrio chlorellavorus* based on genomic annotation. Although the details are beyond our discussion, the figure illustrates the power of genomic sequencing and annotation on piecing together the physiology of an organism. Within the cytoplasmic membrane, the following systems are highlighted: secretion (green), chemotaxis and movement (blue), electron transport (red), ATP-binding cassette transporters (yellow), and permeases/pumps/transporters (orange). Black ovals indicate substrates that enter the glycolysis pathway, while fermentation end products are indicated as black rectangles. Colors of internal compounds correspond to the following: green (amino acids), red (cofactors and vitamins), purple (nucleotides), and orange (non-mevalonate pathway products). Note that genes for synthesis of serine (highlighted in blue) are not present, so presumably it is transported into the cell. Adapted from Soo, R.M., et al. 2015. *Peer J. 3*: e968.

TABLE 10.4 Gene function in some genomes of *Bacteria*

Functional categories	Percentage of genes		
	Escherichia coli (4.64 Mbp)[a]	*Haemophilus influenzae* (1.83 Mbp)[a]	*Mycoplasma genitalium* (0.58 Mbp)[a]
Metabolism	21.0	19.0	14.6
Structure	5.5	4.7	3.6
Transport	10.0	7.0	7.3
Regulation	8.5	6.6	6.0
Translation	4.5	8.0	21.6
Transcription	1.3	1.5	2.6
Replication	2.7	4.9	6.8
Other, known	8.5	5.2	5.8
Unknown	38.1	43.0	32.0

[a]Chromosome size, in megabase pairs. Each organism listed contains only a single circular chromosome.

(such as transport and membrane biosynthesis) than do *Bacteria*. However, this conclusion may also be skewed a bit because the corresponding pathways have been less studied in *Archaea* than in *Bacteria* and many of the relevant archaeal genes remain unidentified.

We now transition to look at the genomes of eukaryotes and their major organelles, structures whose evolutionary roots lie in the *Bacteria*.

Check Your Understanding

- What lifestyle is typical of *Bacteria* and *Archaea* that contain fewer than 500 protein-encoding genes?
- Which is likely to have more genes, a species of *Bacteria* with 8 Mbp of DNA or a eukaryote with 10 Mbp? Explain.
- In prokaryotic cells with the largest genomes, which gene category contains the largest percentage of genes?

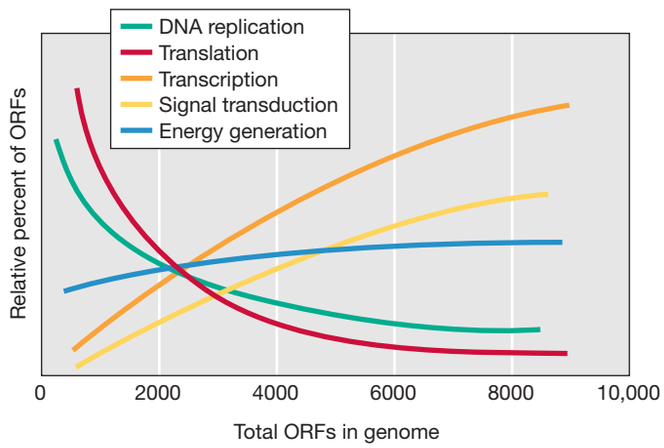


Figure 10.10 Functional category of genes as a percentage of the genome. The percentage of genes encoding products for translation or DNA replication is greater in organisms with small genomes, whereas the percentage of transcriptional regulatory genes is greater in organisms with large genomes.

10.4 Organelle and Eukaryotic Microbial Genomes

Mitochondria and chloroplasts are eukaryotic cell organelles derived from endosymbiotic bacteria (◀ Section 2.14 and ▶ Section 18.1) and thus share many fundamental traits with *Bacteria* to which they are phylogenetically related. The genomes of both organelles encode the machinery necessary for protein synthesis including ribosomes, transfer RNAs, and the other components necessary to drive translation. The genomes of several microbial eukaryotes have also been sequenced (**Table 10.5**), and their size varies widely (Figure 10.2). Certain single-celled protozoans, including the free-living ciliate *Paramecium* (40,000 genes) and the pathogen *Trichomonas* (60,000 genes), have significantly more genes than do humans (Table 10.5). In this section we focus on organellar genomes and the genomes of a few select microbial eukaryotes.

The Chloroplast Genome

Green plant and algae cells contain chloroplasts, the organelles that perform photosynthesis (▶ Section 14.3 and Figure 14.9). Each chloroplast contains several identical copies of the genome. Until recently, it was accepted that all chloroplast genomes were circular DNA molecules. However, with the power of next-generation sequencing, linear and single-stranded plasmid-like chloroplast genomes have also been detected. In fact, most of the chloroplast genomes in corn (maize) are linear in structure. Based on the over 800 chloroplast genomes in the databases, the typical chloroplast genome is about 100–200 kbp and contains two inverted repeats of 6–76 kbp that each encode copies of the three rRNA genes (**Figure 10.11**). As might be expected, many

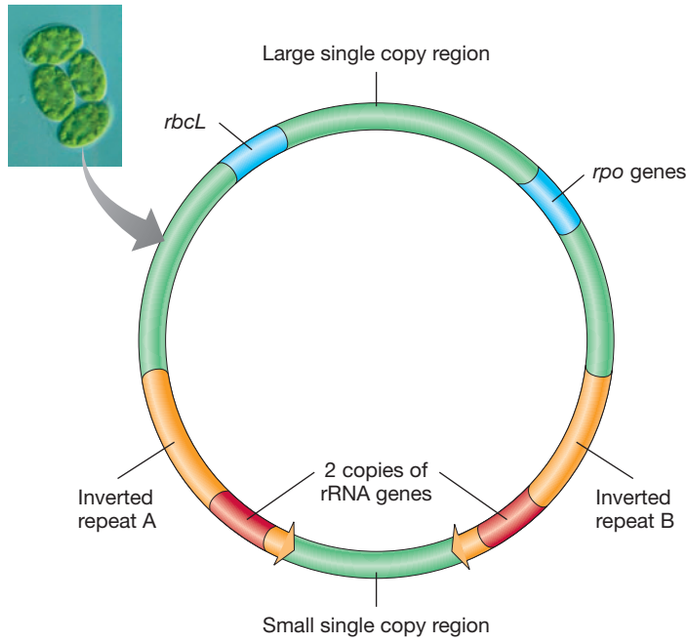


Figure 10.11 Map of a typical chloroplast genome. The inverted repeats each contain a copy of the three genes for rRNA (5S, 16S, and 23S). The large subunit of RuBisCO is encoded by *rbcL* and the chloroplast RNA polymerase by *rpo* genes. Inset: Photo of four cells of the green alga *Makinoella* with chloroplasts clearly visible.

TABLE 10.5 Some eukaryotic nuclear genomes[a]

Organism	Comments	Lifestyle[b]	Genome size (Mbp)	Haploid chromosomes	ORFs
Nucleomorph of *Bigelowiella natans*	Degenerate endosymbiotic nucleus	E	0.37	3	331
Encephalitozoon intestinalis	Smallest known eukaryotic genome, human pathogen	P	2.3	11	1,800
Cryptosporidium parvum	Parasitic protozoan	P	9.1	8	3,800
Plasmodium falciparum	Malignant malaria	P	23	14	5,300
Saccharomyces cerevisiae	Yeast, a model eukaryote	FL	12.1	16	5,400
Ostreococcus tauri	Marine green alga, smallest free-living eukaryote	FL	12.6	20	8,200
Aspergillus nidulans	Filamentous fungus	FL	30	8	9,500
Giardia intestinalis (also called *Giardia lamblia)*	Flagellated protozoan, causes acute gastroenteritis	P	12	5	9,700
Drosophila melanogaster	Fruit fly, model organism for genetic studies	FL	180	4	13,600
Caenorhabditis elegans	Roundworm, model for animal development	FL	97	6	19,100
Mus musculus	Mouse, a model mammal	FL	2,500	23	25,000
Homo sapiens	Human	FL	2,850	23	25,000
Arabidopsis thaliana	Model plant for genetics	FL	125	5	26,000
Paramecium tetraurelia	Ciliated protozoan	FL	72	> 50	40,000
Pinus taeda	Loblolly pine tree	FL	20,000	19	50,000
Trichomonas vaginalis	Flagellated protozoan, human pathogen	P	160	6	60,000

[a]All data are for the haploid nuclear genomes of these organisms in megabase pairs. For most large genomes, both size and ORFs listed are best estimates due to large numbers of repetitive sequences and/or introns in the genomes.
[b]E, endosymbiont; P, parasite; FL, free-living.

chloroplast genes encode proteins for photosynthetic reactions and autotrophy. For example, the enzyme RuBisCO, which is composed of a small and large subunit, catalyzes the first step in CO_2 fixation in the Calvin cycle (◀ Section 3.12 and ▶ Section 14.2). The *rbcL* gene encoding the large subunit of RuBisCO is present on the chloroplast genome (Figure 10.11), whereas the gene for the small subunit, *rbcS*, resides in the plant cell nucleus and its protein product must be imported from the cytoplasm into the chloroplast after synthesis.

The chloroplast genome also encodes tRNAs used in translation, several proteins used in transcription and translation, and some other proteins. Not all chloroplast proteins are encoded by the chloroplast genome; some are nuclear encoded. These are likely genes that migrated to the nucleus as the chloroplast evolved from an endosymbiotic cell into a photosynthetic organelle. Introns, the hallmark of genes in eukaryotes, are common in chloroplast genes and are primarily of the self-splicing type (◀ Section 6.6).

Mitochondrial Genomes and Proteomes

Mitochondria are the eukaryotic cell's respiratory organelles and are present in all but a few eukaryotes (◀ Section 2.14 and ▶ Section 18.1). Mitochondrial genomes primarily encode proteins for oxidative phosphorylation and, like chloroplast genomes, also encode proteins, rRNAs, and tRNAs for protein synthesis. However, most mitochondrial genomes encode far fewer proteins than those of chloroplasts. The largest mitochondrial genome known has only 62 protein-encoding genes, but others contain as few as three. The mitochondria of almost all mammals, including humans, encode only 13 proteins in addition to 22 tRNAs and 2 rRNAs. **Figure 10.12*a*** shows a map of the 16,569-bp human mitochondrial genome. While human mitochondrial genomes are circular, diverse arrangements exist in other organisms. For example, some mitochondrial genomes are linear, including those of certain algae, protozoans, and fungi. Finally, the mitochondria of many fungi and flowering plants contain, in addition to the mitochondrial genome, small circular or linear plasmids (◀ Section 6.2).

Mitochondria require many more proteins than their genome encodes (in particular, proteins needed for translation), and thus many mitochondrial proteins are encoded by genes in the nucleus. The yeast mitochondrion contains as many as 800 different proteins in its proteome (all the proteins encoded by a genome; Section 10.9). However, only eight (~1%) of them are encoded by the yeast mitochondrial genome, the remaining proteins being encoded by nuclear genes (Figure 10.12*b*). The nuclear-encoded proteins required for translation and energy generation in mitochondria are more closely related to their counterparts in *Bacteria* than to those in the eukaryotic cytoplasm, consistent with both the evolutionary history of the mitochondrion (▶ Section 13.4) and with a scenario—like that seen in the chloroplast—of genes having migrated from the original endosymbiont to the host cell nucleus.

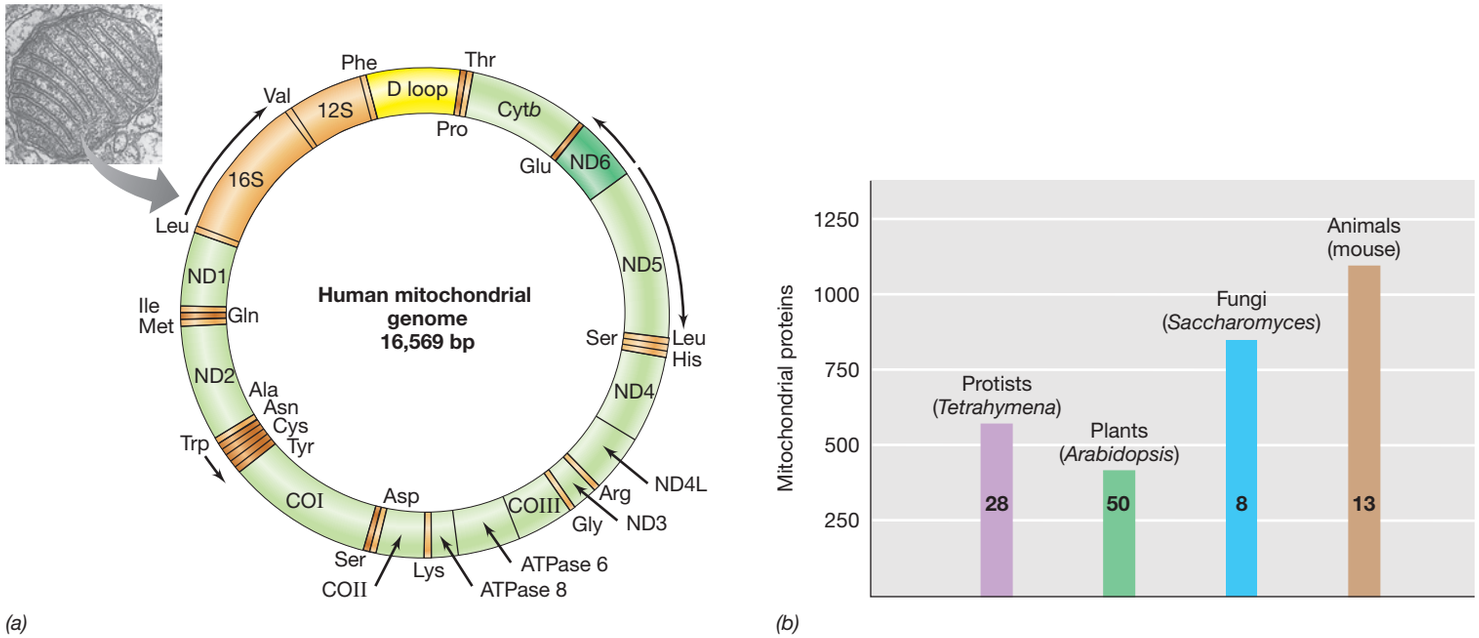


Figure 10.12 **Map of the human mitochondrial genome and the mitochondrial proteome.** *(a)* The genome encodes rRNAs, 22 tRNAs, and several proteins. Arrows show direction of transcription for genes of a given color, and the three-letter amino acid designations for tRNA genes are also shown. The 13 protein-encoding genes are in green. Cyt*b*, cytochrome *b*; ND1–6, components of the NADH dehydrogenase complex; COI–III, subunits of the cytochrome oxidase complex; ATPase 6 and 8, polypeptides of the mitochondrial ATPase complex. The two promoters are in the region called the D loop, which is also involved in DNA replication. Inset: Transmission electron micrograph of a mitochondrion (credit, D.W. Fawcett). *(b)* Mitochondrial proteomes. The numbers in each colored bar are the number of proteins encoded on the mitochondria of some model eukaryotes.

Genomes and Introns in Some Microbial Eukaryotes

Apart from the human pathogenic protozoan *Trichomonas*, which contains almost three times more genes than human cells, parasitic eukaryotic microorganisms typically have relatively small genomes of 10–40 Mbp containing between 4000 and 11,000 genes. For example, *Trypanosoma brucei*, the agent of African sleeping sickness (▶ Section 34.6), has 11 chromosomes, 35 Mbp of DNA, and almost 11,000 genes. The four species of *Plasmodium* that infect humans (causing malaria, ▶ Section 34.5) have genomes ranging from 23 to 27 Mbp arranged in 14 chromosomes containing a total of about 5500 genes.

As in *Bacteria*, the smallest eukaryotic genome belongs to an endosymbiont. Known as a *nucleomorph*, it is the degenerate remains of a eukaryotic endosymbiont of a certain green alga that has acquired the ability to photosynthesize by secondary endosymbiosis (▶ Section 18.1). Nucleomorph genomes range from about 0.37 to 0.85 Mbp. The smallest genome in a *parasitic* eukaryote belongs to *Encephalitozoon intestinalis*, an intracellular pathogen of humans and other animals. *E. intestinalis* even lacks mitochondria, and although its haploid genome contains 11 chromosomes, the genome size is only 2.3 Mbp with approximately 1800 genes (Table 10.5); this is smaller than many bacterial genomes (Table 10.1).

The baker's yeast *Saccharomyces cerevisiae* is widely used as a model eukaryote and its genome contains 16 chromosomes (13.4 Mbp of DNA). Yeast has approximately 6000 ORFs, which is fewer than that of some genomes of *Bacteria* (Tables 10.1 and 10.5). How many of these yeast genes are actually essential? This question has been addressed by systematically inactivating each gene in turn with *knockout mutations* (mutations that completely inactivate genes, ▶ Section 12.4). Knockout mutations cannot normally be obtained in essential genes in a haploid organism. However, yeast can be grown in both diploid and haploid states (▶ Section 18.10). By generating knockout mutations in diploid cells and then investigating whether they can also exist in haploid cells, it is possible to determine whether a particular gene is essential for cell viability. Using knockout mutations, it has been shown that around 900 yeast ORFs (17% of its genome) are absolutely essential. Note that this number of essential genes is much greater than the approximately 300 genes estimated to be the minimal number required in a bacterial cell (Section 10.3).

Being a eukaryote, the yeast genome contains introns (◀ Section 6.6). However, the total number of introns in the protein-encoding genes of yeast is a mere 225. Most yeast genes that contain introns have only a single small intron near the 5′ end of the gene. This situation differs greatly from that seen in more complex eukaryotes (**Figure 10.13**). For example, in the worm *Caenorhabditis elegans*, the average gene has five introns, and in the fruit fly *Drosophila*, the average gene has four. Introns are also common in the genes of plants, averaging around four per gene. The model flowering plant *Arabidopsis* averages five introns per gene, and over 75% of *Arabidopsis* genes have introns. In humans almost all protein-encoding genes have introns, and it is common for a single gene to have 10 or more. Moreover, introns in human genes are typically much longer than exons, the DNA that actually encodes proteins. Indeed, exons make up only about 1% of the human genome, whereas introns account for 24%. The remaining

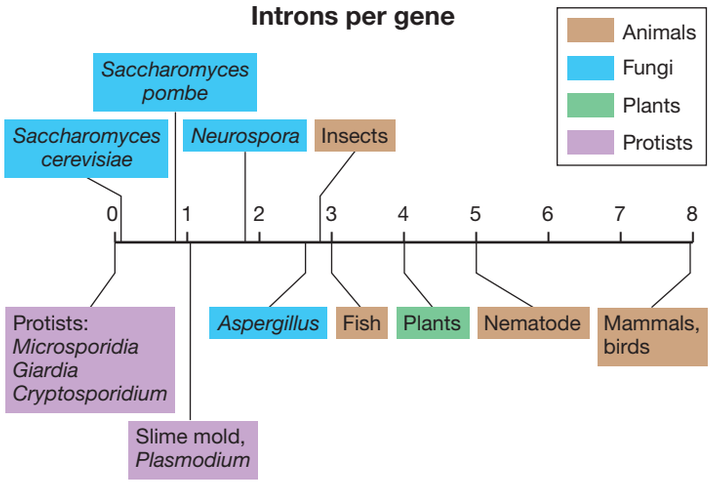


Figure 10.13 Intron frequency in the genes of different eukaryotes. The average number of introns per gene is shown for a range of eukaryotic organisms; microbial species tend to have fewer introns per gene, whereas plants and animals have the most.

DNA is made up of repetitive sequences, noncoding RNA, and regulatory regions.

We will discuss how comparative genomics can be used to determine evolutionary relationships and how genomes evolve in Chapter 13. For now, we turn our focus to how various omic approaches can be used to determine the function of each gene product. The dynamic nature of microbes and how they interact with their environment can be characterized through the use of functional omics.

Check Your Understanding

- What is unusual about the genes that encode mitochondrial proteins?
- What do chloroplast genomes typically encode?
- What is unusual about the genome of the eukaryote *Encephalitozoon*?

II • Functional Omics

Knowing an organism's genome sequence may not reveal what all the genes encode and when and why they are transcribed and translated. These topics require functional analyses of molecular events downstream of the genome itself.

Despite the major effort required to generate an annotated genome sequence, the net result is simply a "list of parts." To understand how a cell *functions*, we need to know more than which genes are present. We must also understand (1) gene expression, (2) the function of gene products, (3) the activity of the proteins made, and (4) the metabolites produced during growth.

In analogy to the term "genome," the entire complement of RNA, proteins, or metabolites produced under a given set of conditions is called the *transcriptome*, *proteome*, and *metabolome*, respectively. The suffix "omic" denotes their corresponding areas of study. Table 10.6 summarizes some of the "omics" terminology used in microbiology today.

10.5 Functional Genomics

As previously discussed (Section 10.2), genome sequencing, assembly, and annotation yields an abundance of information. However, the roles of many open reading frames (ORFs) remain unknown after annotation and are thus classified as encoding "hypothetical proteins." The percentage of hypotheticals in a given microbial genome averages 30% of the total annotated ORFs. This value even holds true for the minimal *Mycoplasma* genome created by synthetic biologists, which possesses a trim 473 genes (▶ Section 12.12). In fact, the function of less than 1% of the approximately 120 million protein sequences that exist in public databases is known! Thus, obtaining a genome sequence is only the *beginning* of teasing apart how a microbe functions and survives in its environment.

In this unit of the chapter we discuss how to gain insight into gene function through the analysis of RNA, protein, and metabolites, and we begin with how comparative genomics, genetic tools, and next-generation sequencing can be used to determine gene function.

UNIT 3

Functional Genomics and Heterologous Expression

How do microbes get selected for genome sequencing? This selection is usually a result of an interesting phenotypic trait displayed by the microbe. Such was the case with the multi-antibiotic-resistant,

TABLE 10.6 Some omics terminology

DNA	**Genome** the total complement of genetic information of a cell or a virus **Metagenome** the total genetic complement of all the cells present in a particular environment **Epigenome** the total possible epigenetic changes **Methylome** the total methylated sites on the DNA (whether epigenetic or not) **Mobilome** the total set of mobile genetic elements in a cell **Resistome** the total set of antibiotic resistance genes in a cell
RNA	**Transcriptome** the total RNA produced in an organism under a specific set of conditions **Exome** the part of the RNA pool encoded by exons that remains after introns are removed
Protein	**Proteome** the total set of proteins encoded by a genome; sometimes also used in place of *translatome* **Translatome** the total set of proteins present under specified conditions **Interactome** the total set of interactions between proteins (or other macromolecules) **Secretome** the total set of proteins secreted by a cell **Kinome** the total set of protein kinases encoded by a genome
Metabolites	**Metabolome** the total complement of small molecules and metabolic intermediates **Glycome** the total complement of sugars and other carbohydrates
Organisms	**Microbiome** the total complement of microorganisms in an environment (including those associated with a higher organism) **Virome** the total complement of viruses in an environment **Mycobiome** the total complement of fungi in a natural environment

gram-positive bacterium *Paenibacillus* species strain LC231 (**Figure 10.14**). While *Bacteria* displaying resistance to multiple drugs is not unique (▶ Section 28.7), strain LC231 was cultured from an underground cave ecosystem that has been isolated from the surface for over 4 million years (Figure 10.14*a*). With no exposure to current pathogens (where it could have picked up genes by horizontal transfer, Chapter 9) or to antibiotics used in clinical or veterinary medicine, this bacterium displayed resistance—surprisingly—to at least 14 different classes of antibiotics! How did such resistance come about?

In an effort to understand the gene products or *resistome* (Table 10.6) responsible for the multidrug resistance of strain LC231, bioinformatics revealed ten ORFs known to encode resistance to seven different types of antibiotics (Figure 10.14*b*). This comparative genome analysis was facilitated by an online program called *Resistance Gene Identifier*, which allows for genome sequences to be searched against a database of known antibiotic resistance genes from other bacteria. However, the mechanism by which LC231 was resistant to seven *other* antibiotic types was not evident from comparative genomics. To attack this question, microbiologists used a common functional genomics approach that employs the model bacterium *Escherichia coli*. This approach is based on *heterologous expression*, which is the process of expressing a gene from one organism in a different, host organism (Chapter 12). To heterologously express LC231 genes in *E. coli*, the LC231 genome was fragmented and inserted into plasmids. The resulting plasmids were transformed into *E. coli* to create a clone library of transformants, with each transformant containing a plasmid with a different piece of the LC231 genome (Figure 10.14*a* and Chapter 12). *E. coli* colonies that resulted from this clone library were then screened and selected for resistance to the antibiotics of interest. To determine the identity of the LC231 genome fragment responsible for conferring antibiotic resistance to

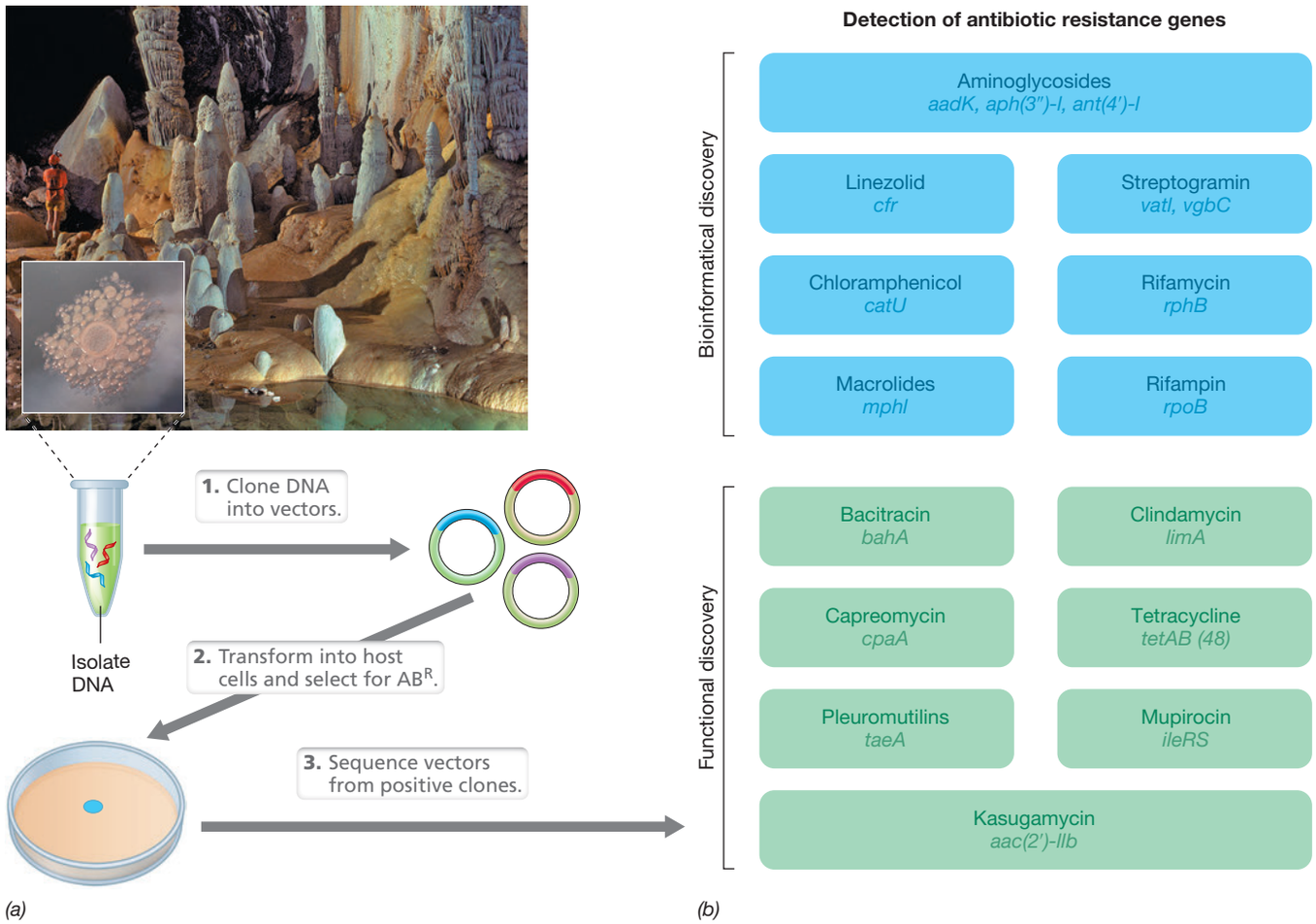


Figure 10.14 Functional genomics and discovery of new antibiotic resistance genes. *(a)* Heterologous expression of *Paenibacillius* strain LC231 DNA and selection of antibiotic-resistant transformants for sequence analysis. DNA from *Paenibacillus* strain LC231, isolated from Lechuguilla Cave, Carlsbad Caverns, New Mexico, USA (inset photo of *Paenibacillus* colonies courtesy of L. Ejim, C. Groves, and G. Wright), is extracted and inserted into plasmids for expression in *Escherichia coli*. Plasmid DNA from antibiotic-resistant *E. coli* colonies is sequenced and analyzed for the presence of new genes conferring antibiotic resistance (AB^R). *(b)* Discovery of genes conferring resistance to 14 different types of antibiotics in *Paenibacillus* strain LC231. Gene names are listed under the type of antibiotic they confer resistance to. Genes discovered by genome database searches are in blue, while genes identified by functional genomics and heterologous expression in *E. coli* from part *a* are in green. Data adapted from Pawlowski, A.C., Wenliang, W., Koteva, K., Barton, H.A., McArthur, A.G., and Wright, G.D. 2016. *Nat. Commun. 7*: 13803.

the transformant, the corresponding plasmid DNA was isolated and sequenced (Figure 10.14*a*).

The functional genomic screen in *E. coli* resulted in "mining" five new antibiotic resistance genes that had not been previously discovered (Figure 10.14*b*). Two of these genes encode novel enzymes that modify the antibiotics bacitracin and capreomycin by an amidohydrolase (an enzyme that removes an amine group) and acetyltransferase (an enzyme that adds an acetyl group), respectively; such modifications inactivate the antibiotics. While its environmental isolation limits strain LC231 from spreading its resistome to pathogens—assuming that deep subsurface materials are not exposed to Earth's surface from natural catastrophes or human activities—comparative genomics has shown that the genomes of at least some subsurface-dwelling bacteria contain novel antibiotic resistance determinants that represent an untouched pool of antibiotic resistance. This discovery highlights the importance not only of monitoring antibiotic use in clinical and veterinary microbiology, but also of developing mechanisms to inhibit newly discovered antibiotic-modifying enzymes. With the abundance of mobile genetic elements, or **mobilome** components (Table 10.6 and ▶ Section 13.9), that exist in microbial genomes in nature, given enough time, these new antibiotic resistance genes will undoubtedly be transferred to other species and could someday emerge in human pathogens.

The Utility of Comparative Genomics: Discovery of New Antiphage Systems

In Chapter 9 we discussed some barriers to horizontal gene transfer that are employed by *Bacteria* and *Archaea*. While the role of many of these microbial "immune systems" was discovered quite by accident, the omics era has allowed for similar defense systems to be identified in thousands of genomes by searching for similar gene sequences in the databases. In the process of annotating these restriction modification, phage exclusion, and CRISPR systems (◀ Section 9.12), computational biologists have noticed that many of the genes encoding these systems are clustered next to each other within the genome on what are called *chromosomal islands* (▶ Section 13.10). Besides containing the systems mentioned above, these chromosomal islands also contain numerous open reading frames (ORFs) of unknown function (Section 10.2).

Using comparative genomics, molecular biologists have aligned the sequences in these chromosomal islands using a process similar to that described for genome assembly and annotation in Section 10.2. This alignment resulted in the discovery of *homologs* (ORFs possessing similar sequence and evolutionary origin; ▶ Section 13.8) to be enriched next to genes encoding known defense systems in the genomes of many species (**Figure 10.15*a***). Besides the similar gene sequence and length, these unknown homologs are often found surrounded by other ORFs oriented in the same direction in many genomes. Both proximity and shared direction of transcription are strong predictors of an operon and suggest that the gene products are dependent on one another. To test if these conserved ORFs did encode antiphage systems, scientists expressed the genes in model bacteria and tested the new host's resistance to bacteriophage infection by using a range of viruses. This study resulted in the discovery of *over ten* new antiphage systems and highlights both the constant arms race between microbes and their viruses and the previously hidden antiviral capacities present in bacterial genomes.

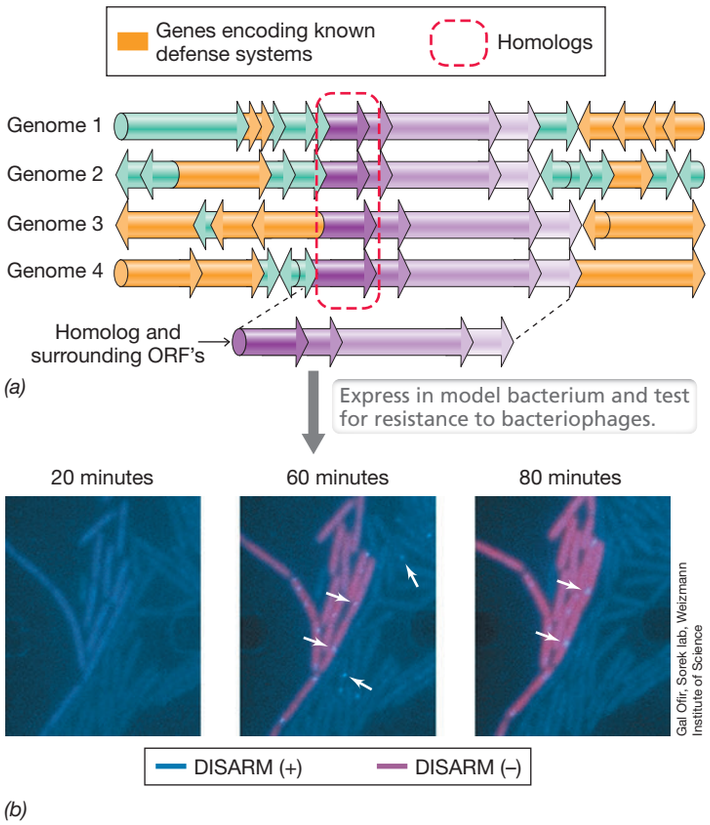


Figure 10.15 Comparative genomics and discovery of the DISARM antiphage system. *(a)* Alignment of a chromosomal island region from four separate genomes containing genes encoding known antiphage systems (yellow). A region containing unannotated homologs sharing strong sequence similarity (denoted by red dashed box) with adjacent ORFs in the same orientation is selected for functional analysis in a model bacterium. *(b)* Expression of the DISARM (*d*efense *i*sland *s*ystem *a*ssociated with *r*estriction–*m*odification) antiphage system in *Bacillus subtilis*. Cells expressing the DISARM system are labeled blue, while those without DNA encoding the resistance system are labeled red. The genome of the bacteriophage added to the cells contains a DNA binding site for a fluorescently tagged protein present in both cell types. As the bacteriophage genome is replicated, more fluorescent protein binds and the blue-white foci enlarge (white arrows; 60- and 80-minute time points). Over time, bacteriophage genomes are prevented from replicating in cells expressing the DISARM system (blue cells) as indicated by the relative absence of blue-white foci.

Figure 10.15*b* illustrates the antiphage activity of the DISARM (*d*efense *i*sland *s*ystem *a*ssociated with *r*estriction–*m*odification) system that was isolated from *Bacillus paralicheniformis*. Heterologous expression of this system in *Bacillus subtilis* renders the modified bacterium resistant to eight distinct tailed bacteriophages (◀ Section 5.2) because their corresponding viral DNA does not get replicated in cells expressing the DISARM system. Conversely, cells not expressing DISARM are still replicating bacteriophage DNA 80 minutes postinfection (Figure 10.15*b*). While the exact mechanism behind the DISARM system is still being elucidated, it does encode a methylase—an enzyme that modifies the *host's* DNA—and hence likely functions in a way similar to other restriction modification systems by degrading the unmethylated bacteriophage DNA (◀ Section 9.12).

Check Your Understanding

- What does the term resistome mean?
- How can genes encoding antibiotic resistance be identified using heterologous expression systems?

10.6 High-Throughput Functional Gene Analysis: Tn-Seq

Besides the heterologous expression and related systems just discussed, if genetic studies can be performed in a microbe whose genome has been sequenced, the functional role of its genes can be characterized by constructing a library of gene mutants that allow for gene analysis on an enormous scale. The most common and random way to generate these mutants is through transposon (Tn) mutagenesis (◀ Section 9.11).

Because transposons encode antibiotic resistance genes along with their insertion sequences, Tn mutants with gene interruptions can be selected using an antibiotic that allows growth of only cells containing the transposon (such cells are resistant to the antibiotic). The application of this technique was illustrated in Figure 9.35 where a gene product that plays a role in biofilm formation was identified in *Pseudomonas aeruginosa*. However, this application required the screening of *individual* transposon mutants. By contrast, the development of next-generation sequencing technology has allowed for *high-throughput* screening of mutants. To be considered high-throughput screening, automated devices are used to gather sequence data on a large set of strains or mutants in parallel. This advancement in screening capabilities combined with transposon mutagenesis has been instrumental in the development of a powerful functional genomics tool called *transposon insertion site sequencing* (Tn-Seq). The utility of Tn-Seq is based on three premises: (1) a transposable element exists that is flanked by a specific nucleotide sequence called a *restriction site* (▶ Section 12.2) that guides a nuclease enzyme to cut at a specific chromosomal sequence outside of the transposon insertion locus; (2) distinct Tn mutants can be generated that display different *fitness profiles* in a pooled culture depending on the genomic insertion, location, and growth condition screened; and (3) it is possible to determine the chromosomal location of each transposon insertion and quantify the number of each Tn mutant in a mixed pool (**Figure 10.16**).

If a gene product of unknown function is beneficial or essential for the survival of a cell under a specific growth condition, its corresponding Tn mutant will display *decreased* fitness and be present at low frequency in a mixed culture of competitive transposon mutants. Conversely, if *elimination* of a gene product is beneficial under certain growth conditions, the corresponding Tn mutant will display *increased* fitness and be present at a high ratio compared to less competitive Tn mutants within the pool. Mutant fitness or abundance is measured by extracting genomic DNA from the pooled culture after exposure to a certain growth condition and digestion with a restriction enzyme (Figure 10.16*c*; ▶ Section 12.2) that recognizes a nucleotide sequence 20 base pairs outside of the transposon. Next-generation sequencing methods are then used to add adapters, amplify, and sequence the short region of chromosomal DNA next to the Tn insertion site (Figure 10.16*c*), which can then be mapped back to the genome.

Tn-Seq has been used to characterize novel genes encoding competence (◀ Section 9.6) in *Streptococcus mutans*, persistence (◀ Section 8.12) in pathogenic *Escherichia coli*, antibiotic resistance in *Enterococcus*, and amino acid biosynthesis in the anaerobic sulfate-reducing bacterium *Desulfovibrio*. Figure 10.16 illustrates a Tn-Seq scheme used to characterize cellular components in *Caulobacter crescentus*, a model bacterium for studying the cell cycle (◀ Section 8.8), that lead to susceptibility to bacteriophage ϕCbK infection. Tn-Seq studies of a pool of Tn mutants subjected to increasing concentrations of ϕCbK resulted in the recovery of Tn mutants with insertions in genes that encode the phage receptor protein with the highest frequency (Figure 10.16*c*, *d*). This phage receptor is essential for the transport of the phage DNA into the host and ultimate infection (Figure 10.16*b* and ◀ Section 5.4). However, mutants with interruptions in genes that encode proteins that help phage to initially "find" and adsorb to the bacterium, such as those encoding the flagellum and pili, decreased in abundance as more phage were added to the culture (Figure 10.16*c*, *d*). Tn mutants recovered at an intermediate level in the pool possessed insertions in genes needed to replicate the ϕCbK genome (Figure 10.16 *c*, *d*). These mutants have the same adsorption and infection rates as the wild type but are less efficient at producing new ϕCbK.

Because of the power of Tn-Seq, scientists have modified the technique for new discoveries. Current fitness studies can miss single-cell phenotypes as some of these Tn mutants can obtain resources from other mutants in the pool. To prevent mutants from sharing cellular metabolites, a technique called *microfluidics* is used to encapsulate individual cells in a manner similar to that discussed for single-cell genomics in Section 10.11. In this way, cross-feeding is prevented, and previously unrecognized phenotypes can be detected.

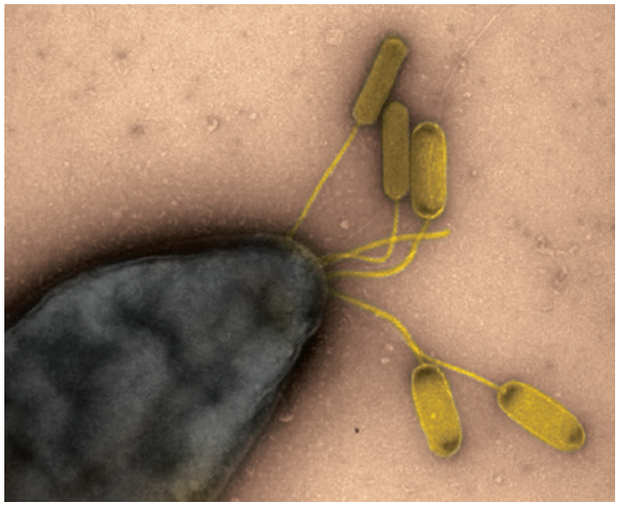
With this background in functional genomics, we now switch gears to study how multiple—as compared to single—genomes can be studied simultaneously using a powerful form of omics that can reveal the diversity of single target genes or the entire genomic complement of virtually any environmental sample.

Check Your Understanding

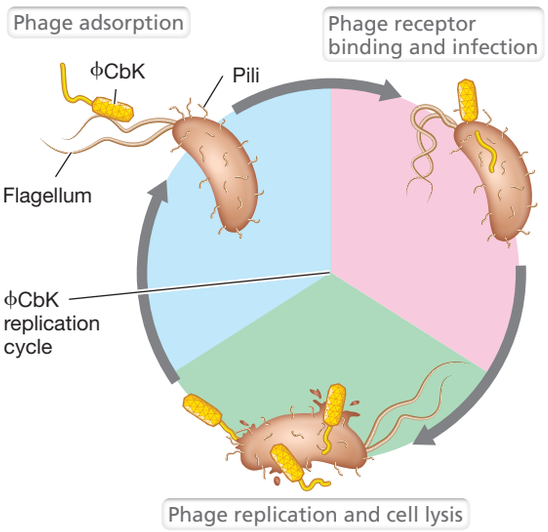
- What are the three premises that Tn-Seq is based on?
- How can a gene essential to a bacterium's survival be identified using Tn-Seq?

10.7 Metagenomics

Microbial communities contain many microbial species, many of which have never been cultured or formally identified. **Metagenomics** is the science that analyzes pooled DNA or RNA from an environmental sample containing organisms that have not been isolated and identified (**Figure 10.17**). Just as the total gene content of an organism is its *genome*, so the total gene content of a microbial community is its **metagenome** (Table 10.6). In addition to metagenomic analyses based on DNA sequencing, analyses based on RNA or proteins—metatranscriptomics (Section 10.8) or metaproteomics (Section 10.9), respectively—may be used to explore the patterns of gene expression in natural microbial communities. With today's molecular technology, these studies can even be done on individual cells (Section 10.11).



(a)



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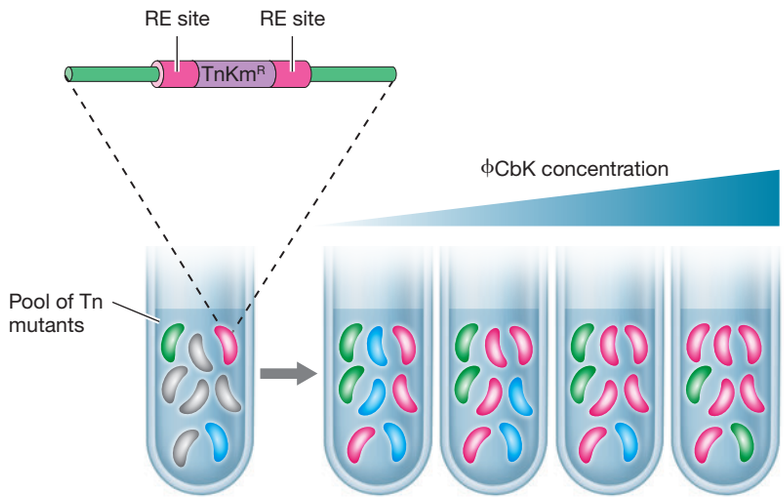
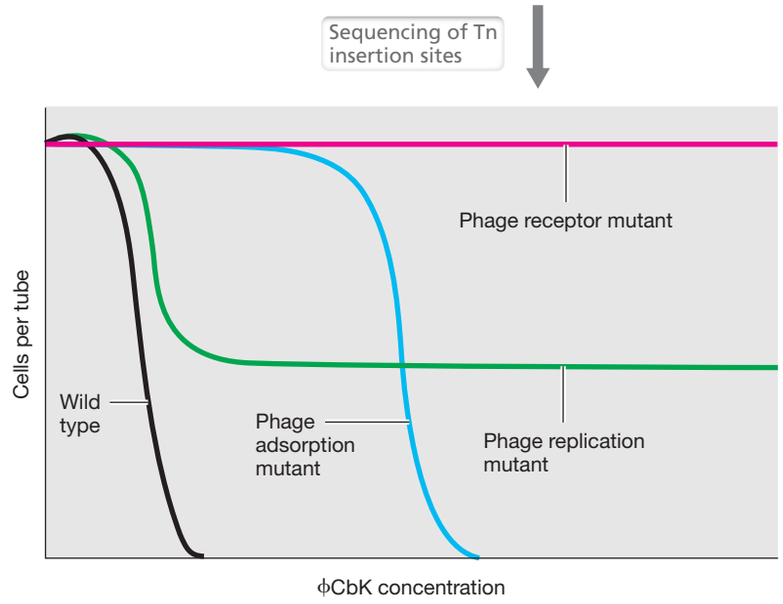


(c)



(d)

Figure 10.16 Tn-Seq and fitness analysis of *Caulobacter crescentus* mutants during bacteriophage infection. *(a)* Electron micrograph of bacteriophage ϕCbK binding to the flagellated cell pole of a cell of *C. crescentus*. *(b)* Replication cycle of ϕCbK. ϕCbK adsorbs to the flagellum and pili of *C. crescentus* and then injects its genome through the bacteriophage-specific receptor. The host cell then replicates the ϕCbK genome and new viral particles are made prior to cell lysis. *(c)* A library of transposon (Tn) mutants (◀ Section 9.11) is constructed and exposed to increasing numbers of ϕCbK particles. These mutants contain a Tn that possesses terminal DNA sequences that are recognized by a special restriction enzyme (RE). This RE cuts the chromosomal DNA at sites outside of the Tn insertion. As the pool of Tn mutants is exposed to increasing concentrations of ϕCbK, mutants that are resistant to the bacteriophage survive at a greater rate and thus display increased fitness. Cell colors denote mutants in gene products necessary for the corresponding ϕCbK replication cycle in part *b*. *(d)* Tn-Seq analysis of Tn mutant pools following infection with ϕCbK. Total DNA from the Tn mutant pools is extracted, cut with the RE mentioned in part *c*, and sequenced by next-generation methods. The chromosomal location of the Tn insertion site determined from sequencing is plotted versus the concentration of ϕCbK. The data show that phage receptor mutants are most resistant. Line colors correspond to Tn mutants in the ϕCbK replication cycle as depicted in part *b*. Data adapted from Christen, M., et al. 2016. *J. Mol. Biol. 428*: 419.

Examples of Metagenomic Studies

While several examples of metagenomic studies are presented in this text, two simple examples are highlighted here. Several environments have been surveyed by large-scale metagenome sequencing projects. Extreme environments, such as highly acidic runoff waters from mining operations (▶ Section 22.2), tend to have low microbial species diversity. Consequently it has been possible to isolate community DNA (and metabolites, Section 10.10) and assemble much of it into nearly complete individual genomes. Conversely, complex environments such as fertile soils or aquatic environments are much more challenging, and complete genome assemblies here are more difficult, yet possible (▶ Section 19.8). A surprising finding that has emerged from metagenomic studies is that the majority of genes recovered from natural habitats do not originate from cells

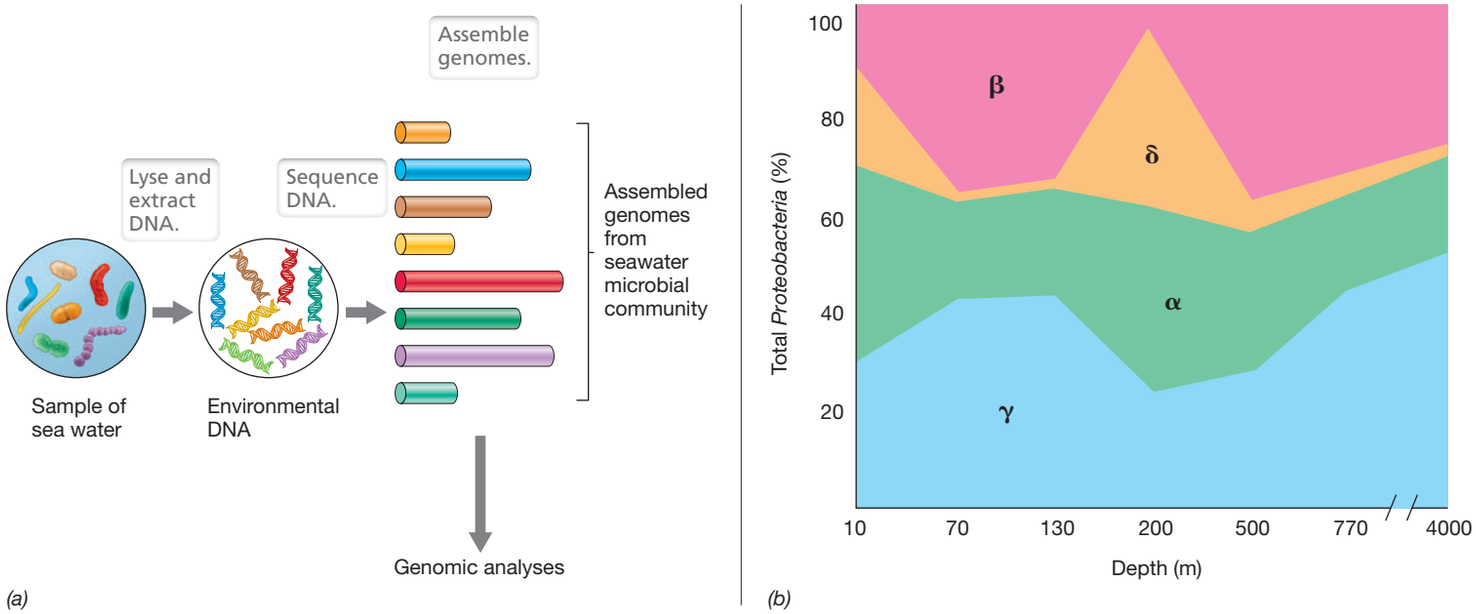


Figure 10.17 Metagenomics and the microbiome. *(a)* Isolation, sequencing, and identification of DNA from a sample of seawater. *(b) Proteobacteria* in the ocean. The distribution with depth of the major subgroups (alpha α, beta β, gamma γ, and delta δ) of *Proteobacteria* in the Pacific Ocean is shown. Many other types of bacteria are also present (not shown). Data adapted from Kembel, S.W., Eisen, J.A., Pollard, K.S., and Green, J.L. 2011. *PLoS One 6*: e23214.

but from viruses.This is discussed further in Chapter 11 where we consider the genomics and phylogeny of viruses.

Even if complete genomes cannot be assembled from environmental DNA, much useful information can be derived from metagenomic surveys (▶ Section 19.8). For example, environments can be analyzed for the presence and distribution of specific microbial groups. These vary greatly in relative abundance in different environments, and Figure 10.17*b* illustrates this for subgroups of *Proteobacteria* (a major phylum of gram-negative *Bacteria*, Chapter 16) at a sampling site in the Pacific Ocean near the Hawaiian Islands. Light, oxygen, nutrients, and temperature all change with depth in a water column, and these factors can be correlated with proteobacterial subgroups to show which are most competitive at each depth (Figure 10.17*b*). One curious observation that has emerged from such metagenomic studies is that much DNA in natural habitats does not reside in *living* cells. Around 50–60% of the DNA in the oceans is extracellular DNA present in deep-sea sediments. Presumably this was DNA deposited when dead organisms from the upper layers of the ocean sank to the bottom and lysed. Because nucleic acids are major reservoirs of phosphate, marine sediment DNA is thought to be a major component of the global phosphorus cycle.

Metagenomics and "Biome" Studies

The human body is estimated to contain about 10 trillion (10^{13}) cells, but each of us also carries around ten times more prokaryotic cells than human ones. This collection of prokaryotic cells is called the human *microbiome* (Chapter 24). Most of these organisms inhabit the large intestine, with the majority belonging to one of two phylogenetic groups of *Bacteria*, the *Bacteroidetes* and the *Firmicutes* (Chapter 16). A fascinating finding is that the composition of the gut microbiome correlates with obesity in both humans and experimental mouse models. The data show that the higher the proportion of *Firmicutes* (mostly species of *Clostridium* and relatives) in the gut, the more obese is the human or mouse. A suggested mechanism that explains this finding is that fermentative species of *Firmicutes* convert more dietary fiber into fatty acids that can be absorbed by the host (▶ Section 24.9). In this way, the obese host gets more usable organic carbon than the thinner host from the same amount of food.

Recent surveys of the human and mouse gut microbiome have also revealed the rather surprising finding that over 60 species of fungi (eukaryotic microbes, Chapter 18) are present (**Figure 10.18**). These constitute the gut *mycobiome* (the prefix "myco" means fungal). Many fungi, typically nonpathogenic yeasts, inhabit the skin, the oral cavity, and virtually all moist surfaces on the human body. Many of these are common and generally harmless yeasts, such as *Saccharomyces, Cladosporium*, and most species of *Candida*. Most of these also are found in the gut, although some gut fungi—such as *Aspergillus* and *Trichosporon*—are potential serious pathogens (Chapter 34). Moreover, although gut fungi constitute less than 1% of the total human microbiome, it is known that certain conditions such as inflammatory bowel disease and some cases of obesity correlate strongly with specific fungal populations. Hence, metagenomics holds great promise for exploring possible connections between specific microbial populations and specific diseases in humans and other animals. Moreover, in cases where a clear cause-and-effect relationship is strongly suspected, metagenomics also holds great promise as a clinical tool for making medical diagnoses.

In the next two sections we travel beyond the genome to explore the technology for examining *gene expression*: omic methods that can reveal the RNAs and proteins encoded by the genome.

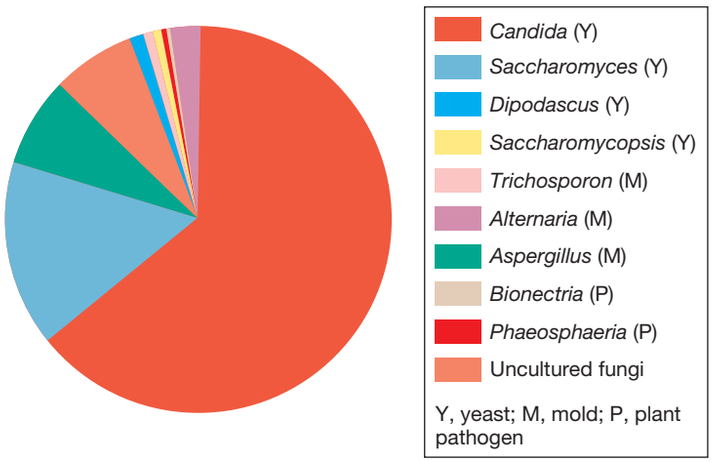


Figure 10.18 **The mouse mycobiome.** The data shown represent the relative amount of different fungal genera of the mouse intestine. The pie chart shows the most common fungi present are yeasts. Data adapted from Iliev, I.D., et al. 2012. *Science 336*: 1314.

Check Your Understanding

- What is a metagenome? The mycobiome?
- How is a metagenome analyzed?

10.8 Gene Chips and Transcriptomics

Once a genome sequence is available, the sequence can be used to synthesize miniature devices that can be used to detect genes from specific microbes, determine genome differences between closely related strains of the same species (for example, the presence of chromosomal islands), identify genome sequences that are bound by specific DNA-binding proteins, and measure gene expression. *Transcriptomics* refers to the study of a cell's global transcription and is done by monitoring the **transcriptome**, the total RNA generated under a chosen growth condition. Besides the aforementioned reasons for doing transcriptomics, the technique can also put a functional label on genes that have been annotated as simply encoding "hypothetical proteins." In these cases, discovering the *conditions* under which these genes are transcribed often yields clues to their function. Two main approaches are used in transcriptomics: *microarrays* and *RNA-Seq*.

Microarrays and the DNA Gene Chip

Microarrays are small, solid supports to which genes or, more often, oligonucleotides corresponding to segments of genes are fixed and arrayed spatially in a known pattern; they are often called **gene chips** (Figure 10.19*a*). Microarrays measure the DNA or RNA that hybridizes to the DNA sequences on the chip. When DNA is denatured (that is, the two strands are separated), the single strands can form hybrid double-stranded molecules with other nucleic acid molecules by complementary or almost complementary base pairing (Figure 10.19*b*; ▶ Section 12.1). This process is called *nucleic acid hybridization*, or **hybridization** for short, and is widely used in detecting, characterizing, and identifying segments of DNA or RNA. The single-stranded segments of nucleic acid, whose identity is already known, are called **nucleic acid probes** or, simply, *probes*. To detect hybridization to the probes, the nucleic acid added to the chip must be labeled with a fluorescent dye and then the hybridized chip is scanned with a laser fluorescence detector that measures which of the probes contain hybridized DNA (Figure 10.19*b*, *c*).

Mastering Microbiology
Art Activity: Figure 10.19 DNA chip design and application

Gene chips are typically about 1 to 2 cm and are inserted into a plastic holder that can easily be manipulated (**Figure 10.20*a***); each chip holds thousands of different DNA fragments in a known order. In practice, each gene is usually represented more than once in the array to increase reliability. Whole genome arrays contain DNA segments that cover the entire genome of an organism. For example, a chip that covers the entire human genome (Figure 10.20*a*) can analyze over 47,000 human transcripts and has room for 6500 additional oligonucleotides for use in clinical diagnostics.

UNIT 3

Measuring Gene Expression and Other Uses of Gene Chips

In a gene expression microarray, the probes are designed and synthesized for each gene based on the genomic sequence. Once attached to the solid support, the DNA segments can be hybridized with labeled RNA from cells grown under specific conditions, and the microarray is then scanned and analyzed by computer. Because mRNA levels are typically too low for use directly, the mRNA

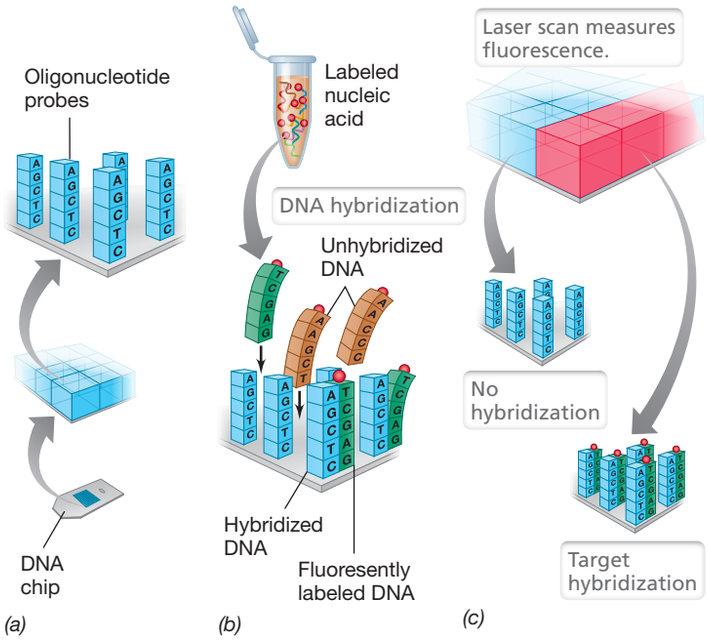
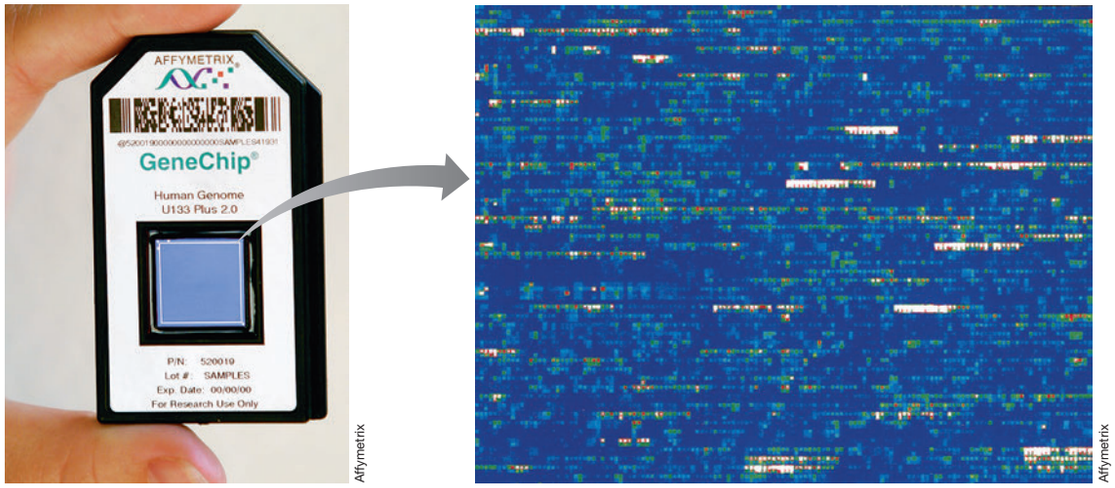


Figure 10.19 **DNA chip design and application.** *(a)* DNA chip design. Short single-stranded oligonucleotides (probes) corresponding to each gene in an organism or to diagnostic sequences corresponding to numerous organisms are synthesized and affixed at known locations to make a microarray. *(b)* Microarray hybridization. The presence of specific DNA or RNA (in the form of cDNA) is assayed by hybridizing fluorescently labeled samples (DNA or cDNA) to the DNA probes on the chip. Labeled DNA or cDNA will bind to the probes on the chip if they possess sequence complementarity. *(c)* Analysis of microarray hybridization. A scanning laser is used to identify regions of the chip where labeled nucleic acid has bound to the probes.



(a) (b)

Figure 10.20 Using gene chips to assay gene expression. *(a)* The human genome chip contains over 47,000 gene fragments. Blowup from part *a* to part *b* indicates location of actual microarray. *(b)* A hybridized yeast chip shows fragments from a quarter of the genome of baker's yeast, *Saccharomyces cerevisiae*. Each gene is present in several copies and has been probed with fluorescently labeled cDNA (derived from mRNA) from yeast cells grown under a specific condition. The background of the chip is blue. Locations where the cDNA has hybridized are indicated by a gradation of colors up to a maximum number of hybridizations, which shows as white. Because the location of each gene on the chip is known, when the chip is scanned, it reveals which genes were expressed.

sequences are first amplified and converted into DNA using a modified version of the polymerase chain reaction (PCR) that converts RNA to *complementary DNA* (cDNA, ▶ Section 12.1).

To monitor global gene expression, total RNA (or cDNA) from a test sample is hybridized to an array of oligonucleotides corresponding to the entire genome. Figure 10.20*b* shows part of a chip containing probes for over 6000 protein-encoding genes of the yeast *Saccharomyces cerevisiae*. After hybridizing yeast cDNA to the chip, a distinct hybridization pattern is observed, and the fluorescence and its intensity reveal both which genes were expressed and at what level (Figure 10.20*b*); these data yield the *transcriptome* of the yeast culture grown under specified conditions (Table 10.6). Using such analyses, gene expression under different growth conditions can be measured. For example, in yeast—which can grow by either fermentation or respiration (Chapter 3)—transcriptome analyses have shown that genes that control production of ethanol (a key yeast fermentation product) are strongly repressed while genes encoding citric acid cycle functions (needed for aerobic growth) are strongly activated when the organism is shifted from fermentative to respiratory conditions. Overall, over 700 genes are turned on and more than 1000 turned off during this metabolic transition. In "shift" experiments of this type, the expression pattern of genes of unknown function is also revealed, and analysis of these expression patterns sometimes yields valuable clues to the cellular function of these unknown proteins.

Microarrays can also be used to identify specific microbes. For example, identification (ID) chips have been used in the food industry to detect DNA sequences unique to specific pathogens, such as the gastrointestinal pathogen *Escherichia coli* O157:H7 (Table 10.1), an occasional foodborne pathogen (▶ Section 33.11). In environmental work, microarrays called *PhyloChips* have been used to assess microbial diversity. These contain oligonucleotides complementary to the 16S rRNA of different bacterial species, a molecule widely used in microbial systematics (▶ Sections 13.11 and 13.12). After extraction of bulk DNA or RNA from an environment, the presence or absence of a given species can be assessed by the hybridization response on the chip (▶ Section 19.7). Although ID chips and PhyloChips can be made highly specific, the inexpensive nature of DNA isolation, sequencing, and analysis have made metagenomic approaches (Section 10.7) to the identification of specific pathogens or phylogenetic groups in natural samples the preferred method of assessment.

RNA-Seq Analysis

RNA-Seq analysis is a transcriptomic method in which all the RNA molecules from a cell are converted to DNA (cDNA, ▶ Section 12.1) and then sequenced. Provided that the genome sequence is available for comparison, RNA-Seq reveals both which genes were transcribed and how many RNA copies of each gene were made. Because RNA-Seq targets *all* transcripts, it is ideal for measuring the expression of mRNA, to identify long untranslated regions, and to discover noncoding RNAs. RNA-Seq requires high-throughput sequencing (second-generation sequencing, Section 10.2) and is complicated a bit by the fact that the most abundant RNA in a cell is ribosomal RNA (rRNA). Nevertheless, methods are available to remove rRNA or enrich mRNA and primary transcripts from a total RNA pool. In addition, recent advancements in sequencing technology may allow sequencing without removing rRNA.

RNA-Seq has overtaken microarray analysis as the method of choice for global studies of gene expression. The data from transcriptomic experiments can be presented in the form of a *heat map*, which uses different colors to show the level of gene expression. For example, **Figure 10.21*a*** identifies gene clusters that are upregulated (more

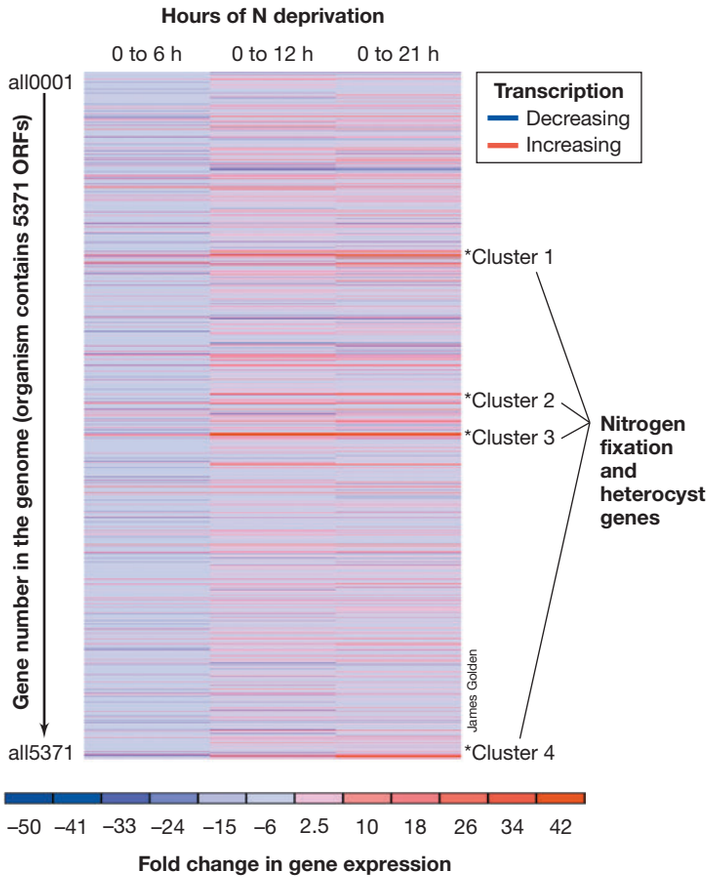


Figure 10.21 RNA-Seq analysis of the heterocyst-forming cyanobacterium *Anabaena* during nitrogen starvation. Cyanobacteria are oxygenic phototrophs and only some species, such as those that form heterocysts, can fix nitrogen under fully oxic conditions. *(a)* Heat map of gene expression 6, 12, and 21 h after cells were starved for fixed nitrogen. Genes that display increased expression are in red, whereas those that display decreased expression are in blue. Gene clusters 1–4 all encode proteins linked to nitrogen fixation. *(b)* Mapping of RNA-Seq reads. The arrows indicate the annotated open reading frames, and the plots underneath correspond to the number of sequencing reads detected for each chromosomal nucleotide position. Note that the negative numbers represent chromosomal positions upstream of the predicted start codon for the genes. The genes *psbB* and *petF* encode key proteins of photosynthesis, and *nrrA* encodes a protein that regulates heterocyst formation (the heterocyst is the site of N_2 fixation, ◀ Section 3.12). The pigments, diversity, and general biology of cyanobacteria are discussed in Sections 14.3, 14.4, 14.6 and 15.3. Parts *a* and *b* modified from Flaherty, B.L., F. van Nieuwerburgh, S.R. Head, and J.W. Golden. 2011. *BMC Genomics 12*: 332.

intensely transcribed) during nitrogen deprivation in the heterocyst-forming cyanobacterium *Anabaena*, a phototroph that can use N_2 as its nitrogen source (nitrogen fixation, ◀ Section 3.12). Gene clusters 1–4 represent increased expression of nitrogen fixation and heterocyst formation genes (heterocysts are the site of N_2 fixation) as the time of nitrogen deprivation increases (Figure 10.21*a*). RNA-Seq data can also be used for transcript mapping by plotting the sequences read against the genome annotation. Figure 10.21*b* illustrates the sequencing coverage at each base along the open reading frame regions for *psbB*, *petF*, and *nrrA*, genes that encode two key proteins needed for photosynthesis and a regulator protein for heterocyst formation in *Anabaena*, respectively (◀ Section 8.9). These plots demonstrated long 5′ untranslated regions present in these transcripts, which may be sites where specific DNA-binding proteins attach in regulatory events (Chapter 7).

Transcript abundance under different culture conditions can also be analyzed using RNA-Seq data, as indicated by a comparison of cultures of a *Clostridium* species in exponential and stationary phase (**Figure 10.22**). Clostridia are gram-positive bacteria that produce endospores, the highly resistant and dormant stage of the cell's life cycle (◀ Section 2.8); the general properties of sporulating clostridia are discussed in Section 16.8. As one might predict, transcription of genes of the glycolytic pathway (the major means by which the organism makes ATP) is elevated during exponential growth, whereas expression of sporulation genes increases in stationary phase, when nutrients become limiting (◀ Section 8.6). RNA-Seq is also being used for microbial community analysis and can provide information on relative transcription levels when a genome sequence is not available for comparison. In this case the sequences detected must be identified by homology with sequences present in databanks.

As discussed in Section 10.7, metagenomics is the genomic analysis of pooled DNA or RNA obtained from organisms in an environment. Metagenomic analysis using RNA-Seq can be exploited for culturing microbes from nature that had previously resisted

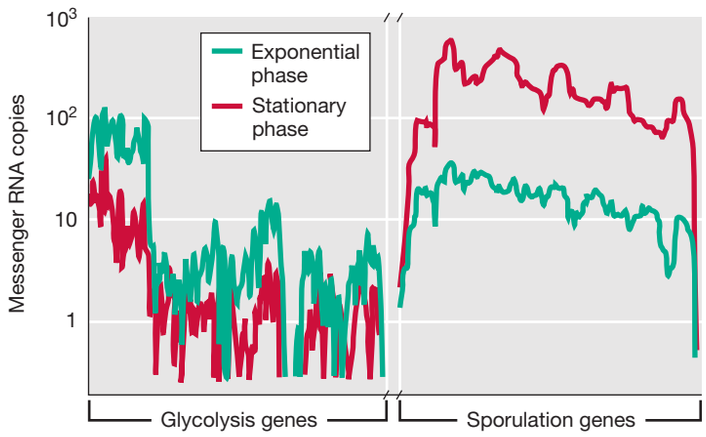


Figure 10.22 Transcriptomic analysis of sporulation genes in *Clostridium*. RNA-Seq analysis of cultures of a *Clostridium* species grown for 4.5 h (cells in exponential phase) or 14 h (cells in stationary phase). Two genomic regions are shown: (left) ~5.4-kb segment surrounding the *gap-pgk-tpi* glycolytic operon, and (right) ~1.2-kb segment surrounding the *cotJC-cotJB* sporulation operon. Production of endospores is triggered by nutrient starvation. Data from Wang, Y., X. Li, Y. Mao, and H.P. Blaschek. 2011. *BMC Genomics 12*: 479.

laboratory culture. This can be accomplished by using RNA-Seq to identify highly transcribed genes in the microbial community that contain the microbe of interest, followed by sequence analyses to identify the proteins encoded by the highly transcribed genes. These data can be used by microbiologists to deduce which nutrients the microbe is most likely using given the predicted enzymatic activities of these proteins. Culture media can then be devised using this information as a guide to successfully culture the previously uncultured.

We transition now from nucleic acid analyses to protein analyses to reveal the importance of other omics to modern-day microbiology and biology in general.

Check Your Understanding

- Why is it useful to survey expression of the entire genome under particular conditions?
- What do microarrays tell you that studying gene expression by assaying individual enzymes cannot?
- What technological advances does RNA-Seq depend on?

10.9 Proteomics and the Interactome

The genome-wide study of the structure, function, and activity of an organism's *proteins* is called **proteomics**. The number and types of proteins in a cell change in response to the cell's environment or other factors, such as developmental cycles. As a result, the term **proteome** has unfortunately become ambiguous. In its wider sense, a proteome refers to *all* the proteins encoded by an organism's genome. In its narrower sense, however, it refers to those proteins present in a cell *at any given time*. The term **translatome** has also been used to describe the latter; that is, it refers to every protein made by a cell *under specific conditions*. Recall from Chapter 7 that the extensive regulatory systems of *Bacteria* and *Archaea* tightly control which proteins are made under any given set of nutrients and environmental conditions. An understanding of which proteins are synthesized under a given set of conditions—the goal of proteomics—complements transcriptomics data and is the "gold standard" for confirming that a transcript detected by transcriptomics was actually translated.

Methods in Proteomics

To identify individual proteins within a mixed pool of proteins, proteomics relies on some form of *mass spectrometry*, a method that can also be used to identify metabolites (Section 10.10). The mass of ^{12}C is defined as exactly 12 molecular mass units (daltons). However, the masses of other atoms, such as ^{14}N or ^{16}O, are not exact integers. Mass spectrometry using extremely high mass resolution techniques, which can distinguish between slightly different mass-to-charge ratios, allows the unambiguous determination of the molecular formula of any small molecule. Thus mass spectrometry can be used to identify the amino acid sequence of several peptides in a sample.

Figure 10.23 illustrates a standard proteomic analysis, which begins with extraction of total protein from a sample. To increase sensitivity, liquid chromatography is typically used to separate protein mixtures. In high-pressure liquid chromatography (HPLC), a protein sample is dissolved in a suitable liquid and forced under pressure through a special column that separates proteins by differences in their chemical properties, such as size, charge, or hydrophobicity. Fractions are collected at the end of the column, the proteins in each fraction are digested by protease enzymes, and the resulting peptides are identified by mass spectrometry. The amino acid sequence of these peptides can then be searched against the translation of a genome to identify the presence of specific proteins (Figure 10.23).

MALDI (*m*atrix-*a*ssisted *l*aser *d*esorption *i*onization) is an advanced version of mass spectrometry that does not require the protein separation and digestion step (Figure 10.23). Instead the sample is affixed to a matrix and then ionized and vaporized by a laser (**Figure 10.24**). The ions generated are accelerated along the column toward the detector by an electric field. The time of flight (TOF) for each ion depends on its mass/charge ratio—the smaller this ratio, the faster the ion moves. The detector measures the TOF for each ion, and the computer calculates the mass and hence the molecular formula. The combination of these two techniques is known as *MALDI-TOF*.

Utility of Proteomics: Profiling Protein Modifications

Not only can proteomics identify proteins that are abundant under certain growth conditions, but it can also be modified to detect post-translational modifications (◀ Section 7.16). For example, because mass spectrometry spectra are known for all proteins of the *Mycobacterium tuberculosis* (the causative agent of tuberculosis, ▶ Section 31.4) proteome, additional chemical groups attached to peptides can also be identified. These chemical modifications include phosphorylation, glycosylation, methylation, pupylation (addition of a ubiquitin-like molecule), lipidation, and acetylation.

Figure 10.25 illustrates the modified proteome of *M. tuberculosis* and the role of the modified proteins in the phenotype of the pathogen. This analysis shows that some modifications can be enriched in functional categories, such as lipidation of proteins that

Figure 10.23 Proteomics. Total protein is isolated from a culture or environmental sample. This protein pool is converted to fractions of small peptides using various denaturation, separation, and digestion methods. Following ionization of the peptide fractions, the ions are characterized using mass spectrometry (M.S). Peaks from the M.S. are compared to standards, and the primary amino acid sequence of the resulting peptides is identified and quantified. The M.S. peaks can also be used to determine if the peptides possess post-translational modifications (◀ Section 7.16 and see Figure 10.25).

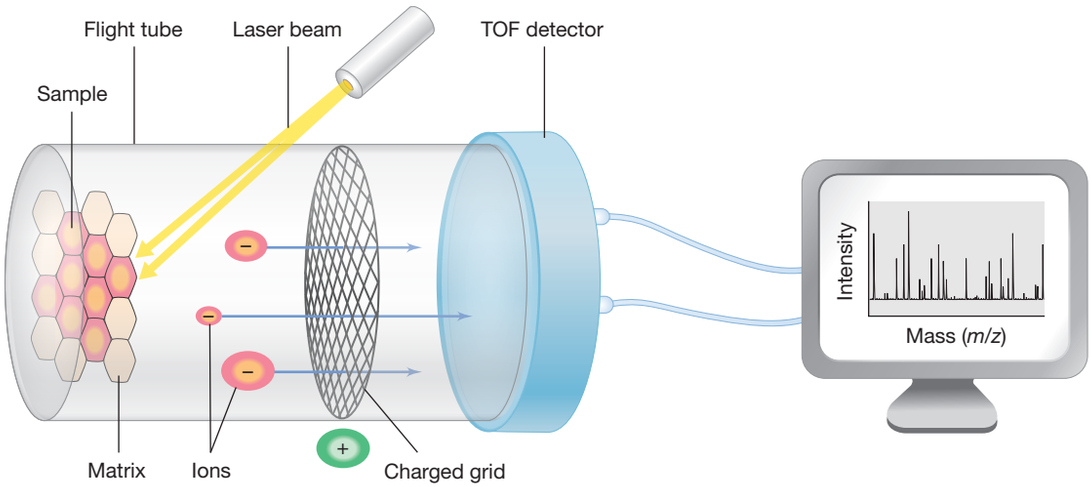


Figure 10.24 MALDI-TOF mass spectrometry. In matrix-assisted laser desorption ionization (MALDI) spectroscopy, the sample is ionized by a laser and the ions travel down the tube to the detector. The time of flight (TOF) depends on the mass/charge (*m*/*z*) ratio of the ion. The computer identifies the ions based on their time of flight, that is, the time it takes to reach the detector.

participate in cell wall biosynthesis (the cell wall of *M. tuberculosis* contains an abundance of lipids that assist in its virulence and play a major role in its acid-fast staining properties, ▶ Sections 16.11 and 31.4). Pupylation, first discovered to occur in *Bacteria* by studying *M. tuberculosis*, is believed to direct proteins to a cellular degradation pathway and is enriched with proteins associated with virulence and metabolism. These protein modifications may play a role in the pathogenesis of *M. tuberculosis* as well as the host's immune response during infection by engaging eukaryotic cell receptors or antigen processing pathways. By studying the modified proteome, immunologists may also be able to identify novel epitopes (portions of an antigen where antibodies attach) as vaccine candidates (▶ Section 28.3).

While proteomic analyses can be performed on pure cultures of specific microbes, *metaproteomics* of environmental samples are particularly insightful for revealing the collective metabolic potential of a microbial community. As will be illustrated in Chapter 19, metaproteomics combined with metagenomic and metatranscriptomic approaches can provide a snapshot of ongoing physiological processes in a microbial community and highlights the power of omics for answering the key questions about the community of "who's there" and "what are they doing" (▶ Figure 19.8).

The Interactome

By analogy with the terms genome and proteome, the **interactome** is the complete set of *interactions* among the macromolecules within a cell (**Figure 10.26**). Originally, the word *interactome* was applied only to interactions between proteins, many of which assemble into complexes. However, it is also possible to consider interactions between different classes of macromolecules, such as between protein and RNA (see the protein–RNA interactome in Figure 10.34*b*).

Interactome data are typically expressed in the form of network diagrams, with each node representing a protein and the connecting lines representing the interactions. Diagrams of whole interactomes

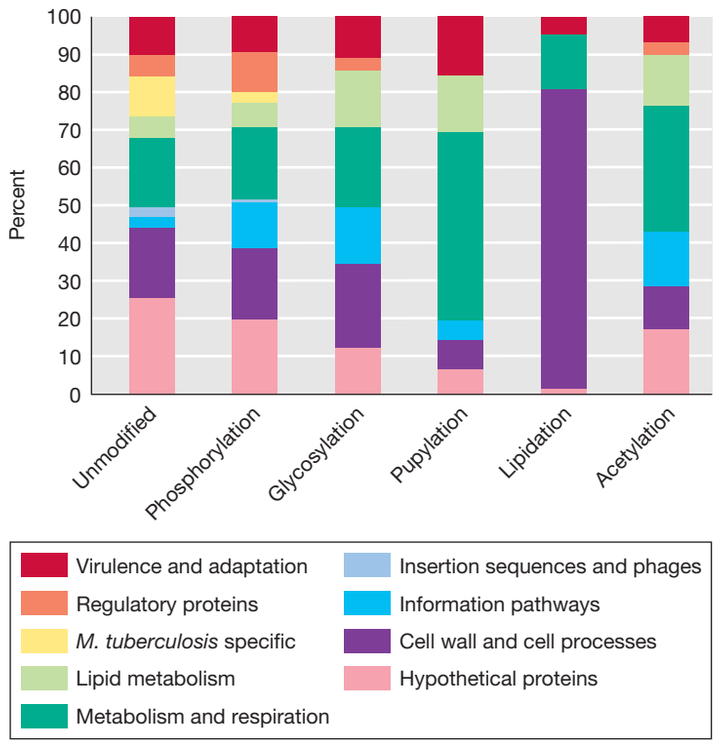


Figure 10.25 Modified proteome of *Mycobacterium tuberculosis*. *M. tuberculosis* is the causative agent of the human disease tuberculosis, and its biology is discussed in ▶ Sections 16.11 and 31.4. Post-translational modifications (◀ Section 7.16) found on different categories of *M. tuberculosis* proteins. The "*M. tuberculosis* specific" category of proteins were first detected in *M. tuberculosis* and possess conserved Pro-Glu/Pro-Pro-Glu amino acid sequence motifs (Section 10.12). Many of these *M. tuberculosis*–specific proteins are believed to be secreted into host cells because they contain an N-terminal signal sequence (◀ Sections 6.12 and 6.13). Data adapted from Banaei-Esfahani, A., Nicod, C., Aebersold, R., and Collins, B.C. 2017. *Curr. Opin. Microbiol. 39*: 64.

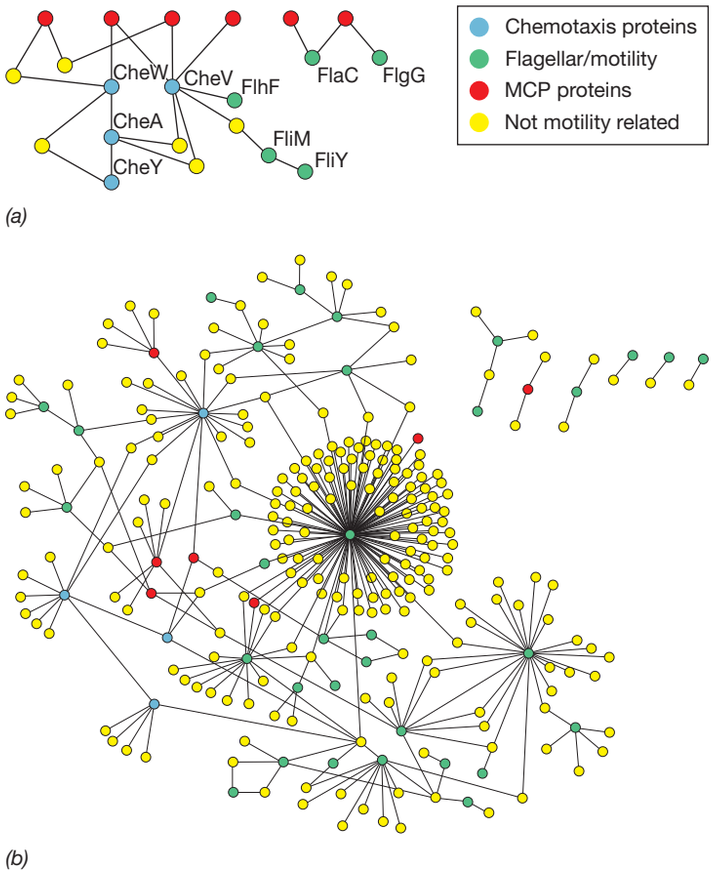


Figure 10.26 Motility protein interactome for *Campylobacter jejuni*. This network illustrates the way in which interactome data are depicted. *(a)* A subsection of the network highlighting the well-known proteins of the chemotaxis signal transduction pathway (CheW, CheA, and CheY) and their partners. MCP, methyl-accepting chemotaxis proteins (◀ Section 7.6). *(b)* High-confidence interactions between all proteins known to have roles in motility. Note the six small networks that fall outside the single large network.

can be extremely complex (see Figure 10.33), and thus more focused interactomes, such as the motility protein network from the bacterium *Campylobacter jejuni* (Figure 10.26), are more instructive. This figure shows the core interactions between well-known components of the chemotaxis system (◀ Sections 2.11 and 7.6), including all other proteins that are known to interact with these.

There is an omics that deals specifically with metabolism, and we tackle that next.

Check Your Understanding

- Why is the term "proteome" ambiguous, whereas the term "genome" is not?
- What are the most common experimental methods used to survey the proteome?
- What is the interactome?

10.10 Metabolomics

The **metabolome** is the complete set of *metabolic intermediates* and small molecules produced by an organism. In other words, the metabolome reflects the enzymatic pathways encoded by the genome. While transcriptomics and proteomics point to the activity of specific pathways, metabolomic data confirm that these potential reactions and pathways are actually occurring in the cell at any given time or under any given set of conditions.

Advances in Metabolomic Techniques: NIMS

Metabolomics has lagged behind other omics due largely to the immense chemical diversity of small metabolites present in cells (Chapters 3 and 14). This makes systematic metabolomic screening technically challenging. Early attempts used nuclear magnetic resonance (NMR) analysis of extracts from cells labeled with ^{13}C-glucose (^{13}C is a heavy isotope of carbon, most of which is ^{12}C). However, this method is limited in sensitivity, and the number of metabolites that can be identified in a mixture simultaneously is too low for resolution of complete cell extracts.

While MALDI-TOF mass spectrometry (Section 10.9) can be used to detect and identify metabolites, *n*anostructure-*i*nitiator *m*ass spectrometry (NIMS) is a more useful technique that can directly analyze biological samples without the need for special analytical preparation (**Figure 10.27**). Thus biofluids, tissues, cultures, or even individual cells can be analyzed. In NIMS, a laser is used to ionize the sample—just as in MALDI-TOF—but the silicon-coated surface used in NIMS does not generate the background interference seen during ionization of a matrix by MALDI-TOF. This allows for the accurate identification of small metabolites present at low concentrations and increased spatial resolution during tissue imaging. Modifications to the silicon surface can also be made to detect notoriously difficult molecules, such as structurally similar carbohydrates or steroids. These traits also make NIMS more sensitive than MALDI, which is illustrated by the ability of NIMS to detect a specific drug in a single human cell in the yoctomole (10^{-24} M) range (Figure 10.27).

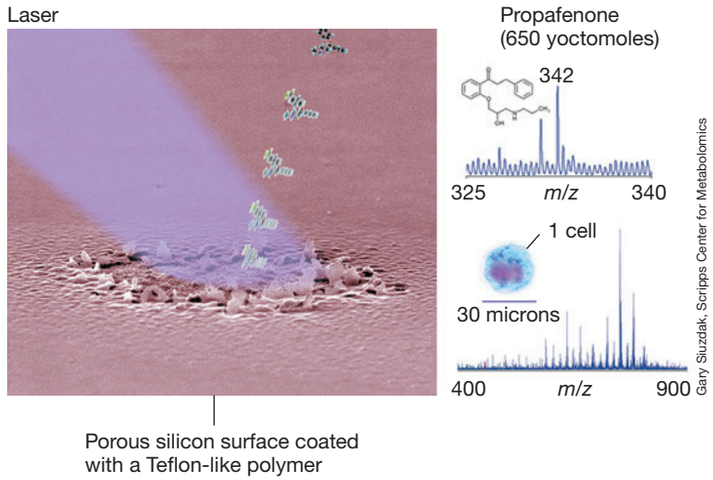


Figure 10.27 NIMS identification of metabolites. In nanostructure-initiator mass spectrometry (NIMS), a cell is placed on the silicon initiator surface and vaporized using a laser. The resulting ionized metabolites within a single cell are then detected using mass spectrometry as indicated by the mass-to-charge plots. Peak 342 represents the metabolite propafenone (1 yoctomole = 1×10^{-24} mole). Because NIMS lacks a matrix, it has extremely high sensitivity and resolution. Ionized metabolites are represented rising from the surface.

Utility of Metabolomics

Metabolome analysis has been particularly useful for the study of plant biochemistry, since plants produce several thousand different metabolites—more than most other types of organisms. These compounds include many so-called *secondary metabolites*, chemicals such as scents, flavors, alkaloids, and pigments, many of which are commercially important. Metabolomic investigations have monitored the levels of several hundred metabolites in the model plant *Arabidopsis*, and significant changes were observed in the levels of many of these metabolites in response to changes in temperature, an alarming hint that global climate change will likely alter plant metabolism in major ways. Metabolomics can also be done on microbial cultures as well as natural microbial communities such as biofilms (◀ Sections 4.9 and 8.10; ▶ Section 20.4). For example, microbiologists have detected over 3500 different metabolites in a relatively simple microbial biofilm growing in extremely acidic (pH ~0.9) and heavy-metal-rich water draining from an abandoned iron mine site in northern California (USA). Many of these metabolites were suspected of being osmolytes and other protective molecules used by the microbial community inhabiting the runoff for combating the osmotic and other life stressors in this extreme environment.

Metabolomics has also been deployed to help characterize the human microbiome (Chapter 24). For instance, our skin epidermis is composed not only of human cells but also of microbes that contribute to epithelial health. Thus metabolites formed by skin cells, associated microorganisms, and personal hygiene products are present. **Figure 10.28** shows results of an actual study of the pattern of metabolites and microbial diversity on the skin of a human male and a female plotted simultaneously on a three-dimensional topographical heat map of the human body. One of the goals of this type of research is to determine if the presence of particular metabolites can be linked to the presence of particular microbial species. Such a picture of the human skin could offer microbiologists as well as medical clinicians a better understanding of the diversity and ecology of the skin microbiome and pave the way for the future use of studies of this type in the diagnosis of skin health or disease.

As expected, the omics study of the skin (Figure 10.28) revealed many microbes known from previous studies of the skin microbiome (▶ Section 24.5). But the study also discovered a diverse mixture of metabolites. While common chemicals produced by human skin cells such as triacylglycerides and diacylglycerides were readily detected, various metabolites resulting from microbial processing of these compounds were also detected. Overall, the level of metabolite diversity detected on specific areas of the body did not correlate well with microbial diversity. Instead, the complexity of the human skin metabolome significantly exceeded the diversity of the microbial profile, and this indicates that each species is likely producing several distinct metabolites. Interestingly, and somewhat surprisingly, the results also showed that personal hygiene products are a major source of metabolites on human skin.

Throughout this chapter we have discussed various omics and their applications as more or less individual entities. We now shift our focus to integrating multiple omics to better understand the entire organism—the major goal of systems biology.

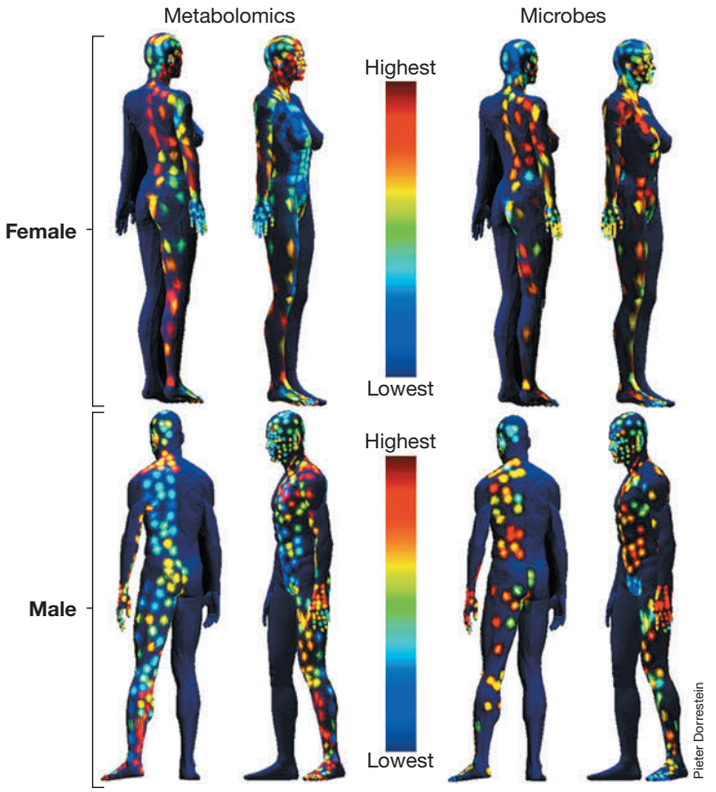


Figure 10.28 Metabolomic and metagenomic mapping of skin. Three-dimensional heat maps represent the diversity of metabolites and microorganisms detected on various areas of the skin on a human male and a female. Red indicates the highest level of diversity and purple indicates the lowest level of diversity.

Check Your Understanding

- What techniques are used to monitor the metabolome?
- What is a secondary metabolite?

III • Systems Biology

The many omics born from the genomics revolution have been used in concert to attack complex problems in both single cells and entire microbial communities. The omics complement each other in ways that allow scientists to develop models that can be tested by laboratory experimentation.

The basic strategy of **systems biology** is to generate comprehensive models for predicting the behavior or properties of an organism that were not obvious from pre-omics era observations. These are referred to as the *emergent properties* of the organism. Understanding an organism's emergent properties provides a deeper insight into its overall biology than can any single omics study by itself. Systems biology integrates the numerous omic datasets covered in Part II of this chapter to create insightful models of the biological workings of an individual organism or an entire microbial community (**Figure 10.29**). We begin by seeing how all of these can come together in ecological studies of individual cells in a microbial community.

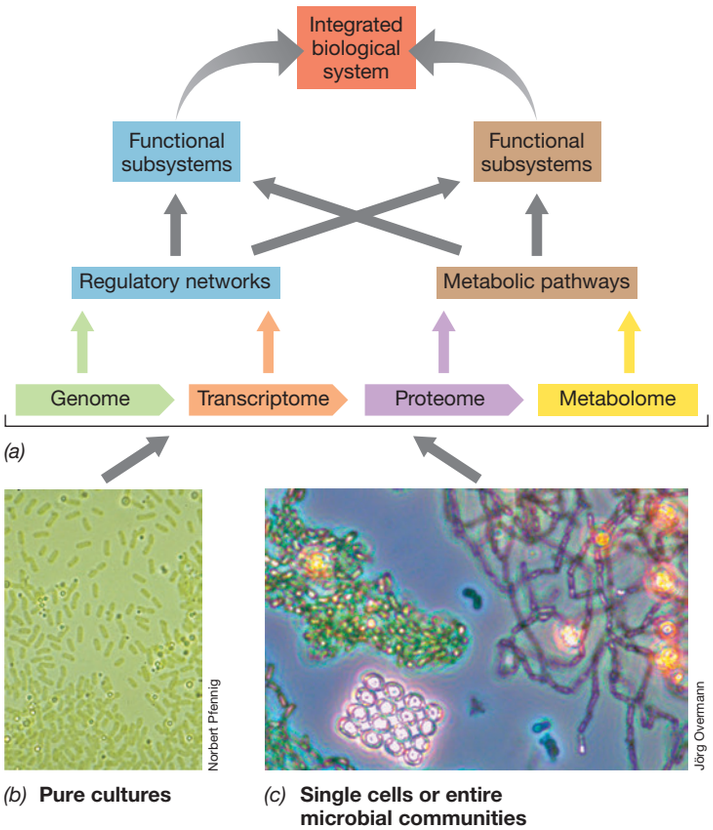


Figure 10.29 The components of systems biology. *(a)* The results of various "omics" analyses are combined and successively integrated into higher-level views of the entire biology of a pure culture, such as *(b)* that of the green sulfur bacterium *Chlorobium*; or of a mixed microbial community, such as *(c)* that of phototrophic sulfur bacteria obtained from a lake; or of a single cell isolated from a microbial community (see Figure 10.30).

10.11 Single-Cell Genomics

Besides sequencing total environmental DNA as described for metagenomics (Section 10.7), the genomes of individual cells can also be sequenced—a technique called *single-cell genomics* (*SCG*) (**Figure 10.30**). This is possible because of the ability to amplify tiny amounts of DNA. Single-cell genomics is critical for studying the metabolic potential of microorganisms in natural microbial communities. While metagenomic analysis of a microbial community can detect the presence of genes specific to certain pathways, it is difficult to discern whether the pathways are contained within the same organism. Besides genome sequencing, transcriptome and proteome analyses can also be performed on individual cells, leading to a comprehensive omics study on one component of a microbial ecosystem.

Cell Isolation and Sample Preparation

The ability to isolate cells is obviously essential for single-cell genomics, and various physical techniques including dilution in microwells (▶ Section 19.3), encapsulation, and *f*luorescence-*a*ctivated *c*ell *s*orting (FACS) have been used to do so. For encapsulation, the sample is diluted and added to sterile oil to form microdroplets. Approximately 30% of the resulting droplets from this technique will contain only one cell (**Figure 10.31**). Combining droplet encapsulation with FACS, which is able to optically detect single cells, allows droplets that do not contain a cell to be rejected. This method of single-cell isolation has also been shown effective in isolating single virions for viral omics analyses.

Sequencing DNA from single cells relies on a modified version of the polymerase chain reaction (PCR, ▶ Section 12.1) called *multiple displacement amplification* (*MDA*) (Figure 10.31; ▶ Section 19.12 and Figure 19.43). This PCR technique uses a highly sensitive viral DNA polymerase to amplify the femtogram (10^{-15} g) quantities of DNA present in a single bacterial cell into the micrograms (10^{-6} g) of DNA required for sequencing (a billionfold amplification). However, because of its very sensitivity, contamination is one of the biggest problems with MDA. Contaminating DNA can originate from the sample itself or from the laboratory equipment and reagents. If great care is not taken in isolating the cell and amplifying its genome, contaminating DNA can make up half or more of the reaction products and create major problems for genome assembly and further genomic analyses. In addition to analysis of a cell's genome, its RNA can also be analyzed using RNA-Seq following amplification to form cDNA by a modified version of PCR (Section 10.8). Single-cell proteomic analyses are trickier than

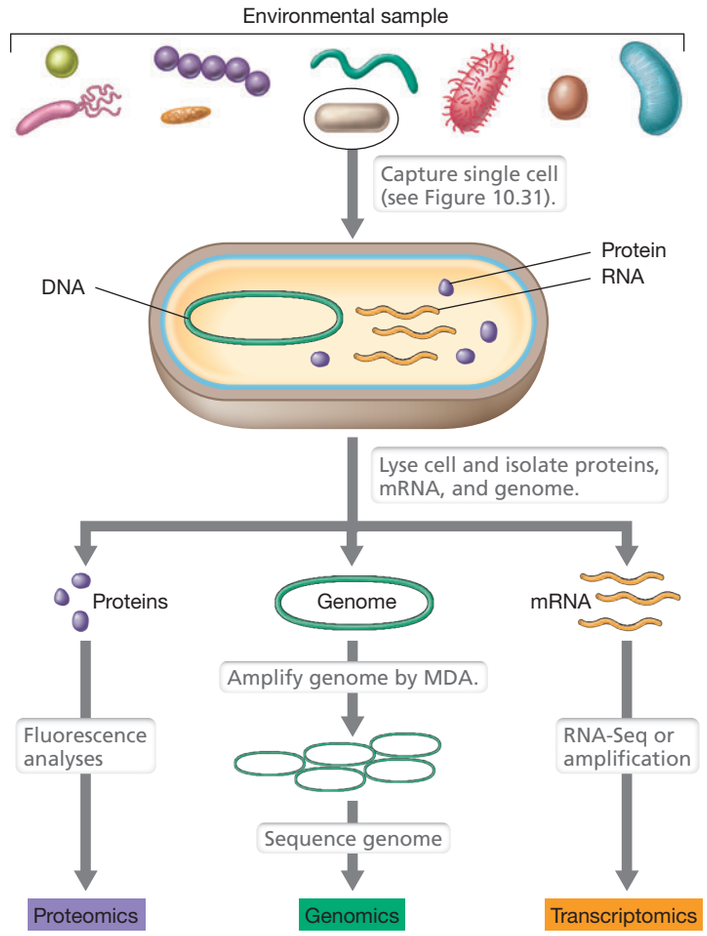


Figure 10.30 Single-cell genomics. A single cell isolated from an environmental sample can be the source of a diversified omics study. MDA, multiple displacement amplification (▶ Section 19.12).

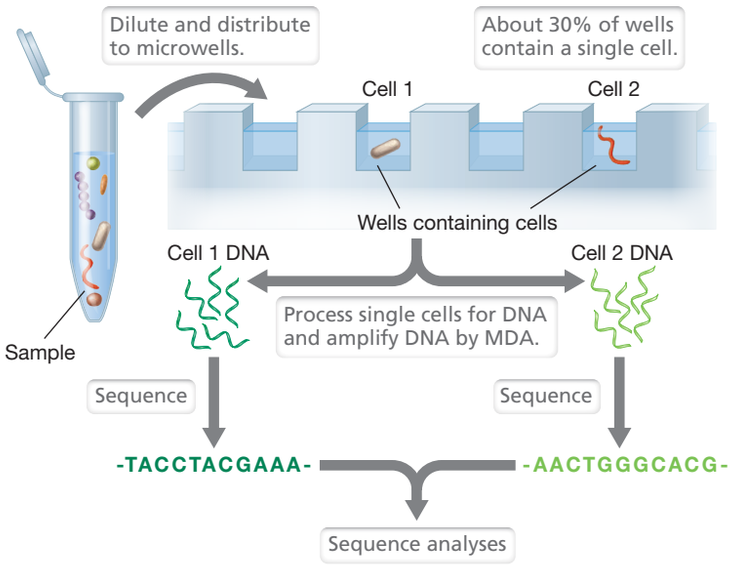


Figure 10.31 Isolation and sequencing of single cells. Droplets of a diluted sample are added to microfluidic wells at a concentration such that a single well will contain no more than a single cell. Wells containing cells are then subject to multiple displacement amplification (MDA) for DNA sequencing.

nucleic acid studies because amplification is not employed, but analyses using extremely sensitive fluorescence methods are available for this purpose (Figure 10.30).

Applications of Single-Cell Omics

Single-cell omics have the unique power to probe several facets of an organism's biology in an individual cell rather than on a cell population basis. Using SCG, metabolic genes present in a microbial community (Figure 10.29*c*) not only can be identified but can be assigned to particular species; such information reveals which particular organisms are degrading which particular nutrients. For example, single-cell genomics has been used to analyze hydrocarbon degradation by bacteria in polluted environments, leading to a better understanding of which organisms are doing what in the overall process. Similarly, plasmids and viruses can be allocated to their correct host when the genome of a single cell is sequenced.

Single-cell genomics has also been used to explore the genomes of phyla of *Bacteria* and *Archaea* that are detected in environmental 16S rRNA gene surveys but for which no laboratory cultures are available (these are called *candidate phyla*, ▶ Section 13.12). Collectively, these yet-to-be-cultivated microbes have been called *microbial dark matter*. One dark matter study applied SCG to over 200 uncultivated archaeal and bacterial cells from nine different environments. The results revealed several surprising findings including the following: Some archaeal RNA polymerase sigma factors are similar to their bacterial counterparts (◀ Sections 6.5 and 6.6); some mRNA stop codons (◀ Section 6.9 and Table 6.4) have been reassigned to incorporate actual tRNAs with their attached amino acids; the genomes of some bacterial cells encode an oxidoreductase enzyme previously seen only in eukaryotes; some *Archaea* contain genes encoding a bacterial-like stringent response (◀ Section 7.9); and some *Archaea* produce an enzyme that functions in peptidoglycan synthesis (recall that peptidoglycan is a "signature molecule" of *Bacteria* and is not known from any *Archaea*, ◀ Section 2.3). Many other examples have emerged as well.

Single-cell genomics is an excellent example of serendipity, the "pleasant surprise" of finding one thing while working on another. In this case, the serendipity occurred when omic methods designed with one goal in mind—analysis of the biology of *cell populations*—were refined to probe the biology of a *single cell* in ways never before thought possible. Single-cell genomics is poised to complement the *Earth Microbiome Project*, a sequencing endeavor to archive the genome sequence of all cultured bacterial and archaeal type strains (http://www.earthmicrobiome.org/). For those species of *Bacteria* and *Archaea* that reside in the microbial dark matter, SCG offers a way to include these species in the archive while simultaneously revealing valuable clues that will one day bring these organisms into laboratory culture.

We close out this chapter with two final sections, each focused on combining previously discussed omics methods to solve a specific problem. The first section explores the system biology of a notorious bacterial pathogen, whereas the second section is devoted to the entire human body.

UNIT 3

Check Your Understanding

- How are single cells isolated from a mixed population?
- What must be done before minute amounts of DNA can be sequenced?

10.12 Integrating *Mycobacterium tuberculosis* Omics

Mycobacterium tuberculosis is a pathogen that infects one-third of the world's population and kills approximately 2 million people every year (▶ Section 31.4). Multidrug resistance and the ability to temporarily enter dormancy in response to stress (◀ Section 8.12) are two characteristics that contribute to the persistence of *M. tuberculosis*. Thus, the identification of new treatment methods is critical for combating *M. tuberculosis*. This challenge is being tackled using a systems biology approach to understand how *M. tuberculosis*—an intracellular pathogen—adapts to the oxygen deficiency (hypoxia, a condition that develops in tuberculosis) in host cells and to identify potential drug targets for therapeutic design.

Tuberculosis Gene Expression and Regulatory Networks

As we discussed in Section 10.8, RNA-Seq can be used to characterize an organism's complete expression profile. A modification of this technique termed *dual RNA-Seq* can be used to simultaneously profile the transcriptomes of a pathogen *and* its host cell. This approach allows for the responses of both the pathogen and host to be captured during the infection process and has been especially useful for understanding how *M. tuberculosis* evades the host's defense systems. Data from dual RNA-Seq and the integration of over 600 separate expression experiments have facilitated the construction of gene expression models. These models have identified the primary energy source of intracellular *M. tuberculosis* cells as host cholesterol, and they have shown that the amino acid aspartate produced by the host is used by *M. tuberculosis* not only as a nitrogen source but also as

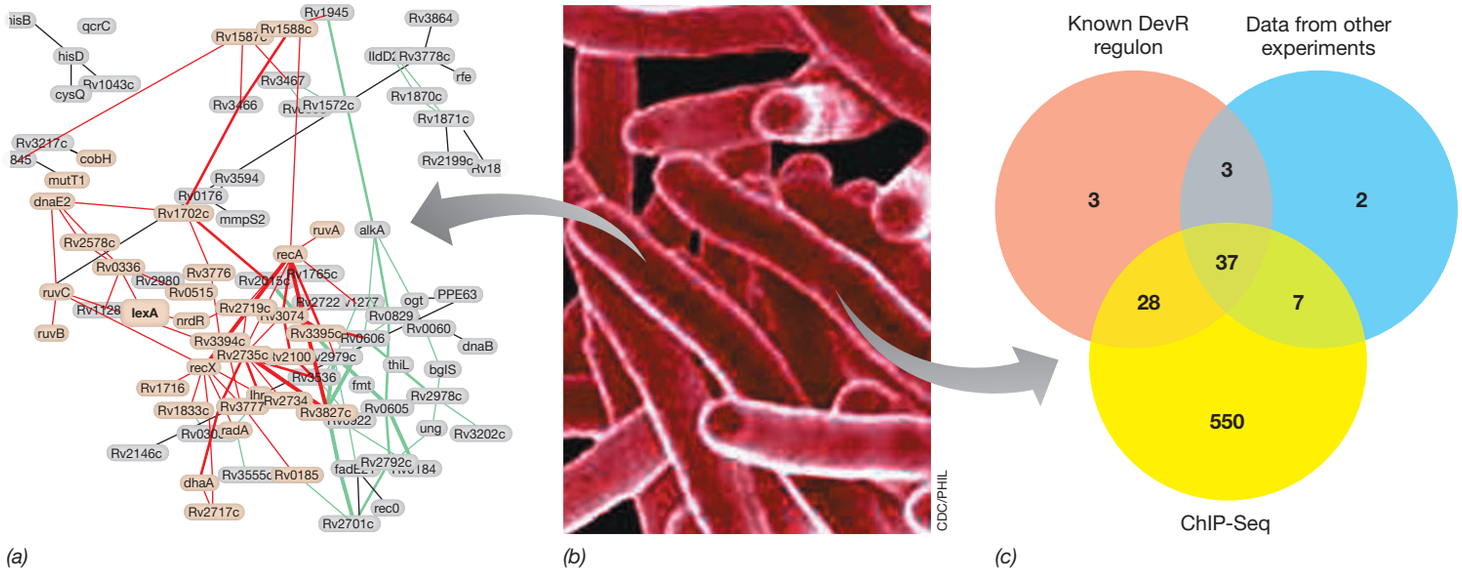


Figure 10.32 **Genes controlled by the regulators LexA and DevR in *Mycobacterium tuberculosis*.** *(a)* A snapshot of the interactome of some of the gene clusters that participate in DNA repair as identified from their expression pattern. Members of the LexA regulon are in red, while green connectors indicate interactions of other DNA repair genes. *(b)* Colorized scanning electron micrograph of cells of *M. tuberculosis*. *(c)* Venn diagram of results of various studies of the DevR regulon. Three methods of assessing genes in the regulon were employed and the number of genes identified from each detection method is indicated. A total of 622 genes were identified by ChIP-Seq analysis; of these, only 37 were identified by every method. Data adapted from van Dam, J.C.J., et al. 2014. *BMC Syst. Biol. 8*: 111.

protection against reactive oxygen species (◀ Section 4.16) produced by macrophages (a type of phagocytic cell, ▶ Section 26.1) trying to kill *M. tuberculosis* cells.

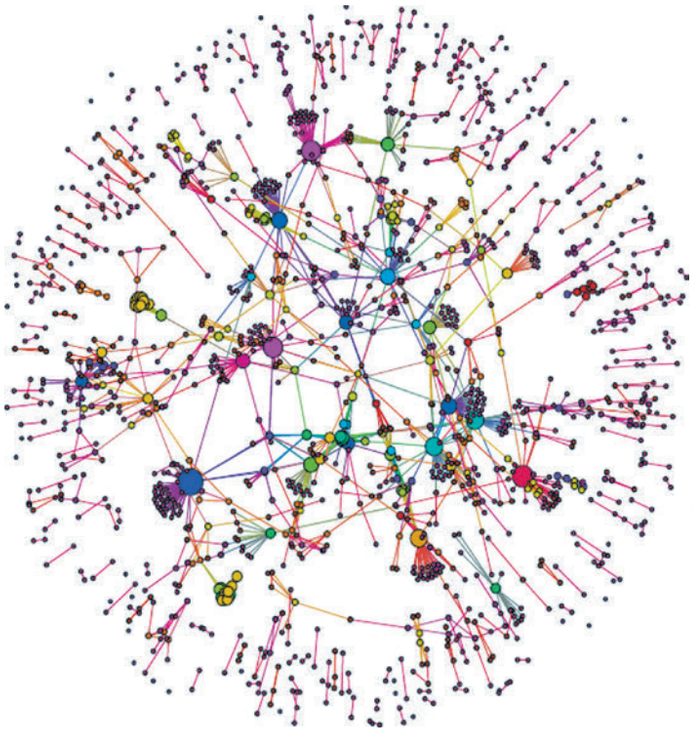
The *M. tuberculosis* research has integrated transcriptome and other omics datasets including ChIP-Seq, a method where an antibody that combines with a specific DNA-binding protein is used to trap the protein bound to its DNA, after which the DNA is removed and sequenced. From ChIP-Seq analyses, several transcription factors and regulatory networks critical to the pathogenesis of *M. tuberculosis* have been identified. These include regulation by the major bacterial cell regulator LexA (◀ Section 9.4) of certain genes for DNA damage response, and control by members of the DevR regulon of the entry of *M. tuberculosis* into dormancy (**Figure 10.32**). Additional potential drug targets have also been identified through the screening of over 1000 different mutant strains of *M. tuberculosis*. This analysis mapped 18 previously uncharacterized genes to the persistence response of *M. tuberculosis* (◀ Section 8.12), illustrating the power of integrating omics with mutant analyses to identify important pathogenicity genes in *M. tuberculosis*.

Tuberculosis Proteomics and Metabolomics

As was illustrated in Figure 10.25, proteomics has been used to identify *M. tuberculosis* protein modifications under certain conditions. The power of proteomics has been used to identify proteins essential to the *M. tuberculosis* hypoxia response. While expected proteins that combat reactive oxygen species such as superoxide dismutase (◀ Section 4.16) were detected, so were toxin–antitoxin systems (◀ Section 8.12) and proteins that participate in the biosynthesis of the unique lipoproteins of the *M. tuberculosis* cell envelope (▶ Section 16.11). Production of nitrate and nitrite transporters also increased as *M. tuberculosis* switched to anaerobic respiration (◀ Section 3.10) for energy metabolism. Moreover, at least 160 different uncharacterized proteins containing the N-terminal amino acid sequence "Pro-Glu/Pro-Pro-Glu" (amino acids and their abbreviations are listed in Figure 6.27) were detected in the pathogen. If drugs could be developed that specifically target this peptide, they might be effective treatments for tuberculosis.

Online fundraising and social media have even been combined for the common goal of identifying new drugs to treat tuberculosis. The *Connect to Decode* initiative (http://c2d.osdd.net) is composed of more than 800 researchers whose collective goal is to identify new tuberculosis drug targets by mapping the entire interactome of *M. tuberculosis*. By analyzing over 10,000 *M. tuberculosis* experimental datasets, the researchers have now annotated 87% of the proteins encoded by the genome. This is a vast improvement over the 52% of genes annotated in the original genome sequence. This collective effort has also resulted in a map of the complete *M. tuberculosis* interactome, with over 1400 proteins connected by more than 2500 functional relationships (**Figure 10.33**). Thus far, nearly 20 potential drug targets and their interactomes have been identified by this initiative. These targets lack homology to human proteins or to proteins of the human oral and gastrointestinal microbiome, thus increasing the probability of identifying drugs with the least side effects.

While tuberculosis metabolomics is in its infancy, studies comparing virulent versus attenuated *Mycobacterium* strains have identified over 1000 different lipids—many of them unique lipoproteins—that are likely quite important to the biology of *M. tuberculosis*. Thus, the *M. tuberculosis* lipid metabolism proteome may reveal several potential therapeutic targets for tuberculosis. Finally, by profiling the *secretome* (an inventory of metabolites secreted) of *M. tuberculosis*, molecules unique to virulent strains (such as the nucleotide



Vashisht, R. et al. 2012. *PLos One 7 (7)*: e39809

Figure 10.33 ***Mycobacterium tuberculosis* protein interactome.** Node color indicates proteins of the same category, connecting lines indicate interactions, and node size indicates relative number of interactions.

1-tuberculosinyladenosine) have been discovered. This modified nucleotide is present in the urine of humans infected with *M. tuberculosis*, and its discovery illustrates the power of omics to link a particular pathogen to a specific molecule—be it a gene, protein, or metabolite—and yield new disease markers for use in clinical diagnostics. Moreover, if this modified nucleotide turns out to be essential to the survival of *M. tuberculosis* cells, drugs that interfere with its synthesis or activity are potential anti-tuberculosis drugs.

Check Your Understanding

- How does dual RNA-Seq differ from traditional RNA-Seq?
- How has systems biology provided new approaches to treating the disease tuberculosis?

10.13 Systems Biology and Human Health

In 2003 the sequence of the 3-billion-base-pair human genome was completed and released in an international effort that cost nearly $3 billion. With the advent of next-generation sequencing, the cost to sequence a human genome has fallen under $1000, triggering an onslaught of human genome sequence data. How can the human genome and systems biology be tailored to benefit human health? By comparing genome sequences in over 2500 people from different continents, scientists have already identified over 88 million sites in the human genome that are subject to variation. These genomic variants include single nucleotide differences, insertions and deletions, and rearrangements, each of which may turn out to be harmless, to be beneficial, to contribute to noninfectious "lifestyle" diseases such as obesity, diabetes, and heart disease, or to govern susceptibility to certain cancers. Understanding if and how genomic variants contribute to disease can be used to improve diagnostics, treatments, and prevention.

The omics revolution has opened the era of *personalized medicine*, where genomic, transcriptomic, proteomic, metabolomic, and pharmacogenomic (omics responses to drugs) data are exploited to generate a snapshot of normal and disease states along with immune processes that occur in between. The first step in personalized medicine is the generation of an *i*ntegrative *P*ersonal *O*mics *P*rofile (iPOP). Tracking changes in this profile during healthy and disease states can aid in assessing medical risks and diagnosing and treating patients. The utility of personalized medicine can be illustrated by an actual study where blood samples taken from a male subject 20 times over a period of two years were subjected to proteomic and metabolic profiling of about 5000 proteins and 4000 metabolites (**Figure 10.34**). The results suggested that the subject was at risk for coronary artery disease—which was not surprising based on his medical history and familial incidence—but also indicated that he was at high risk for type 2 diabetes. While this was a surprising finding considering his overall condition and familial health history, the

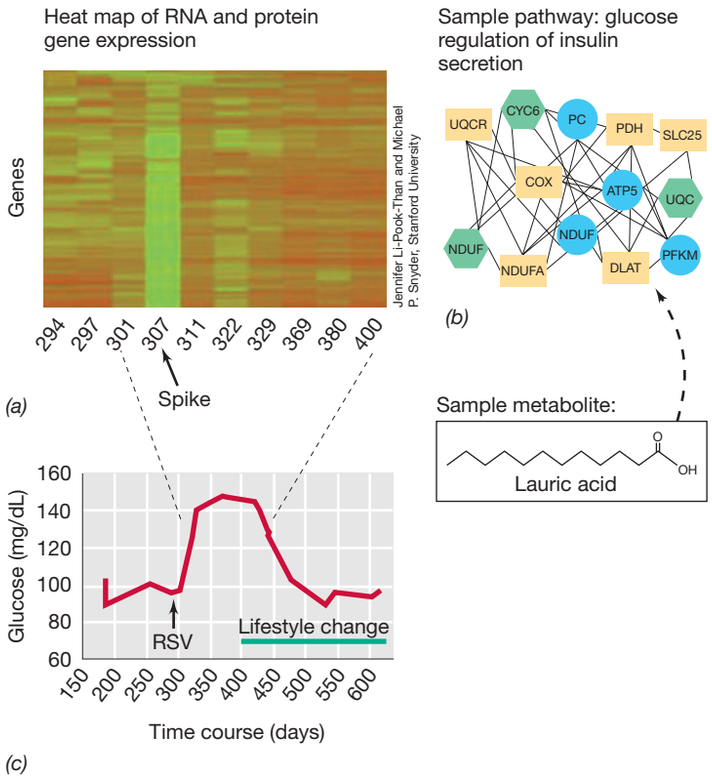


Figure 10.34 **An integrated personal omics profile (iPOP).** *(a)* Partial heat map of RNA and protein expression. Data plotted are from days 294–400 of the two-year study, with an increase in RNAs and proteins related to diabetes spiking on day 307. *(b)* Glucose regulation interactome. RNAs are indicated with blue circles, proteins with yellow squares, and RNA and its corresponding protein with green hexagons. A metabolite from the interactome is also shown. *(c)* Blood glucose levels during the time course of the study. The time of contracting a respiratory virus (RSV) is indicated as well as the time frame in which the patient made lifestyle and diet changes. Parts *b* and *c* adapted from Jennifer Li-Pook-Than and Michael P. Snyder, Stanford University.

prediction was based on profiling the subject's immune response to a viral infection. During this infection, changes in both gene expression and protein profiles related to the insulin response were detected. Just as this subject's personalized medicine profile predicted, he developed diabetes over the course of the two-year study (Figure 10.34). By tracking his iPOP, the onset of diabetes was revealed by the increasing production of RNAs, proteins, and metabolites, such as hemoglobin A1c and lauric acid, related to glucose metabolism (Figure 10.34).

This case study illustrates the potential of iPOPs in monitoring the immune response and disease states. In fact, the omics revolution now allows for genomics, transcriptomics, proteomics, and metabolomics to be performed on different types of immune system cells. The wealth of data from these analyses has led to the development of new immunotherapy options (▶ Sections 12.8 and 28.4). However, problems such as error rates, the analysis and storage of such large datasets, assessment of complicating factors (such as additional diseases during the course of the study, Figure 10.34), and ethical issues need to be addressed before iPOPs become routine tools in clinical medicine.

Microbiology as well as biology in general has been forever changed by the genomics age. Our journey through this chapter has only scratched the surface in describing how omics can be used to address previously intractable scientific questions. Much more is almost certainly in store, as major leaps forward in the omics field so far have typically been only one technological advancement away. In Chapter 11 we continue our genomics theme but change our focus from cells to viruses—the most abundant and genetically diverse microbes on Earth.

Check Your Understanding

- What are genomic variants?
- What is an iPOP?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Genomics

10.1 Small viruses were the first organisms whose genomes were sequenced, but now many prokaryotic and eukaryotic cellular genomes have been sequenced.

Q What is one discovery resulting from the availability of a microbial genome?

10.2 DNA sequencing technology is advancing quickly. These advances have greatly increased the speed of DNA sequencing. Computer analysis of resulting sequencing data is also a vital part of genomics. Computational tools are used not only to annotate genomes but also to analyze sequences and the structures of biological macromolecules.

Q How can protein homology assist in genome annotation?

10.3 Sequenced genomes of *Bacteria* and *Archaea* range in size from 0.11 to 16 Mbp. The smallest are smaller than those of the largest viruses, whereas the largest have more genes than some eukaryotes. Gene content in prokaryotic cells is typically proportional to genome size. Many genes can be identified by their sequence similarity to genes found in other organisms. However, a significant percentage of sequenced genes are of unknown function.

Q Approximately how many genes are predicted to be necessary for a microbial cell to have a free-living existence?

10.4 Virtually all eukaryotic cells contain mitochondria, and in addition, plant cells contain chloroplasts. Although the genomes of organelles are independent of the nuclear genome, the organelles themselves are not. Many genes in the nucleus encode proteins required for organelle function. The complete genomic sequence of many microbial eukaryotes has also been determined, and the number of genes ranges from 1000 (less than many bacteria) to 60,000 (more than twice as many as humans).

Q Which genomes are larger, those of chloroplasts or those of mitochondria? How does your genome compare with that of yeast in overall size and gene number?

II • Functional Omics

10.5 Once a microbial genome has been sequenced, assembled, and annotated, the role of many genes remains unknown. To characterize new gene products, several functional genomics tools can be applied. These include in particular comparative genome analyses and heterologous gene expression.

Q How can the abundance of available microbial genomes be used to identify new systems that prevent viral infection or horizontal transfer of DNA?

10.6 Transposon mutagenesis has been harnessed for identifying gene functions using a high-throughput sequencing methodology. The technique, Tn-Seq, has been used to pinpoint genes in many different bacteria that would be more difficult and laborious to discover using other functional genomic methods.

Q If a gene in *Escherichia coli* was absolutely essential for growth under all conditions, how could you determine this using Tn-Seq?

10.7 Most microorganisms in the environment have never been cultured. Nonetheless, analysis of DNA samples has revealed enormous sequence diversity in most habitats. The concept

of the metagenome embraces the total genetic content of all the organisms in a particular habitat.

Q How do the human microbiome and mycobiome differ?

10.8 Microarrays consist of oligonucleotide probes corresponding to genes or gene fragments attached to a solid support in a known pattern; mRNA, cDNA, or DNA can then be labeled and hybridized to the gene chip to determine patterns of gene expression or the presence or absence of specific organisms. RNA-Seq combined with sequencing of cDNA can be used to profile the entire transcriptome of an organism.

Q Besides gene expression, what else can be assayed using gene chips?

10.9 Proteomics is the analysis of all the proteins present in an organism. The ultimate aim of proteomics is to understand the structure, function, and regulation of these proteins. The interactome is the total set of interactions between macromolecules inside the cell.

Q Besides determining the identity of proteins present under a specific condition, what else can proteomics determine about a microbial proteome?

10.10 Metabolomics profiles the complete set of metabolic intermediates produced by an organism. This analysis can determine active metabolic pathways and potential cross-feeding (one organism supplies a nutrient for another organism) in community samples.

Q Why is investigation of the metabolome lagging behind that of the proteome?

III • Systems Biology

10.11 With advances in molecular techniques, the genomes of single cells can be sequenced. Expression and protein profiles of single cells can also be determined. These techniques are instrumental for studying as yet uncultured microbes.

Q How can single-cell genomics be used to address microbial dark matter?

10.12 By integrating multiple omics datasets in a systems biology approach, computer models predicting molecular activities and interactions in cells can be generated. For example, potential drug targets for the treatment of *Mycobacterium tuberculosis* have been identified using systems biology.

Q How can systems biology be used to discover new diagnostic markers for disease?

10.13 Systems biology can also be applied to personal medicine. Besides detecting genetic variants, disease risks can be predicted by profiling a person's genome, transcriptome, proteome, and metabolome.

Q How can omics be used to design immunotherapy strategies?

APPLICATION QUESTIONS

1. Apart from genome size, what factors make complete assembly of a eukaryotic genome more difficult than assembly of a genome from a species of ***Bacteria*** or ***Archaea***?
2. Describe how one might determine which proteins in ***Escherichia coli*** are repressed when a culture is shifted from a minimal medium (containing only a single carbon source) to a rich medium containing many amino acids, bases, and vitamins. How might one study which genes are expressed during each growth condition?
3. Describe how you could use systems biology to discover a new biologically produced antibiotic.

CHAPTER GLOSSARY

Bioinformatics the use of computational tools to acquire, analyze, store, and access DNA and protein sequences

Codon bias the nonrandom usage of multiple codons encoding the same amino acid; the relative proportions of different codons encoding the same amino acid vary in different organisms. Same as codon usage

Gene chip small, solid supports to which genes or portions of genes are affixed and arrayed spatially in a known pattern (also called microarrays)

Genome the total complement of genes contained in a cell or virus

Genomics the discipline that maps, sequences, analyzes, and compares genomes

Hybridization the joining of two single-stranded nucleic acid molecules by complementary base pairing to form a double-stranded hybrid DNA or DNA–RNA molecule

Interactome the total set of interactions between proteins (or other macromolecules) in an organism

Metabolome the total complement of small molecules and metabolic intermediates of a cell or organism

Metagenome the total genetic complement of all the cells present in a particular environment

Metagenomics the genomic analysis of pooled DNA or RNA from an environmental sample containing organisms that have not been isolated; same as environmental genomics

Microarray small, solid supports to which genes or portions of genes are affixed and arrayed spatially in a known pattern (also called gene chips)

Mobilome the mobile genetic elements in a genome

Nucleic acid probe a strand of nucleic acid that can be labeled and used to hybridize to a complementary molecule from a mixture of other nucleic acids

Open reading frame (ORF) a sequence of DNA or RNA that could be translated to give a polypeptide

Proteome (1) the total set of proteins encoded by a genome or (2) the total protein complement of an organism under a given set of conditions, also called the translatome

Proteomics the genome-wide study of the structure, function, and regulation of the proteins of an organism

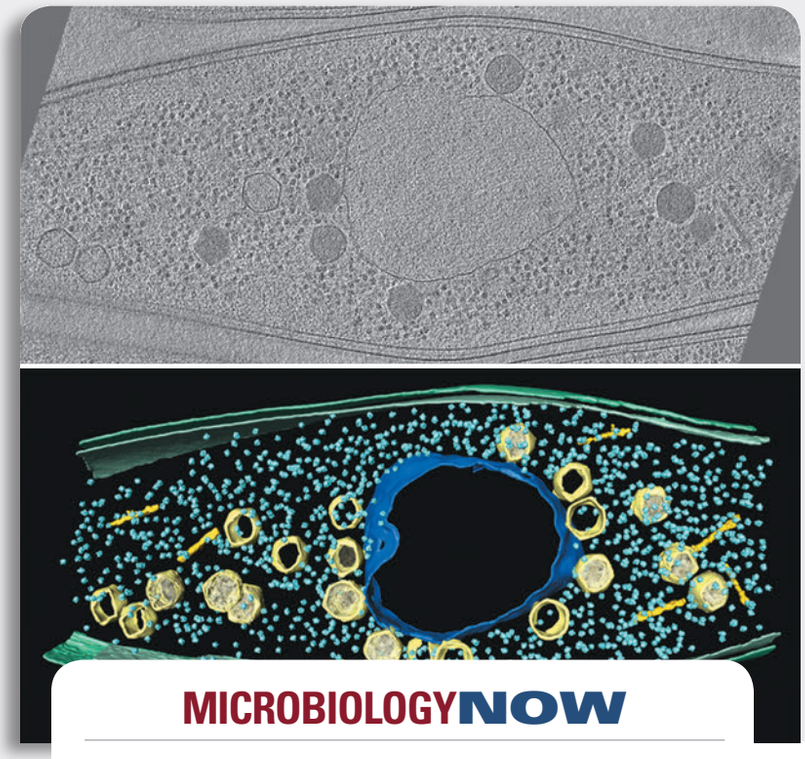
Sequencing deducing the order of nucleotides in a DNA or RNA molecule by a series of chemical reactions

Systems biology the integration of data from genomics and other "omics" areas to build an overall picture of a biological system

Transcriptome the complement of all RNA produced in an organism under a specific set of conditions

Translatome the total set of proteins produced by an organism under a specific set of conditions

Viral Genomics and Diversity 11



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MICROBIOLOGYNOW

Bacteriophages Mimicking Eukaryotes—Discovery of a Phage-Encoded Nucleus and Spindle

A membrane-bound nucleus is a defining feature of eukaryotic cells that differentiates them from their prokaryotic counterparts. Remarkably, molecular biologists have discovered a group of large *Pseudomonas* bacteriophages whose genomes encode structures resembling the eukaryotic nucleus and mitotic spindle. The obvious question is why, and to understand these remarkable events, scientists needed to understand the roles of both eukaryotic-like structures.

Using fluorescent labeling and cryo-electron microscopy, it was determined that the phage-encoded nucleus contains enzymes specific for replicating and transcribing phage DNA. Once empty phage capsids are assembled at the host membrane, they move rapidly toward the phage nucleus to be filled with phage DNA. The top photo shows a cryo-tomogram slice of a *Pseudomonas chlororaphis* cell infected with the phage 201ϕ2-1 (phage virions are about 0.1 μm in diameter), and the bottom image highlights the following structures in color: nucleus-like structure (blue), capsids (light yellow), phage tails (bright yellow), cytoplasmic and outer membranes (green), and ribosomes (light blue).

How do the capsids move specifically toward the nucleus? The phage-encoded spindle structure (not shown) consists of tubulin filaments that use a treadmill action to advance the empty capsids to the nucleus for DNA packaging. Thus, the phage-encoded nucleus appears to function as a compartment similar to that of eukaryotic viral factories. Once fully assembled, the mature bacteriophages lyse their bacterial host and hunt for new bacterial prey.

Because of the large genomes of the phages that produce these eukaryotic-like structures, it is possible that the events described here increase the efficiency of large bacteriophage multiplication such that they can better compete with smaller bacteriophages that are completely reliant on the host cell machinery for replication. This discovery also begs one of the most fascinating of viral genomics questions: Was the nucleus actually a viral invention?

Sources: Chaikeeratisak, V., et al. 2017. Assembly of a nucleus-like structure during viral replication in bacteria. *Science 335*: 194. Chaikeeratisak, V., et al. 2019. Viral capsid trafficking along treadmilling tubulin filaments in bacteria. *Cell 177*: 1771.

I • Viral Genomes and Classification

Viruses break several of the molecular rules that govern cells, but perhaps the greatest of these is in the nature of their genomes. In some viral genomes, DNA—the genetic blueprint of cells—is replaced by RNA and the double helix is supplanted by a single strand of nucleic acid.

We learned in Chapter 5 that viruses have either DNA or RNA genomes that can be either single-stranded or double-stranded. Thus, compared with cells, viral genomes can create some unusual challenges for genetic information flow. In this chapter, we take a more detailed look at viral biology. We begin by grouping viruses by their genome structure rather than by the hosts they infect, because viruses with the same genome structure face common problems in genetic information flow. We then consider which viruses infect cells in each of the domains of life and conclude with a discussion of infective agents that are neither cells nor viruses.

11.1 Size and Structure of Viral Genomes

Viral genomes vary almost a thousand-fold in size from smallest to largest and are typically smaller than those of cells. DNA viruses exist along this entire gradient from the tiny circovirus, whose 1.75-*kilobase* single-stranded genome, which contains three genes, pales in comparison to that of the 2.5-*megabase*-pair double-stranded DNA genome of *Pandoravirus*, which contains 2500 genes (**Figure 11.1**). The genome of the latter is over twice that of the previously known largest virus (*Mimivirus*, see Figure 11.5*a*) and is larger than the genomes of several species of *Bacteria* and *Archaea* (◀ Table 10.1). *Pandoravirus* infects certain marine amoebae, and with virion dimensions of 1 μm × 0.5 μm, it is also larger than some bacterial cells (Figure 11.1).

RNA virus genomes, whether single- or double-stranded, are typically smaller than DNA viruses and contain 2–40 genes. Although some viral genomes are larger than those of some prokaryotic cells, genomes of *Bacteria* and *Archaea* are typically much larger than those of viruses (Figure 11.1), and genomes of eukaryotes are the largest of all. Viroids, naked infectious RNAs that cause certain plant diseases (Section 11.12), have the smallest genomes of all microbes (Figure 11.1).

Whether a viral genome is large or small, once a virus has infected its host, transcription of viral genes must occur and new copies of the viral genome must be made. Only later, once viral proteins begin to appear from the translation of viral mRNA, can viral assembly begin. For certain RNA viruses, the genome is also the mRNA. For most viruses, however, viral mRNA must first be made by transcription of the DNA or RNA genome, and we now consider the variations on how this occurs.

The Baltimore Scheme: DNA Viruses

The American virologist David Baltimore, who shared with the American Howard Temin and the Italian American Renato Dulbecco the Nobel Prize for Physiology or Medicine in 1975 for the discovery of retroviruses and their key enzyme, reverse transcriptase, developed

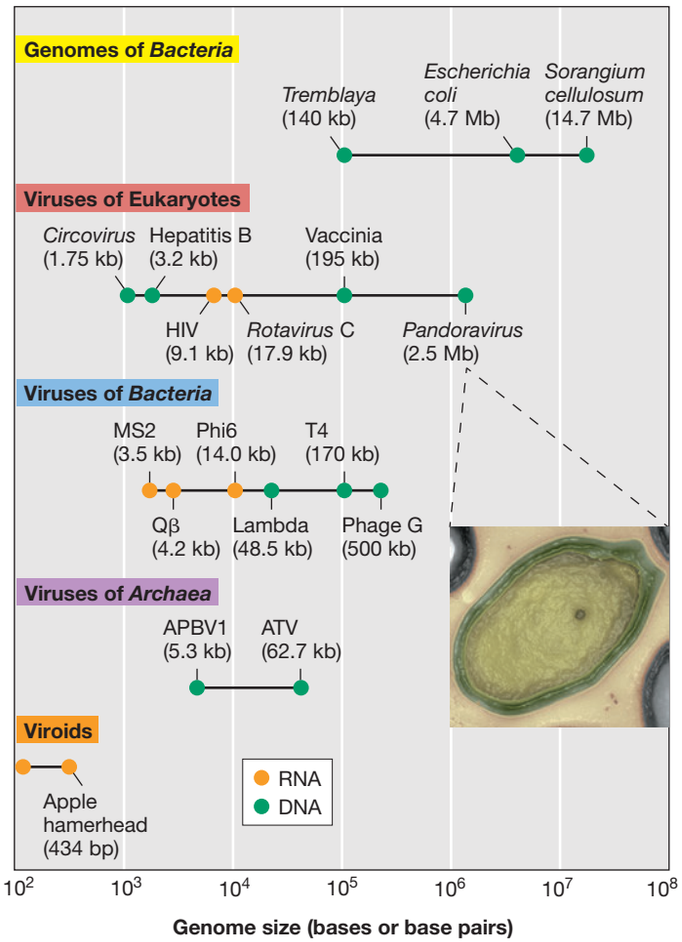


Figure 11.1 Comparative genomics. Genome sizes of select viroids, viruses, and archaeal and bacterial cells. Inset: Micrograph of *Pandoravirus*, ~1 μm in length. Image courtesy of Chantal Abergel, IGS, UMR7256 CNRS-AMU. Archaeal viruses APBV1 and ATV infect *Aeropyrum pernix* and *Acidianus convivator*, respectively. Bacteriophage phi6 and phage G infect *Pseudomonas* and *Bacillus* species, respectively; other bacterial viruses infect *Escherichia coli*.

a classification scheme for viruses. The scheme is based on the relationship of the viral genome to its mRNA and recognizes seven classes of viruses; three classes have DNA genomes and four have RNA genomes (**Figure 11.2**). By convention in virology, viral *mRNA* is always considered to be of the *plus* (+) configuration. Thus, to understand the molecular biology of a particular class of virus, one must know the nature of the viral genome and the steps necessary to produce (+) mRNA from it (Figure 11.2).

Double-stranded DNA viruses are in Baltimore class I. The mechanism of mRNA production and genome replication of class I viruses is the same as that used by cells (Chapter 6). A virus containing a single-stranded genome may be either a **positive-strand** virus (also called a plus-strand virus) or a **negative-strand** virus (also called a minus-strand virus). Class II viruses contain single-stranded plus-strand DNA genomes. Transcription of such a genome would yield (−) mRNA, and thus, before (+) mRNA can be produced from class II viruses, a complementary DNA strand must first be made to form a double-stranded DNA intermediate; this is called the **replicative**

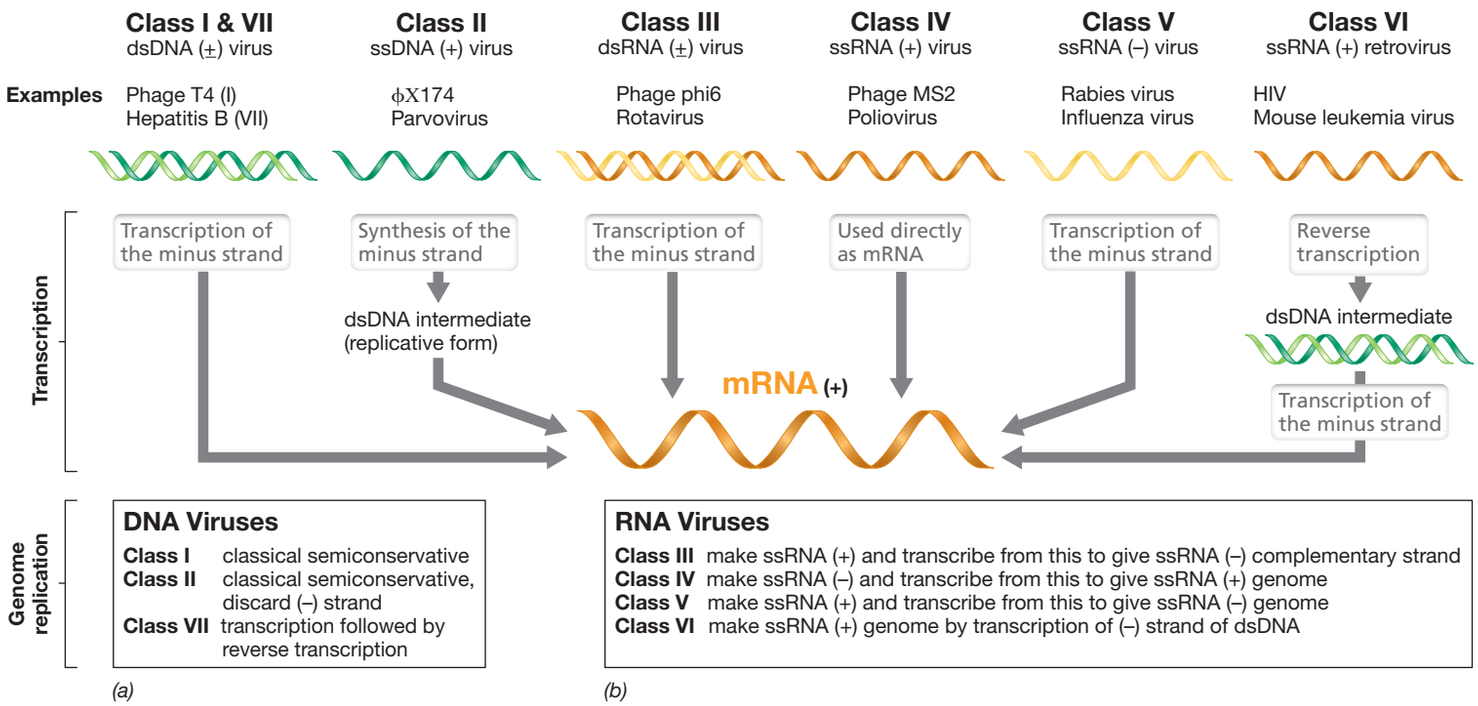


Figure 11.2 The Baltimore classification of viral genomes. Seven classes of viral genomes are known. The genomes can be either *(a)* DNA or *(b)* RNA, and either single-stranded (ss) or double-stranded (ds). With the exception of classes V and VI viruses, where the only known examples infect eukaryotic hosts, the top example listed is a bacterial virus and the bottom example an animal virus. The path each viral genome takes to form its mRNA and the strategy each uses for replication is shown.

form. The latter is used to produce (+) mRNA, and as the source of new genome copies, the plus strand becomes the genome while the minus strand is discarded (Figure 11.2). With only one known exception, all single-stranded DNA viruses are positive-strand viruses.

The Baltimore Scheme: RNA Viruses

The production of mRNA and genome replication will obviously be different for RNA viruses than for DNA viruses. Cellular RNA polymerases do not catalyze the formation of RNA from an RNA template, but instead require a DNA template. Therefore, depending on the virus, RNA viruses must either carry in their virions or encode in their genomes an RNA-dependent RNA polymerase called *RNA replicase*. RNA replicases replicate the viral RNA genome and produce viral-specific mRNA. With positive-strand RNA viruses (class IV), the genome is also mRNA. But for negative-strand RNA viruses (class V), RNA replicase must synthesize a plus strand of RNA from the negative-strand template, and the plus strand is then used as mRNA. The plus strand is also used as a template to make more negative-strand genomes (Figure 11.2). RNA viruses of class III face a similar problem but start with double-stranded (+/−) RNA instead of only a positive or negative strand.

Retroviruses are animal viruses whose genomes consist of single-stranded RNA of the plus configuration but replicate through a double-stranded DNA intermediate (class VI). The process of copying the information found in RNA into DNA is called **reverse transcription** and is catalyzed by an enzyme called *reverse transcriptase*. Human immunodeficiency virus (HIV, the causative agent of AIDS) is a retrovirus. Finally, class VII viruses are those highly unusual viruses (human hepatitis B virus is an example) whose genomes consist of double-stranded DNA but which replicate through an RNA intermediate. As we will see later, these viruses also use reverse transcriptase (Section 11.11).

Hosts for Viruses of Each Baltimore Class

Only certain of the Baltimore classes of viruses are known to infect cells of a particular phylogenetic domain. For example, only two classes are known in *Archaea* and only four in *Bacteria*; only in animals do we find examples of all seven Baltimore classes of viruses (**Figure 11.3*a***). Examples of viruses from each of the Baltimore classes are drawn to scale in Figure 11.3*b*.

Double-stranded DNA viruses (class I) are the primary viruses infecting prokaryotic cells, while single-stranded plus-sense RNA viruses (class IV) are the major viral predators of eukaryotic cells (Figure 11.3*a*). As far as is known, fungi are only infected by RNA viruses of classes III and IV, whereas the vast majority of class I viruses that infect eukaryotes replicate in animal hosts rather than plants. By contrast, plants serve as hosts to many more class II viruses than do animals, whereas virtually all class V viruses (viruses with a single-strand minus-sense genome) infect animals rather than plants. And finally, retroviruses (class VI) are known only from animal hosts, while class VII viruses, which like retroviruses depend on reverse transcriptase to replicate their genome, are much more common in plants than in animals (Figure 11.3*a*). Although the reasons that different genomic classes of virus show specific host preferences is unclear, the fact that *Bacteria* and *Archaea* are hosts for only a relatively small group of viruses suggests that some viral classes may have evolved later in the course of evolution when more complex eukaryotic hosts were available for infection. However,

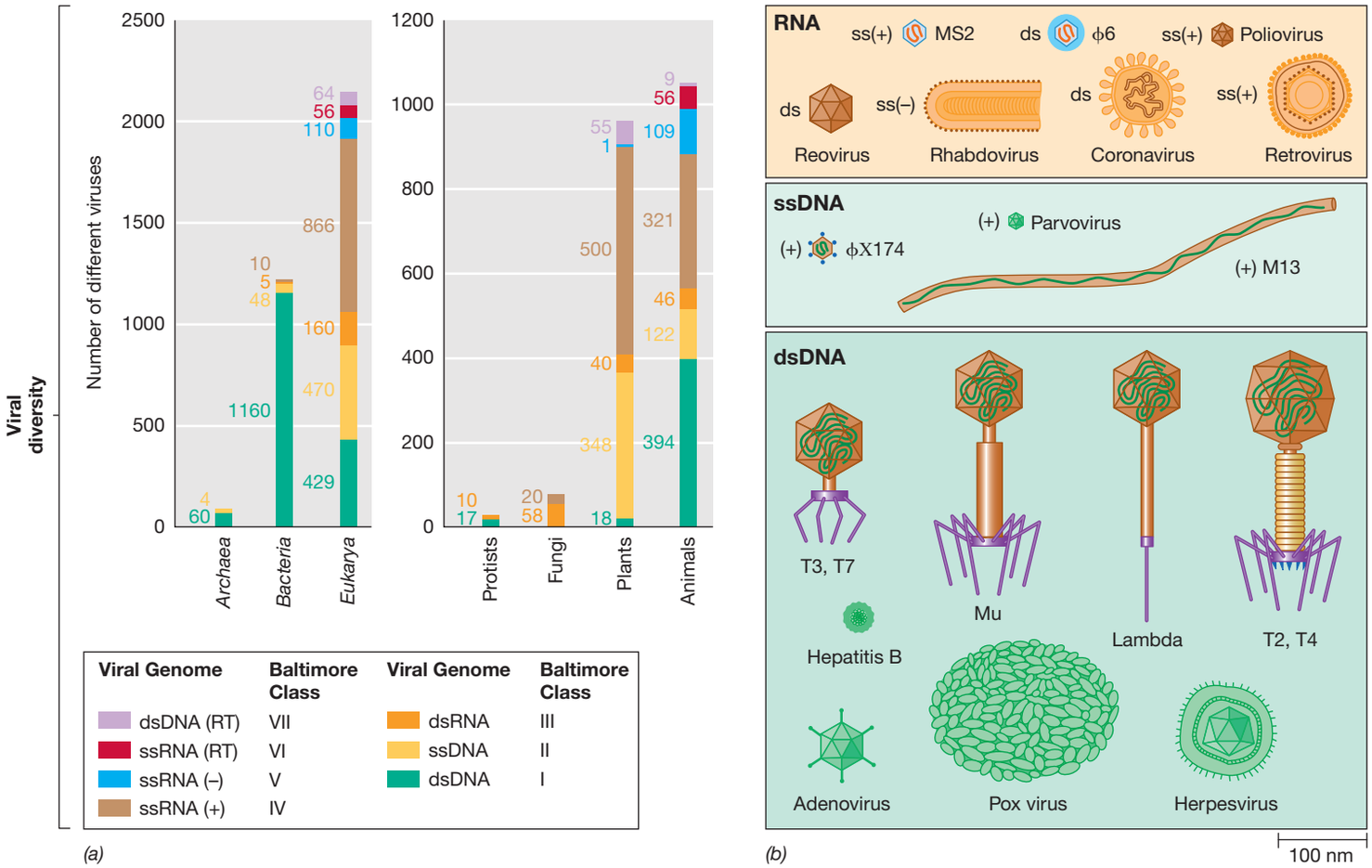


Figure 11.3 Viral hosts and viral diversity. *(a)* Virus host preferences by cellular domain (left graph) and in different major groups of eukaryotes (right graph). RT, reverse transcription. Data adapted from Nasir, A., and G. Caetano-Anollés. 2015. *Sci. Adv. 1*: e1500527. *(b)* Drawings to scale of several viruses discussed in this chapter. The symbols + and − next to viral names indicate the complementarity of the single-stranded DNA or RNA genome of that virus. By convention, all viral mRNAs are designated as + in sequence complementarity.

computational analyses (to be discussed in Chapter 13) suggest that this hypothesis may be incorrect, in that most viral groups, especially the RNA viruses, appear to be of ancient origin.

Viral Protein Synthesis

During the course of viral replication, once (+) mRNA is made (Figure 11.2), viral proteins can be synthesized. In all viruses, these proteins can be grouped into two broad categories: (1) proteins synthesized soon after infection, called *early proteins*, and (2) proteins synthesized later in the infection, called *late proteins*. Both the timing and amount of viral protein synthesis is highly regulated. Early proteins are typically enzymes and are therefore synthesized in relatively small amounts. These include not only nucleic acid polymerases but also proteins that function to shut down host cell transcription and translation. By contrast, late proteins are typically structural components of the virion and other proteins that are not needed until virion assembly begins, and these are made in much larger amounts (◀ Section 5.5).

Virus infection upsets the regulatory mechanisms of the host because there is a marked overproduction of viral nucleic acid and protein in the infected cell. Eventually, when the proper proportions of viral genome copies and virion structural components have been synthesized, new virions are assembled—typically spontaneously—and exit the host cell by either lysing and killing it or by budding or secretion processes in which the host cell may remain alive.

Check Your Understanding

- Distinguish between a positive-strand RNA virus and a negative-strand RNA virus.
- Contrast mRNA production in the two classes of single-stranded RNA viruses.
- What is unusual about genetic information flow in retroviruses?

11.2 Viral Taxonomy and Phylogeny

Because of the remarkable diversity and abundance of viruses, the Baltimore scheme and host specificity are the easiest way to classify viruses. However, these classifications do not consider relationships between viruses. For example, both rabies and influenza viruses are negative-strand RNA viruses (Baltimore class V), but they possess very different morphology, infection strategies, and genomes. While

we will discuss the evolutionary timeline for viral appearance on Earth and their relationship to cells in Chapter 13, here we focus on taxonomic and phylogenetic relationships between viruses.

Viral Taxonomy

Like microorganisms, viruses can be characterized using a polyphasic approach that includes phenotypic, genotypic, and phylogenetic analyses, or *taxonomy* (▶ Section 13.12). Within viral taxonomy there are orders, families, subfamilies, genera, and species. The International Committee on Taxonomy of Viruses or ICTV is composed of virologists that oversee the classification and naming of viruses (http://ictv.global). ICTV uses a combination of host range, structural morphology, replication cycle, nucleic acid sequence, and pathogenicity to classify viruses. ICTV puts the Baltimore class V rabies and influenza viruses mentioned above in the orders *Mononegavirales* and *Articulavirales*, respectively. Another example of the utility of viral taxonomy is the classification of archaeal viruses. While only two Baltimore classes of viruses have been described to infect *Archaea* (classes I and II; Figure 11.3*a*), genomic and morphological diversity of these viruses (Section 11.5) places them into 17 different families within unclassified orders.

Figure 11.4 illustrates the distribution of seven major orders of cultured viruses among the three domains of life. While the number of bacteriophages isolated outnumber those of archaeal viruses by a factor of ~20 (Figure 11.3*a*), 90% of bacteriophages belong to the *Caudovirales* order. Members of the *Caudovirales* are the tailed bacteriophages with double-stranded DNA genomes (Figure 11.3*b* and Section 11.4). In contrast, only 13% of cultured archaeal viruses belong to this order with over 60% of archaeal viruses belonging to unclassified orders (Figure 11.4). The high number of unclassified archaeal viruses stems from the diverse morphology displayed by those in culture (Section 11.5). Eukaryotic viral classification shares the same diversity issue, with most viruses comprising unclassified orders (Figure 11.4).

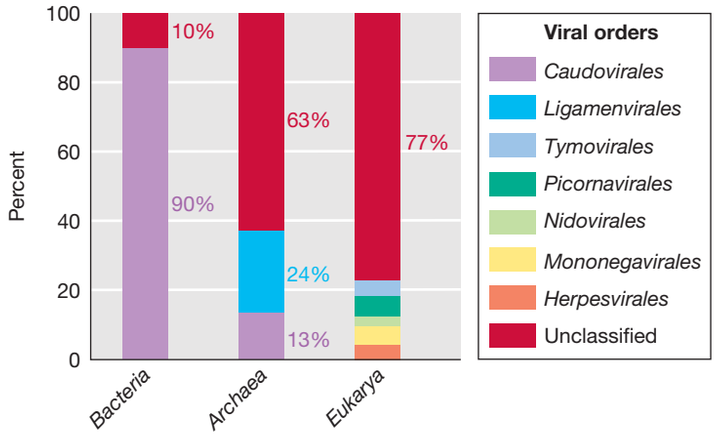


Figure 11.4 Taxonomic classification of viruses infecting the three domains of life. The International Committee on Taxonomy of Viruses assignment of viruses into orders based on host. Data adapted from Mahmoudabadi, G., and Phillips, R. 2018. *eLife* 7: e31955.

Viral Phylogeny

Evolutionary relationships (phylogeny, Chapter 13) among viruses can be difficult to map because they do not possess the ribosomal RNA molecules used to make phylogenetic trees of cells (◀ Section 1.15). Viruses also possess high rates of mutation, especially those with RNA genomes, compared to that of cells. To determine phylogenetic relationships between viruses, the sequences of conserved proteins have been compared along with some structural features. However, in only a few groups of viruses has it been possible to reliably trace phylogenies, and in these cases, trees have been assembled from sequences of a group of genes or proteins shared in common among the group. One such example is *Mimivirus* and its relatives, one of the larger known viruses (**Figure 11.5**; Chapter 5). *Mimivirus* capsids are multilayered and icosahedral. The virion is surrounded by spikes and is nearly 0.75 μm in diameter (Figure 11.5*a*), larger than some prokaryotic cells. *Mimivirus* contains a 1.2-megabase double-stranded DNA genome. *Mimivirus* infects the protozoan *Acanthamoeba* and belongs to a group of giant viruses with large genomes called *nucleocytoplasmic large DNA viruses* (NCLDV) (Figure 11.5*b*). The NCLDV comprise several virus families, including pox viruses (Section 11.6), iridoviruses, and certain plant viruses. These viruses share a set of highly homologous proteins, most of which function in DNA metabolism. A phylogenetic tree of these viruses constructed from DNA sequences encoding these proteins shows how they have diverged from a common ancestor (Figure 11.5*b*). It is thus possible to track the phylogeny of particular viral groups with some confidence, but to do so, one needs to start with a group that is already known to share a number of properties in common.

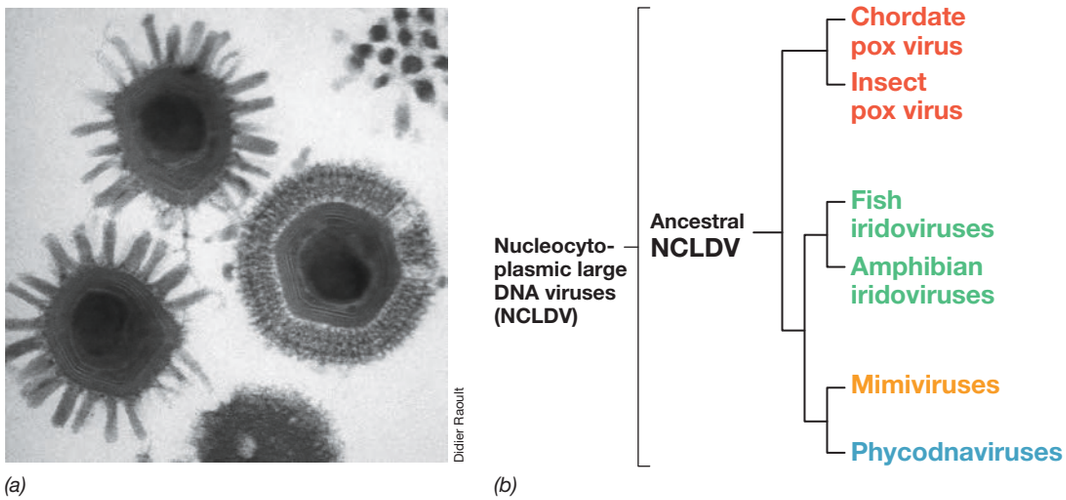


Figure 11.5 Phylogeny of nucleocytoplasmic large DNA viruses (NCLDV). *(a)* Transmission electron micrograph of *Mimivirus*, a member of the NCLDV group. A virion is about 0.75 μm in diameter. *(b)* Phylogeny of major groups of NCLDV based on comparative sequences of several proteins of DNA metabolism. See Chapter 5 for additional coverage of large viruses.

It is clear that diversity in the viral world is enormous and that obtaining a detailed phylogenetic tree of all viruses will remain a challenge. The continual isolation of highly unusual new viruses makes this difficult task even more challenging. For example, only about 7% of the genes of *Pandoravirus* (Figure 11.1) have gene homologs in existing genomic databases. What this means is that over 90% of the genome of this giant virus will likely be new to biology—a striking example of what awaits discovery in the fascinating world of viruses.

Check Your Understanding

- What type of viruses make up the *Caudovirales* order?
- Lacking ribosomes, how can viruses be placed on the universal tree of life?

II • DNA Viruses

Prokaryotic cells are infected primarily by double-stranded DNA viruses, whereas eukaryotic cells host viruses of all genomic variants. Some DNA viruses are tiny while others are huge, and some DNA animal viruses have novel replication steps or cause tumors in their hosts.

DNA viruses infect a wide variety of organisms, in particular, species of *Bacteria* and *Archaea*. In fact, the majority of viruses that infect prokaryotic cells are DNA viruses, mainly of the double-stranded variety (Figure 11.3). We examine several of these here along with some DNA viruses that infect eukaryotes, and we keep our focus on the genetic information flow that occurs during replication of DNA viruses of different genomic makeups.

11.3 Single-Stranded DNA Bacteriophages: ϕX174 and M13

In this section we discuss two single-stranded DNA bacteriophages, ϕX174 and M13. Many single-stranded DNA plant and animal viruses are also known, and because these share with bacterial viruses the fact that their genomes are of the plus complementarity (Baltimore class II, Figure 11.2*a*), many molecular events are similar. Hence, our focus here will be on the phages.

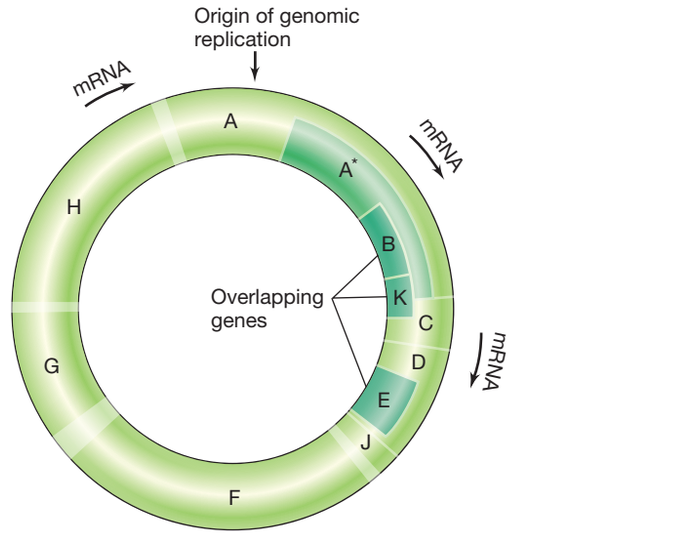
Bacteriophage ϕX174

Bacteriophage ϕX174 contains a circular genome of 5386 nucleotides inside a tiny icosahedral virion, about 25 nm in diameter. Phage ϕX174 has only a few genes and shows the phenomenon of **overlapping genes**, a condition in which there is insufficient DNA to encode all viral-specific proteins; to overcome this problem, parts of the genome are transcribed in more than one reading frame. For example, in the ϕX174 genome, gene B resides within gene A, and gene K resides within both genes A and C (**Figure 11.6*a***). Genes D and E also overlap, gene E being contained completely within gene D. Also, the termination codon of gene D overlaps the initiation codon of gene J.

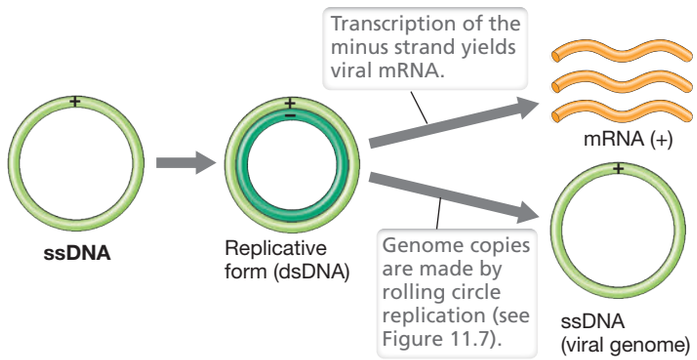
Mastering Microbiology
Art Activity: Figure 11.7 Rolling circle replication in phage ϕX174

The distinct gene products from overlapping genes are made by reinitiating transcription in a different reading frame within a gene to yield a second (and distinct) transcript. In addition to overlapping genes, a small protein in ϕX174 called A*, which functions to shut down host DNA synthesis, is synthesized by the reinitiation of *translation* (not transcription) within the mRNA for gene A. The A* protein is read from the same mRNA reading frame as the A protein but has a different in-frame start codon and is thus a shorter protein.

As we saw earlier (Section 11.1), before a single-stranded DNA genome can be transcribed, a complementary strand of DNA must be synthesized, forming the double-stranded replicative form. This can then be used as a source of both (+) mRNA and genome copies. Upon infection of an *Escherichia coli* cell by ϕX174, the viral DNA is separated from the protein coat and the genome is converted into the replicative form by host enzymes. From this, several copies are made by semiconservative replication, and phage-specific transcripts are made by transcription of the negative strand of the replicative



(a) **Genetic map of ϕX174**



(b) **Flow of events during ϕX174 replication**

Figure 11.6 Bacteriophage ϕX174, a single-stranded DNA phage. *(a)* Genetic map. Note regions of gene overlap. Protein A* is formed using only part of the coding sequence of gene A by reinitiation of translation. The key indicates the functions of the proteins encoded by each gene. Unlabeled parts of the chromosome are regions of noncoding DNA. *(b)* Genetic information flow in ϕX174. Progeny single-stranded DNA is produced from the replicative form by rolling circle replication (see Figure 11.7).

form (Figure 11.6*b*). The replicative form is also the starting point for making copies of the phage genome by a mechanism called **rolling circle replication** (Figure 11.7). This same mechanism is used by phage lambda, a widely used phage in molecular biology and biotechnology (Section 11.4).

In the synthesis of the ϕX174 genome, the rolling circle facilitates the continuous production of positive strands from the replicative form. To do this, the positive strand of the replicative form is nicked and the 3′ end of the exposed DNA is used to prime synthesis of a new strand (Figure 11.7). Cutting of the plus strand is accomplished by the A protein (Figure 11.6*a*). Continued rotation of the circle leads to the synthesis of a linear ϕX174 genome. Note that rolling circle synthesis differs from semiconservative replication (◀ Section 6.3) because only the negative strand serves as a template.

When the growing viral strand reaches unit length (5386 residues for ϕX174), the A protein cleaves it and then ligates the two ends of the newly synthesized single strand to give a single-stranded DNA circle. Ultimately, assembly of mature ϕX174 virions occurs and cell lysis follows. The E protein (Figure 11.6*a*) promotes cell lysis by inhibiting the activity of an enzyme in peptidoglycan synthesis (◀ Section 8.5) in the host cell. Because of the resulting weakness in newly synthesized cell wall material, the host cell ruptures, releasing the phage virions.

Bacteriophage M13

Bacteriophage M13 is a filamentous virus with helical symmetry; the virion is long and thin and attaches to the pilus of its host cell (◀ Figure 5.14). Filamentous phages such as M13 have the unusual property of being released from the host cell without the cell undergoing lysis; infected cells continue to grow, and typical viral plaques (◀ Section 5.3 and Figure 5.10) are not observed. To facilitate the nonlytic release, M13 DNA is covered with coat proteins as it exits across the cell envelope. Four minor coat proteins cover the tips of the virion while the major coat protein covers the sides (Figure 11.8). Thus, mature M13 virions do not accumulate in the cell as occurs

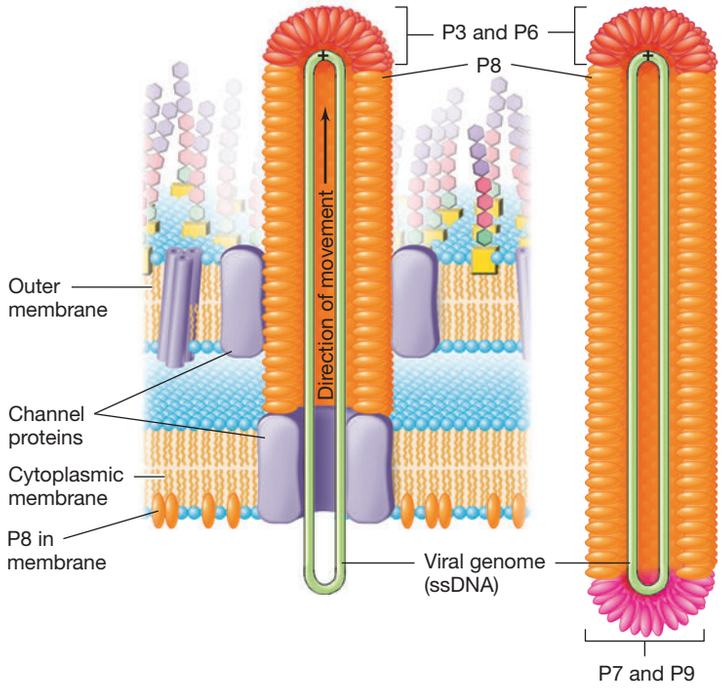


Figure 11.8 Release of phage M13. The virions of phage M13 exit infected cells without lysis. *(a)* Budding. The virus DNA crosses the cell envelope through a channel constructed from virus-encoded proteins. As this occurs, the DNA is coated with phage proteins that have been embedded in the cytoplasmic membrane. *(b)* Complete virion. The two ends of the virion are covered with small numbers of the minor coat proteins P3 and P6 (front end) or P7 and P9 (rear end). P8, major coat proteins. Because bacteriophage M13 is a single-stranded DNA phage, it was widely used in the past as a tool for molecular cloning and DNA sequencing (Chapters 10 and 12).

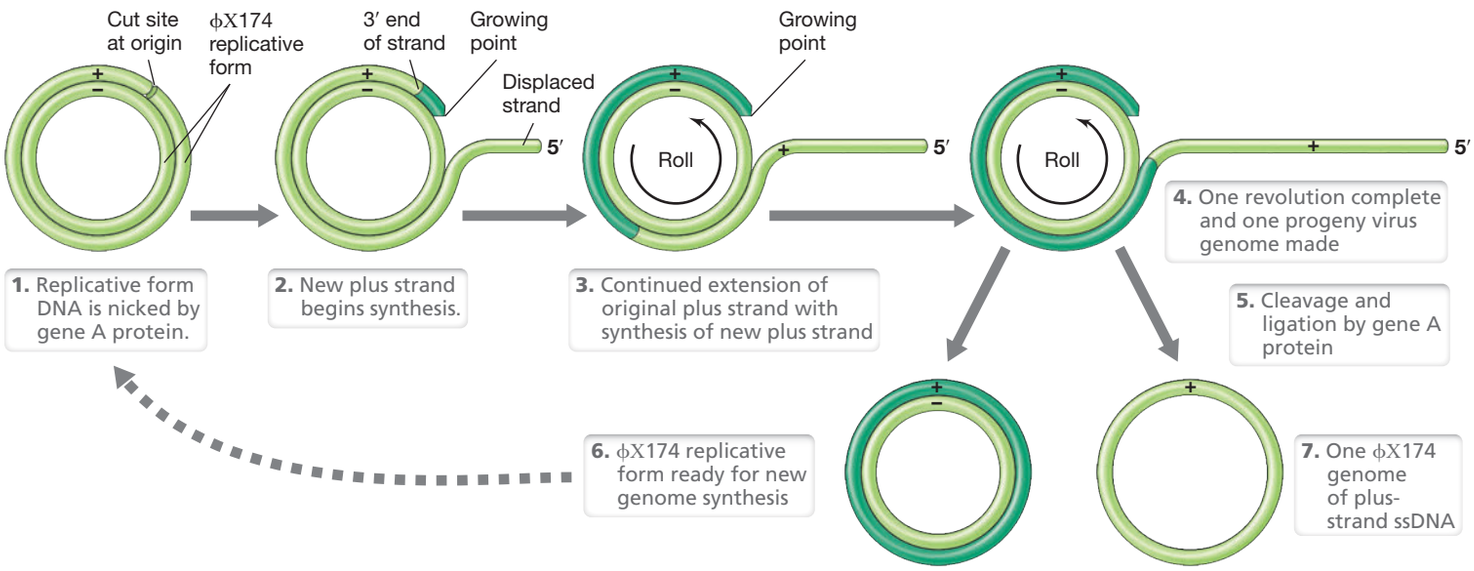


Figure 11.7 Rolling circle replication in phage ϕX174. Replication begins at the origin of the double-stranded replicative form with the cutting of the plus strand of DNA by gene A protein (both strands of DNA are shown in light green here for simplification). After one new progeny strand has been synthesized (one revolution of the circle), the gene A protein cleaves the new strand and ligates its two ends.

with typical lytic bacteriophages. Instead, these filamentous bacteriophages cause chronic infections.

Several features of phage M13 have made it useful as a cloning and DNA sequencing vehicle in the past. For example, many aspects of DNA replication in M13 are similar to those of ϕX174 and the genome is very small; this facilitates sequencing efforts. Second, a double-stranded form of genomic DNA essential for cloning purposes is produced naturally when M13 produces its replicative form. Third, as long as infected cells are kept growing, phage can be produced indefinitely, yielding a continuous source of the cloned DNA. These and other features of M13 made this phage a workhorse of the genetic engineering field for many years, although today M13 has been replaced for most genetic engineering tasks by a variety of even more convenient and useful tools, as we will see in Chapter 12.

We transition now from *single-stranded* DNA bacteriophages to *double-stranded* DNA bacteriophages, where, because of the nature of the genome, replication events resemble those in cells but have some unique features as well.

Check Your Understanding

- Why is formation of the replicative form of ϕX174 necessary in order to make phage-specific mRNA?
- In the ϕX174 genome, describe the difference between how the gene B and gene A* proteins are made.
- How can M13 virions be released without killing the infected host cell?

11.4 Double-Stranded DNA Bacteriophages: T4, T7, and Lambda

The double-stranded DNA (dsDNA) (Baltimore class I, Figure 11.2*a*) bacteriophages are among the best studied of all viruses, and we have already discussed two important ones, T4 and lambda, in Chapter 5. Because of their importance in molecular biology, gene regulation, and genomics, we consider details regarding their replication and also that of bacteriophage T7.

Bacteriophage T4

Bacteriophage T4 is a large, tailed, double-stranded DNA virus of *Escherichia coli* that possesses complex morphology (90 nm × 200 nm; ◀ Figure 5.7). As a *virulent* bacteriophage, T4 always kills its host cell following infection (◀ Section 5.5). The T4 genome differs from many other bacteriophage genomes in that its DNA contains the modified cytosine base 5-hydroxymethylcytosine in place of cytosine (**Figure 11.9*a***); this modification is catalyzed by a phage-encoded enzyme and further glycosylated (a glucose molecule is added) to produce a viral nucleotide that cannot be degraded by the cell's restriction modification systems (◀ Sections 5.5 and 9.12).

Because of its large, 170-kilobase-pair linear genome, bacteriophage T4 encodes its own DNA polymerase. Other proteins that function in viral DNA replication such as primases and helicases (◀ Section 6.3) are also encoded by the T4 genome. In fact, T4 produces its own eight-protein DNA replisome complex (◀ Section 6.4) to facilitate phage-specific genome synthesis. However, the T4 genome does not encode its own RNA polymerase to express these genes. Instead T4-specific proteins modify the specificity of the host RNA polymerase so that it recognizes only phage promoters; these modification proteins are encoded by T4 genes as part of the "early proteins" in the replication cycle (◀ Figure 5.16).

To facilitate expression of only T4-encoded genes, a phage-encoded anti-sigma factor (◀ Section 7.16) is produced that binds to the host RNA polymerase sigma factor and prevents it from recognizing promoters on host genes. This effectively switches the activity of host RNA polymerase from transcribing host-specific genes to transcribing T4-specific genes. Later in the infection process, other phage proteins modify the host RNA polymerase so that it now

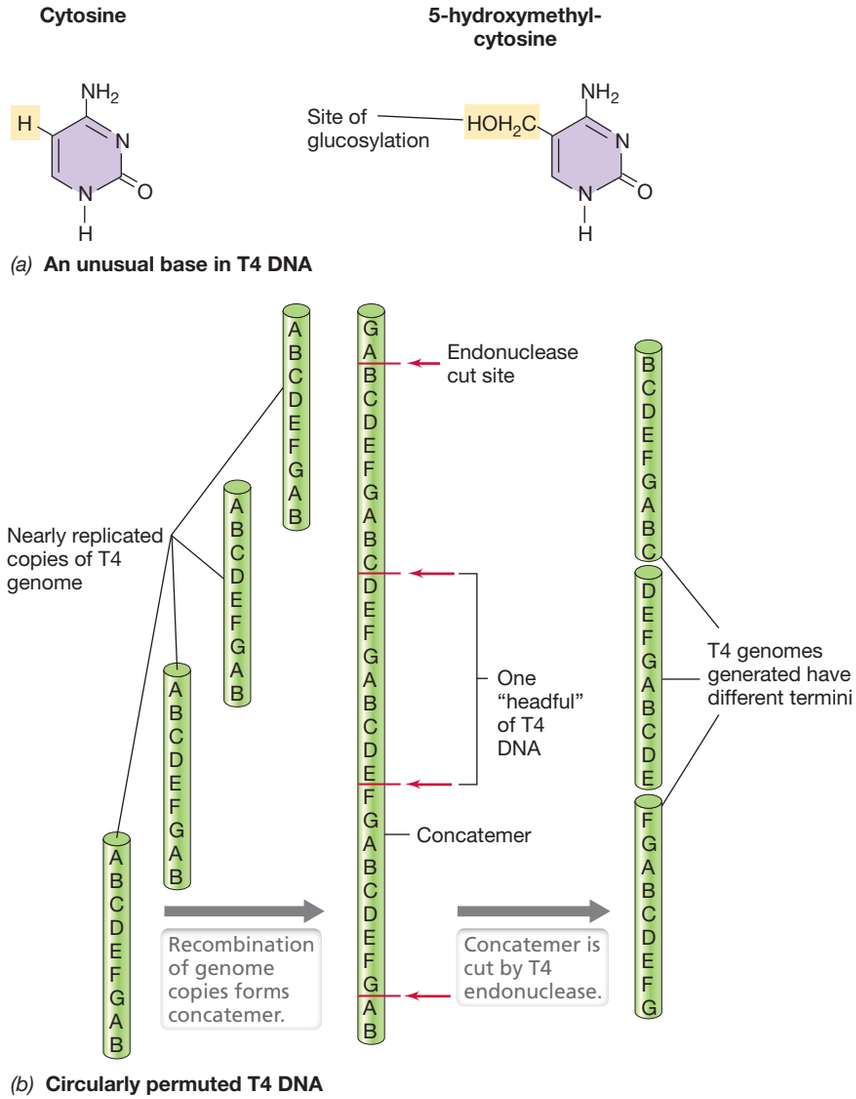


Figure 11.9 Circular permutation and the unique DNA of bacteriophage T4. *(a)* The unique base 5-hydroxymethylcytosine in the DNA of bacteriophage T4. Once this base is glucosylated, the T4 DNA is resistant to restriction enzyme attack. *(b)* Generation of virus-length T4 DNA molecules with permuted sequences by an endonuclease that cuts off constant lengths of DNA from a concatemer regardless of their sequence.

recognizes T4 promoters of genes encoding middle proteins. Finally, transcription of T4 late genes begins, and this requires a new T4-encoded sigma factor that directs host RNA polymerase to promoters for these genes only. At this point, viral assembly can begin.

Besides encoding an unusual nucleotide and its own replication machinery, the T4 genome has another unusual feature: In a population of T4 virions, although each copy of the genome contains the same set of genes, they are arranged in a different order. This is a phenomenon called *circular permutation*, which is a feature of many virus genomes. The term circular permutation is derived from the fact that DNA molecules that are circularly permuted appear to have been linearized by opening identical circular genomes at different locations. Circularly permuted genomes are also *terminally redundant*, meaning that some DNA sequences are duplicated on both ends of the DNA molecule as a result of the mechanism that generated them.

The T4 genome is first replicated as a unit and then several genomic units are recombined end to end to form a long DNA molecule called a **concatemer** (Figure 11.9*b*). When the T4 DNA is packaged into capsids, the concatemer is not cut at a specific sequence; instead linear segments of DNA just long enough to fill a phage head are generated. This is called *headful packaging* and is common among bacteriophages. However, because the T4 head holds slightly more than a genome length, the headful mechanism generates terminal repeats of about 3–6 kbp at each end of the DNA molecule (Figure 11.9*b*).

Bacteriophage T7

Bacteriophage T7 is a relatively small virulent DNA virus that infects *Escherichia coli* and a few related enteric bacteria. The virion has an icosahedral head and a very short tail (Figure 11.3*b*), and the T7 genome is a linear double-stranded DNA molecule of about 40 kilobase pairs.

When a T7 virion attaches to a host cell and the DNA is injected, early genes are quickly transcribed by host RNA polymerase and then translated. One of these early proteins inhibits the host restriction system, a mechanism for protecting the cell from foreign DNA (◀ Sections 5.5 and 9.12). This occurs very rapidly, as the T7 antirestriction protein is made and becomes active before the entire T7 genome has entered the cell. Other early proteins include a T7 RNA polymerase and proteins that inhibit host RNA polymerase activity. T7 RNA polymerase recognizes only T7 gene promoters distributed along the T7 genome. This transcriptional strategy differs from that of phage T4 because, as we saw, T4 uses the host RNA polymerase throughout its replication cycle but modifies the host polymerase to recognize only phage genes.

Genome replication in T7 begins at an origin of replication within the molecule and proceeds bidirectionally from this point (**Figure 11.10*a***). Phage T7 uses its own DNA polymerase, which is a composite protein including one polypeptide encoded by the phage

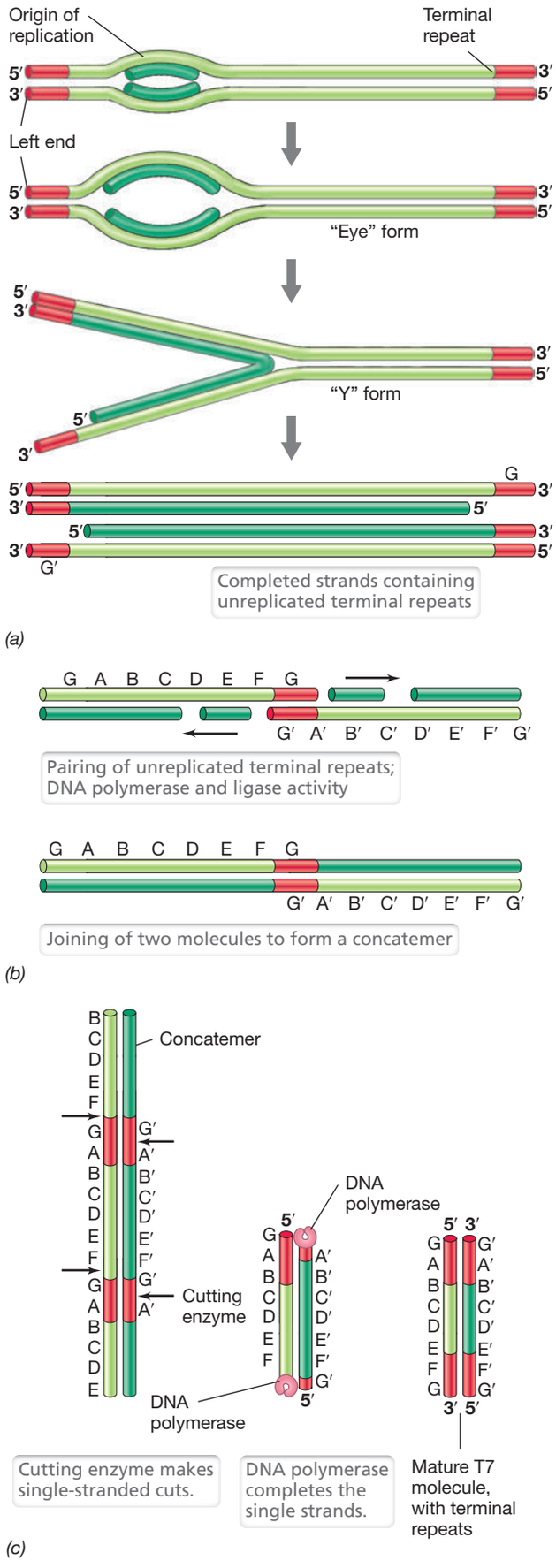


Figure 11.10 Replication of the bacteriophage T7 genome. *(a)* The linear, double-stranded DNA undergoes bidirectional replication, giving rise to intermediate "eye" and "Y" forms (for simplicity, both template strands are shown in light green and both newly synthesized strands in dark green). *(b)* Formation of concatemers by joining DNA molecules at their unreplicated terminal ends. *(c)* Production of mature viral DNA molecules from T7 concatemers by activity of the cutting enzyme, an endonuclease.

UNIT 3

Mastering Microbiology
Art Activity: Figure 11.10 Replication of the bacteriophage T7 genome

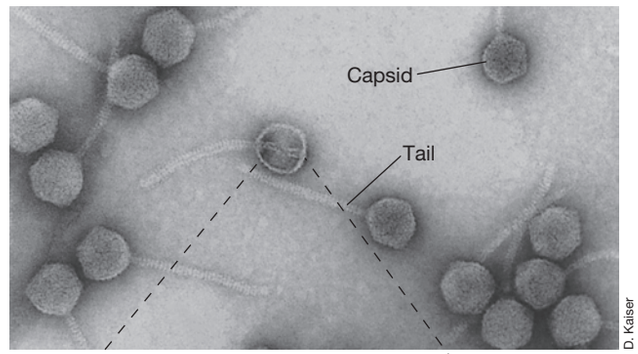
and one by the host. As in phage T4, T7 DNA contains terminal repeats at both ends of the molecule and these are eventually used to form concatemers (Figure 11.10*b*). Continued replication and recombination leads to concatemers of considerable length, but ultimately a phage-encoded endonuclease cuts each concatemer at a specific site, resulting in the formation of linear DNA molecules with terminal repeats that are packaged into phage heads (Figure 11.10*c*). However, because T7 endonuclease cuts the concatemer at specific sequences, the DNA sequence in each T7 virion is identical. This differs from the situation in phage T4 discussed above, where DNA concatemers are processed using a "headful mechanism" that generates circularly permuted genomes.

Bacteriophage Lambda

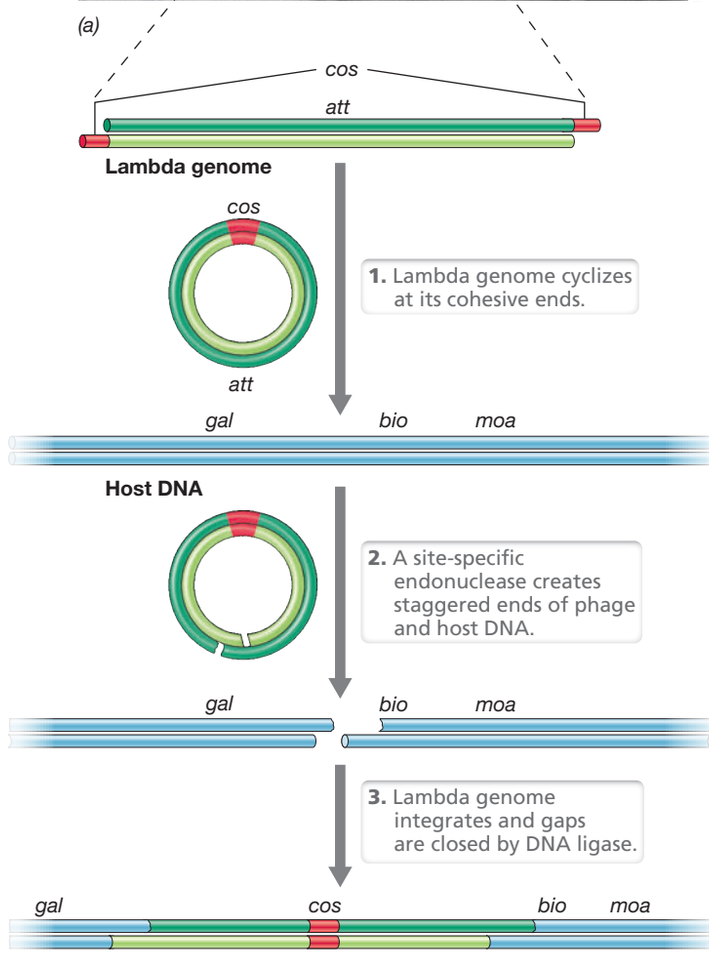
Bacteriophage lambda, which also infects *Escherichia coli*, is a double-stranded DNA virus with a head and tail (**Figure 11.11*a***). Lambda is a *temperate* bacteriophage and thus has two possible pathways—lytic or lysogenic. During the lytic cycle, lambda kills its host cells in the same manner as T4 and T7. However, in *lysogeny* lambda differs from T4 and T7 by maintaining a stable relationship with its host. Inside its capsid, the 48.5-kilobase lambda genome is linear and possesses a single-stranded region 12 nucleotides long at the 5′ end of each DNA strand. These single-stranded "cohesive" ends are complementary in base sequence; when lambda DNA enters the host cell, they base-pair to form the *cos* site and cyclize (circularize) the genome (Figure 11.11*b*).

If lambda enters the *lytic* pathway, long, linear concatemers of genomic DNA are synthesized by the rolling circle replication mechanism described for ϕX174 (Section 11.3). The double-stranded concatemer is then cut into genome-sized lengths at the *cos* sites and the resulting genomes packaged into lambda phage heads (Figure 11.11*c*). Once the tail has been added and mature lambda virions have been assembled (Figure 11.11*a*), cell lysis occurs and the virions are released. In its role as a lytic phage, lambda can also package a few chromosomal genes from its lysed host in newly synthesized virions and then transfer these to a second host cell, a process called *transduction*. Transduction is an important means of horizontal gene transfer in nature and is also an important tool in bacterial genetics (◀ Section 9.7).

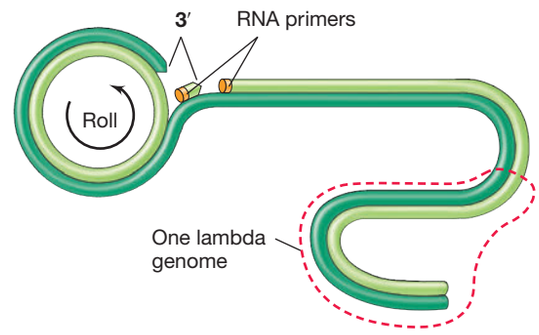
Instead of the lytic pathway, if lambda takes the *lysogenic* route, its genome integrates into the *E. coli* chromosome. This requires a protein called *lambda integrase*, a phage-encoded enzyme that recognizes the phage and bacterial genome attachment sites (*att* in Figure 11.11*b*) and facilitates integration of the lambda genome. From this relatively stable state, certain events such as host DNA damage can initiate the lytic cycle once again. After such a trigger, a



(a)



(b) **Integration of lambda DNA into the host**



(c) **Rolling circle replication of lambda genome**

Figure 11.11 Bacteriophage lambda: virions, integration of viral DNA and rolling circle replication. *(a)* Transmission electron micrograph of phage lambda virions. The head of each virion is about 65 nm in diameter and contains linear dsDNA. *(b)* Lambda DNA integrates at specific attachment (*att*) sites on both the host and phage genomes. Host genes near *att* include *gal*, galactose utilization; *bio*, biotin synthesis; and *moa*, molybdenum cofactor synthesis. Lambda integrase is required, and specific pairing of the complementary ends results in integration of lambda DNA. *(c)* During rolling circle replication, as one strand (dark green) rolls out, it is both replicated at its opposite end and serves as a template for synthesis of the complementary strand.

lambda excision protein excises the lambda genome from the host chromosome, transcription of lambda DNA begins, and lytic events unfold. With these two contrasting events—lysis and lysogeny—in mind, what controls the path that phage lambda will take?

Lysis or Lysogeny: Regulation of the Lambda Lifestyle

Whether lysis or lysogeny occurs in a lambda infection depends in large part on the levels of two key repressor proteins that can accumulate in the cell following infection: the *lambda repressor*, also called the *cI protein*, and a second repressor called *Cro*. In a nutshell, the first repressor that accumulates will control the outcome of the infection.

If genes encoding the cI protein are rapidly transcribed following infection and cI accumulates, it represses the transcription of all other lambda-encoded genes, including Cro. When this happens, the lambda genome integrates into the host's genome and becomes a prophage (**Figure 11.12**). The host continues to grow, yielding more lysogens, until an event that triggers lysis is encountered. Cro, on the other hand, represses expression of a protein called cII whose function is to activate the synthesis of cI. Hence, following infection, if cI is present at insufficient levels to repress expression of phage-specific genes, Cro accumulates in the cell; if this happens, lambda travels the lytic pathway.

Control of these alternative lifestyles—lysis or lysogeny—of lambda has been likened to a "genetic switch," where a defined series of events must occur for one pathway to be favored over the other. Although infection of an *E. coli* cell by a lambda virion typically results in the lytic cycle, as we have said, lytic events can be switched off if sufficient concentrations of cII are present to ensure adequate levels of cI (Figure 11.12). But how does this come about? Levels of protein cII are controlled by the relative activity of a protease in the cell that slowly degrades cII and by levels of yet another protein, cIII, whose function is to stabilize cII and protect it from protease attack. We thus have a cascade of regulatory events here: cIII controls cII, which in turn controls cI. But even this is not the end of the story. Several other proteins not described here also play a role in the lambda lytic/lysogenic "decision," and hence the progress of a lambda infection is a highly intricate series of events.

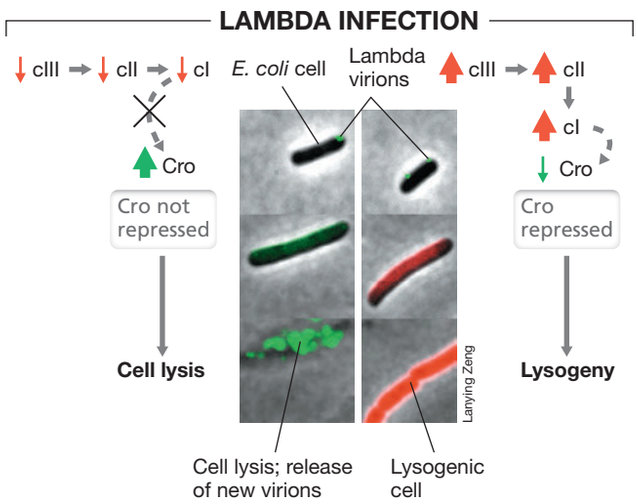


Figure 11.12 Regulation of lytic and lysogenic events in phage lambda. The photomicrographs show time courses of cells of *Escherichia coli* following a course of lytic (left panel, green) or lysogenic (right panel, red) events, as controlled by various repressors. The colors originate from genetically engineered lambda phage that trigger the production of specific fluorescent proteins when either lytic genes (green) or lysogenic genes (red) are expressed. Lytic cells are killed whereas *E. coli* lysogens continue to grow and divide.

Indeed, this tiny bacteriophage employs some of the most elaborate regulatory systems known in virology. One of the few lambda proteins expressed during lysogeny prevents the growing lysogen from entering dormancy during stressful conditions (◀ Section 8.12). This regulatory mechanism helps ensure the continued spread of the lambda virus in a mixed population of *Escherichia coli* lysogens and nonlysogens.

Cells of both *Bacteria* and *Archaea* are prokaryotic, and one might predict that what is true for bacterial viruses is also true of archaeal viruses. We see next that this is definitely not the case.

Check Your Understanding

- What is a concatemer?
- How does the mechanism used by bacteriophage T7 to evade the host cell's restriction system differ from that of T4?
- What commits lambda to the lytic versus the lysogenic pathway?

11.5 Viruses of *Archaea*

Many bacteriophages and archaeal viruses have been isolated and characterized thus far. For *Bacteria*, these include both DNA and RNA phages, some with single-stranded and others with double-stranded genomes. However, all characterized archaeal viruses have DNA genomes, and with rare exception, double-stranded DNA genomes (Figure 11.3*a*).

DNA Archaeal Viruses

Several DNA viruses have been discovered that infect species in the two main phyla of *Archaea*, the *Euryarchaeota* and the *Crenarchaeota* (Chapter 17). Most viruses that infect species of *Euryarchaeota*, including both methanogenic and halophilic *Archaea*, are of the "head and tail" type, resembling the structurally complex phages that infect enteric bacteria, such as phage T4 (◀ Section 5.5; Section 11.4). Besides two special cases discussed later, all other characterized archaeal DNA viruses contain double-stranded and typically *circular* DNA genomes.

The most morphologically distinctive archaeal viruses infect hyperthermophilic *Crenarchaeota*. For example, the sulfur chemolithotroph *Sulfolobus* is host to several structurally unusual viruses. One such virus, called *SSV*, forms spindle-shaped virions that often cluster in rosettes (**Figure 11.13*a***). Such viruses are widespread in acidic hot springs worldwide. Virions of SSV contain a circular DNA genome of about 15 kilobase pairs. A second morphological type of *Sulfolobus* virus forms a rigid, helical rod-shaped structure (Figure 11.13*b*). Viruses in this class, nicknamed *SIFV*, contain linear DNA genomes about twice the size of that of SSV. Many variations on the spindle- and rod-shaped patterns have been seen in archaeal viral isolation studies, and a few species of *Crenarchaeota* are also infected by filamentous viruses.

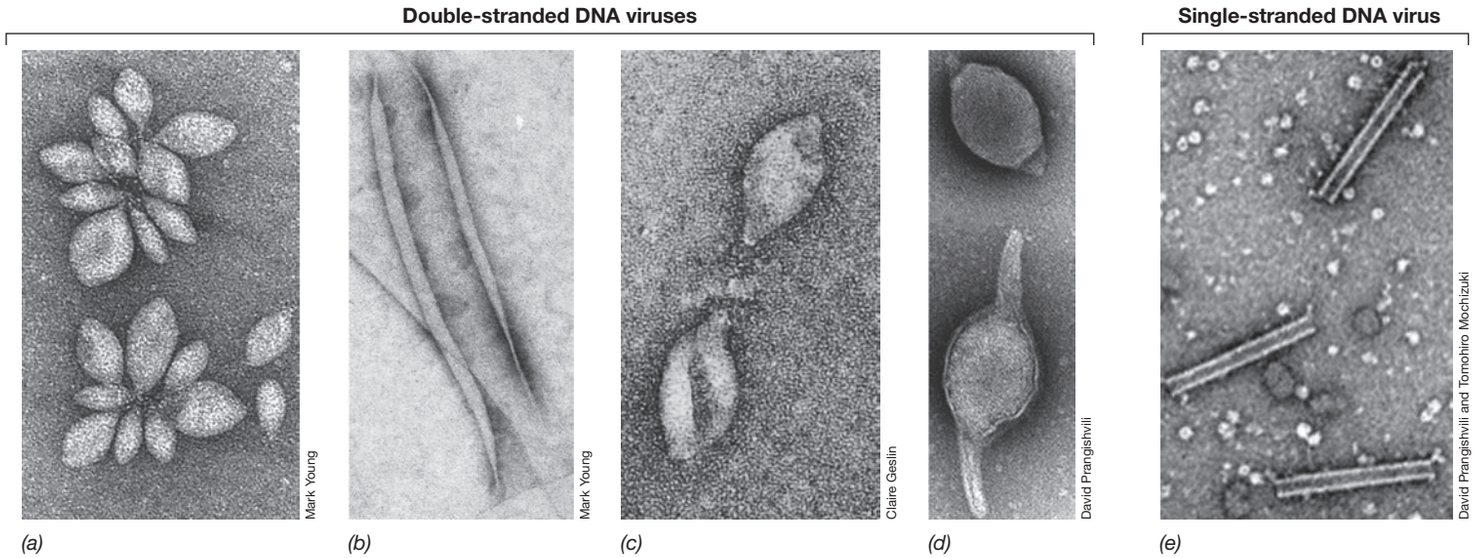


Figure 11.13 Archaeal viruses. Electron micrographs of viruses of *Crenarchaeota* (parts *a*, *b*, *d*, *e*), and a virus of a euryarchaeote *(c)*. *(a)* Spindle-shaped virus SSV1 that infects *Sulfolobus solfataricus* (virions are 40 × 80 nm). *(b)* Filamentous virus SIFV that infects *S. solfataricus* (virions are 50 × 900–500 nm). *(c)* Spindle-shaped virus PAV1 that infects *Pyrococcus abyssi* (virions are 80 × 120 nm). *(d)* ATV, the virus that infects the hyperthermophile *Acidianus convivator*. When released from the cell the virions are lemon-shaped (left), but they proceed to grow appendages on both ends (right). ATV virions are about 100 nm in diameter. *(e)* Filamentous virus ACV of *Aerophilum pernix*. ACV virions are about 220 nm long.

A spindle-shaped virus that infects the hyperthermophile *Acidianus* displays a novel behavior. The virion, called *ATV*, contains a circular genome of about 68 kilobase pairs and is lemon-shaped when first released from the host cells. However, shortly after release from its lysed host cell, the virion produces long, thin tails, one at each end (Figure 11.13*d*). The tails are actually tubes, and as they form, the virion becomes thinner and its volume is reduced. Remarkably, this is the first example of virus development in the complete absence of host cell contact. It is thought that the extended tails of ATV help the virus in some way survive in its hot (85°C), acidic (pH 1.5) environment or perhaps assist in attachment to a new host cell. This unusually shaped virus is also lysogenic, a property rarely seen in other archaeal viruses.

A spindle-shaped virus also infects the hyperthermophile *Pyrococcus* (*Euryarchaeota*). This virus, named *PAV1*, resembles SSV but is larger and contains a very short tail (Figure 11.13*c*). PAV1 has a small circular DNA genome and is released from host cells without cell lysis, probably by a budding mechanism similar to that of the *Escherichia coli* bacteriophage M13 (Section 11.3). *Pyrococcus* has a growth temperature optimum of 100°C and thus PAV1 virions must be extremely heat-stable. Despite their similar morphologies, genomic comparisons of PAV1 and SSV-type viruses show little sequence similarity, indicating that the two types of viruses do not have common evolutionary roots.

Although double-stranded DNA genomes seem to predominate in the *Archaea*, two archaeal viruses have been described that possess single-stranded DNA genomes. Pleomorphic virus 1 (HRPV-1) infects the extreme halophile *Halorubrum* and is unusual not only because of its circular single-stranded DNA genome (of the + complementarity, a property it shares with bacteriophage ϕX174, Figure 11.6), but also due to its enveloped nonuniform morphology. Thus, the virus is released by secretion versus the host cell lysis mechanism common to virulent bacteriophage. Over half of the 7048-nucleotide HRPV-1 genome encodes viral proteins that have also been found in the double-stranded DNA haloarchaeal virus His2. This highlights the challenges of classifying viruses based on genome type and the Baltimore scheme (Section 11.1) but also indicates the likely relationships that exist between archaeal viruses of quite different genomic structure. A second archaeal virus possessing a single-stranded DNA genome is the *Aeropyrum c*oil-shaped *v*irus (ACV) that infects the hyperthermophile *Aeropyrum pernix* (Figure 11.13*e*). A filamentous capsid surrounds this circular single-stranded 24.9-kilobase viral genome, which is double the size of the second largest known single-stranded DNA viral genome. Thus, much diversity obviously exists among archaeal viruses.

Genome Content of DNA Archaeal Viruses

While over 2000 bacteriophage genomes have been sequenced, only a little over 100 archaeal viral genome sequences are available in the databases. This disparity in number is due to the difficulty in culturing many archaeal hosts versus their bacterial counterparts. Metagenomic approaches (◀ Section 10.7) are helping expand this number and identify viruses that are not in culture (see discussion of archaeal RNA viruses in next subsection). On average, archaeal virus genomes are smaller than bacteriophage genomes (65 versus 101 open reading frames). Comparative analysis of viral genomes suggests that the genome replication strategies of many archaeal viruses are more similar to those employed by eukaryotic viruses than those used by bacteriophages. Unlike many bacteriophages such as phage T7 (Section 11.4), archaeal viral genomes do not encode a virus-specific RNA polymerase. Thus, archaeal viruses are dependent on the host cell's RNA polymerase

for gene expression, and transcription factors predicted to commandeer the archaeal RNA polymerase have been annotated in many archaeal viral genomes.

Besides the genes for transcription factors mentioned above and some characterized structural proteins, analyses indicate that up to 90% of the genes within many archaeal viral genomes encode proteins of unknown function unrelated to any other proteins in the databases. However, some have been characterized. For example, a unique method of viral release occurs with two archaeal viruses that infect *Sulfolobus islandicus*. Not only do the *S. islandicus* rod-shaped virus 2 (SIRV2) and turreted icosahedral virus (STIV) genomes encode structural proteins for their capsids, they also encode a protein called *PVAP* (*p*rotein forming *v*irus-*a*ssociated *p*yramid) that facilitates viral release from the cell. PVAP assembles into seven-fold pyramid structures that rupture the host cell's S-layer (◀ Section 2.5) and open outwards to create a portal for viruses to exit the host cell (**Figure 11.14*a, b***). Heterologous expression (◀ Section 10.5 and Chapter 12) of PVAP in the bacterium *Escherichia coli* also resulted in the formation of pyramid structures that protruded into the periplasmic space (Figure 11.14*c*), thus indicating that a single archaeal viral protein alone is sufficient for producing these structures. The large number of unique archaeal viral proteins that remain to be characterized will undoubtedly lead to even more remarkable discoveries.

RNA Archaeal Viruses

Thus far, RNA viruses that can replicate in the laboratory on archaeal hosts are unknown, despite the fact that a variety of RNA viruses infect *Bacteria* and eukaryotes (Figure 11.3). Although no concrete examples of archaeal RNA viruses have emerged, environmental genomics have shown that they almost certainly exist. In some acidic hot springs of Yellowstone National Park (USA) that support large communities of *Crenarchaeota*, a large number of unusually shaped and structurally tough archaeal viruses have been discovered (**Figure 11.15**) and grown in the laboratory. Thus far, all of these have been DNA viruses. However, using the powerful tools of metagenomics (◀ Section 10.7), researchers studying these hot springs have discovered viral RNAs whose RNA sequences bear no resemblance to those of any known RNA viruses that infect *Bacteria*. Because these springs are too hot for eukaryotes and the cell numbers of *Bacteria* are few, the unusual RNA is almost certainly from RNA archaeal viruses that are yet to be propagated in the laboratory.

Sequence analyses of the hot spring viral RNA show that it originated from single-stranded plus-sense RNA viruses (Baltimore class IV, Figure 11.2*b* and Section 11.8). These viral genomes also encode an RNA replicase—a hallmark of RNA viruses—and are likely to replicate by way of polyprotein formation, a replication mechanism employed by some class IV viruses of eukaryotes, such as poliovirus (Section 11.8). Replication steps of the putative RNA archaeal viruses, including important molecular details such as the extent to which viral (rather than host) polymerases participate in the replication process, are unclear and await laboratory cultivation of the viruses. However, now that scientists know that such viruses almost certainly exist, they can be on the lookout for them in viral enrichment and isolation studies.

We move on now to consider animal viruses—a group in which there is no shortage of genomic diversity and fascinating replication schemes, many unique to biology.

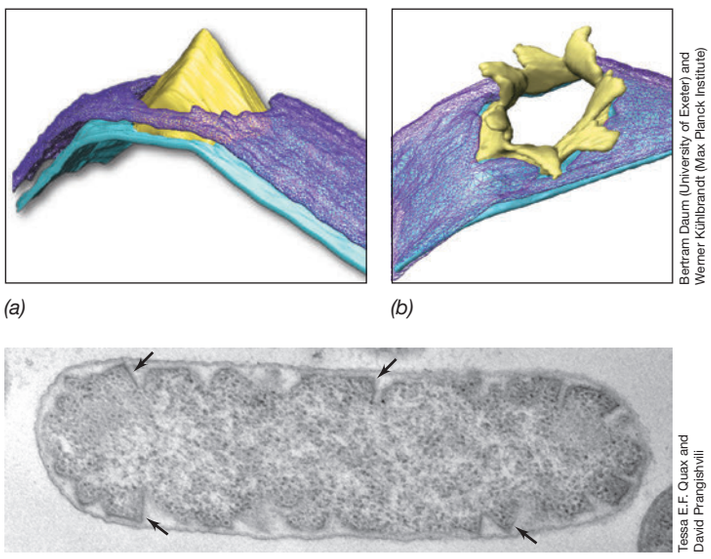


Figure 11.14 Virus-associated pyramids (VAPs). Electron cryotomograph showing a *Sulfolobus islandicus* cell with SIRV2 VAP *(a)* closed and *(b)* open. The VAPs open to allow release of virions from the *Sulfolobus* host cell. *(c)* Transmission electron micrograph showing heterologous expression of the protein for archaeal VAP formation (PVAP) in *Escherichia coli*. The arrows point to pyramid structures formed in the periplasm. The *E. coli* cell is about 0.7 μm in diameter.

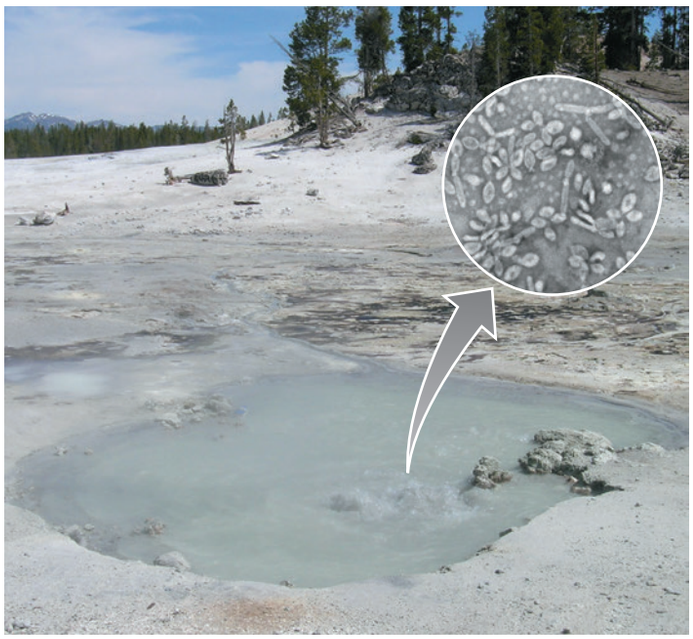


Figure 11.15 An acidic Yellowstone hot spring and its archaeal viruses. This spring is a boiling mudpot with a pH of about 2 and is loaded with hyperthermophilic and acidophilic *Crenarchaeota* (▶ Section 17.10). Inset: Transmission electron micrograph of a mixture of archaeal viruses from the spring. Compare with Figure 11.13.

Check Your Understanding

- What type of genome is seen in most archaeal viruses?
- Compared with other archaeal viruses, what are two unusual features of the virus that infects *Acidianus*?
- Describe the unique mechanism some *Sulfolobus* viruses use to exit the host cell.

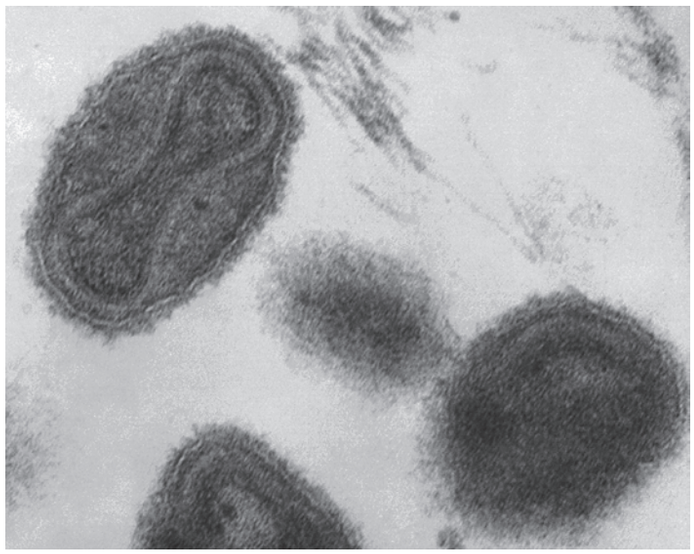
11.6 Uniquely Replicating DNA Animal Viruses

Unusual replication strategies are seen in two groups of double-stranded DNA (Baltimore class I, Figure 11.2*a*) animal viruses: pox viruses and adenoviruses. Pox viruses are unique because all replication events, including DNA replication, occur in the host *cytoplasm* instead of the nucleus, and adenoviruses are unique because the replication of their genome proceeds in a leading fashion on *both* DNA template strands.

Pox Viruses

Pox viruses have been important historically as well as medically. Smallpox virus was the first virus to be studied in any detail and was the first virus for which a vaccine was developed (over 200 years ago the British physician Edward Jenner was the first to protect people from infection by smallpox virus by exposing them to the similar but much less virulent cowpox virus). Pox viruses are among the largest of all viruses, the brick-shaped vaccinia virions measuring almost 400 nm in diameter (**Figure 11.16**). Because it closely resembles the smallpox virus but is not pathogenic, vaccinia is used as a smallpox vaccine today and as a safe laboratory model for smallpox virus molecular biology.

Mastering Microbiology
Art Activity: Figure 11.17*b* Adenoviruses and their genomic replication



CDC/PHIL, Fred Murphy and Sylvia Whitfield

Figure 11.16 Smallpox virus. Transmission electron micrograph of a negatively stained thin section of smallpox virus virions. The virions are approximately 350 nm (0.35 μm) long. The dumbbell-shaped structure inside the virion is the nucleocapsid, which contains the double-stranded DNA genome. All replication functions for pox virus occur in the host cytoplasm.

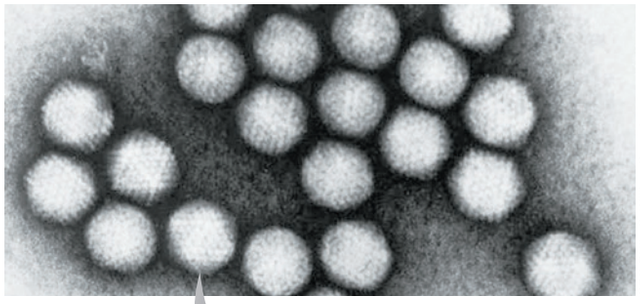
The vaccinia virus genome consists of linear double-stranded DNA of about 190 kilobase pairs encoding about 250 genes. Following attachment, vaccinia virions are taken up into host cells and the nucleocapsids (Figure 11.16) are liberated in the cytoplasm; all replication events take place in the cytoplasm. Uncoating of the viral genome requires the activity of a viral protein that is synthesized after infection (the gene encoding this protein is transcribed by a viral RNA polymerase contained within the virion). In addition to this uncoating gene, a number of other viral genes are transcribed, including genes that encode a DNA polymerase that synthesizes copies of the viral genome. These are then incorporated into virions that accumulate in the cytoplasm, and the virions are released when the infected cell lyses.

Vaccinia virus has been genetically engineered to contain certain proteins from other viruses for use in *recombinant vaccines* (▶ Section 12.8). A vaccine is a substance capable of eliciting an immune response in an animal that protects the animal from future infection with the same agent (Chapter 28). Vaccinia virus causes no serious health effects in humans but elicits a strong immune response. Therefore, as a carrier of proteins from pathogenic viruses, vaccinia virus is a relatively safe and effective tool for stimulating an immune response against these pathogens. Success has been obtained with vaccinia virus vaccines against the viruses that cause influenza, rabies, herpes simplex type 1, and hepatitis B. We discuss all of these viruses in upcoming sections.

Adenoviruses

Adenoviruses are a group of small and naked icosahedral viruses (**Figure 11.17*a***) that contain linear double-stranded DNA genomes. Adenoviruses are of minor health importance, causing mild respiratory infections in humans, but they have unique stature in virology because of the mechanism by which they replicate their genomes. Attached to the 5′ end of adenoviral genomic DNA is a protein called the adenoviral *terminal protein*, and it is essential for replication of the adenoviral genome. The complementary DNA strands also have inverted terminal repeats that play a role in the replication process (Figure 11.17*b*).

Following infection, the adenoviral nucleocapsid is released into the host cell nucleus, and transcription of the early genes proceeds by activity of the host RNA polymerase. Most early transcripts encode important replication proteins such as the terminal protein and a viral DNA polymerase. Replication of the adenoviral genome begins at either end of the DNA genome and the terminal protein facilitates this process because it contains a covalently bound cytosine that functions as a primer for DNA polymerase (Figure 11.17*b*). The products of this initial replication are a completed double-stranded viral genome and a single-stranded minus-sense DNA molecule. At this point, a unique replication event occurs. The single DNA strand cyclizes by means of its inverted terminal repeats, and a complementary (plus-sense) DNA strand is synthesized beginning from its 5′ end (Figure 11.17*b*). This mechanism is unique because double-stranded DNA is replicated *without the formation of a lagging strand*, as occurs in conventional semiconservative DNA replication (◀ Section 6.3). Once sufficient copies of the adenoviral genome have formed and virion structural components accumulate in the host cell, mature adenoviral virions are assembled and released from the cell following lysis.



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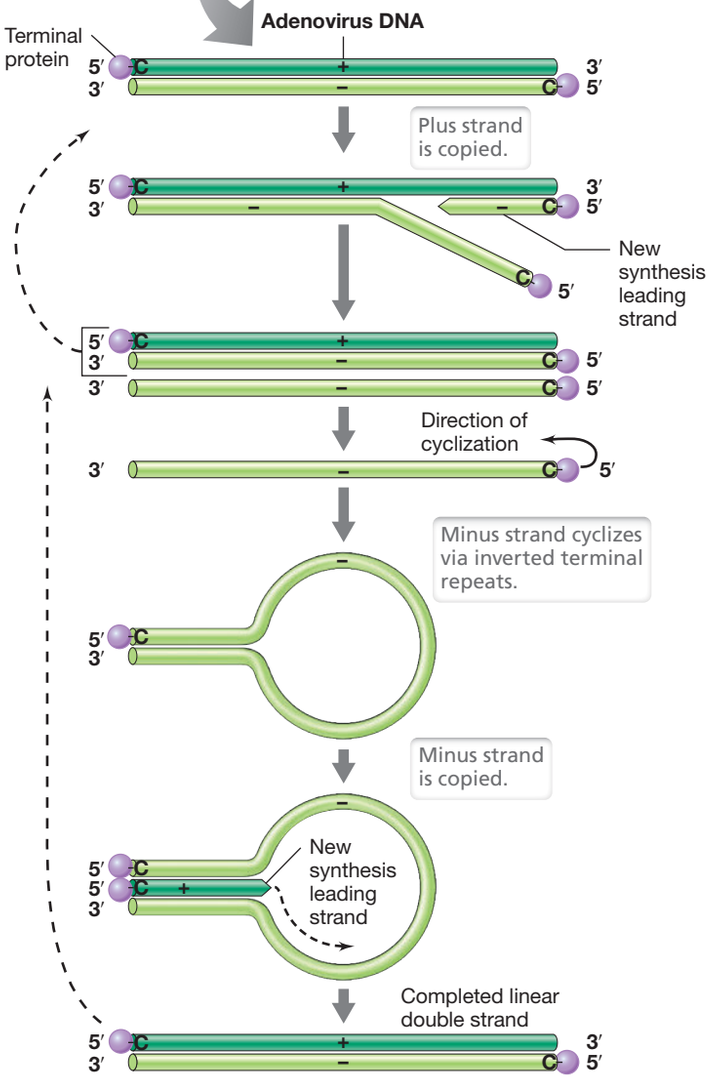


(b)

Figure 11.17 Adenoviruses and their genomic replication. *(a)* Transmission electron micrograph of adenoviral virions. Note the icosahedral structure. *(b)* Adenoviral genome replication. Because of loop formation (cyclization), there is no lagging strand; DNA synthesis is leading on both strands. A cytosine (C) is attached to the terminal protein. Adenoviruses are one of several classes of human viruses that cause upper respiratory infections such as the common cold (▶ Section 31.7). Rhinoviruses (single-stranded plus-sense RNA viruses, Section 11.8) cause the vast majority of colds.

In addition to infectious diseases, some animal viruses cause cancer, and we consider these now.

Check Your Understanding

- What is unusual about genome replication in pox viruses?
- What is unusual about genome replication in adenoviruses?
- Why is the adenovirus terminal protein essential for replicating its genome?

11.7 DNA Tumor Viruses

Besides catalyzing lytic events or becoming integrated into a genome in a latent state, some DNA animal viruses can induce the formation of tumors. These include viruses of the polyomavirus family and some herpesviruses, both of which contain double-stranded DNA genomes (Baltimore class I, Figure 11.2*a*).

UNIT 3

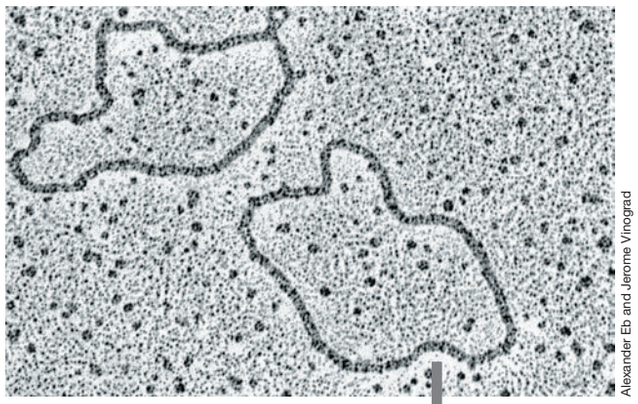
Polyomavirus SV40

Polyomavirus SV40 is a naked icosahedral virus that can cause tumors in small mammals, such as hamsters and rats. Its circular genome consists of double-stranded DNA (**Figure 11.18*a***). The genome is too small (5.2 kb) to encode its own DNA polymerase, so host DNA polymerases are used and SV40 DNA is replicated in a bidirectional fashion from a single origin of replication. Because of the small genomes of polyomaviruses, the strategy of overlapping genes, typical of many small bacteriophages (Sections 11.3 and 11.8), is also employed here. Transcription of the viral genome occurs in the nucleus, and the mRNAs are exported to the cytoplasm for protein synthesis. Eventually SV40 virion assembly occurs (in the nucleus) and the cell is lysed to release the new virions.

When SV40 infects a host cell, one of two outcomes can occur, depending on the host cell. In *permissive* hosts, virus infection results in the usual formation of new virions and the lysis of the host cell. In *nonpermissive* hosts, lytic events do not occur; instead, the viral DNA becomes integrated into host DNA, genetically altering the cells in the process (Figure 11.18*b*). Such cells can show loss of growth inhibition and become malignant, a process called *transformation* (◀ Figure 5.22). As in certain tumor-causing retroviruses (Section 11.11), expression of specific SV40 genes is required to convert the cell to the transformed state. These tumor-inducing proteins bind to and inactivate host cell proteins that control cell division, and in this way, they promote uncontrolled cell development.

Herpesviruses

Herpesviruses are a large group of double-stranded DNA viruses that cause a variety of human diseases, including fever blisters (cold sores), venereal herpes, chicken pox, shingles, and infectious mononucleosis. An important group of herpesviruses cause cancer. For example, Epstein–Barr virus causes Burkitt's lymphoma, a tumor endemic in children of central Africa and New Guinea. A widespread herpesvirus is *Cytomegalovirus* (CMV), present in nearly three-quarters of all adults in the United States over 40 years of age. For healthy individuals, infection with CMV comes with no apparent symptoms or long-term health consequences. However, CMV can cause



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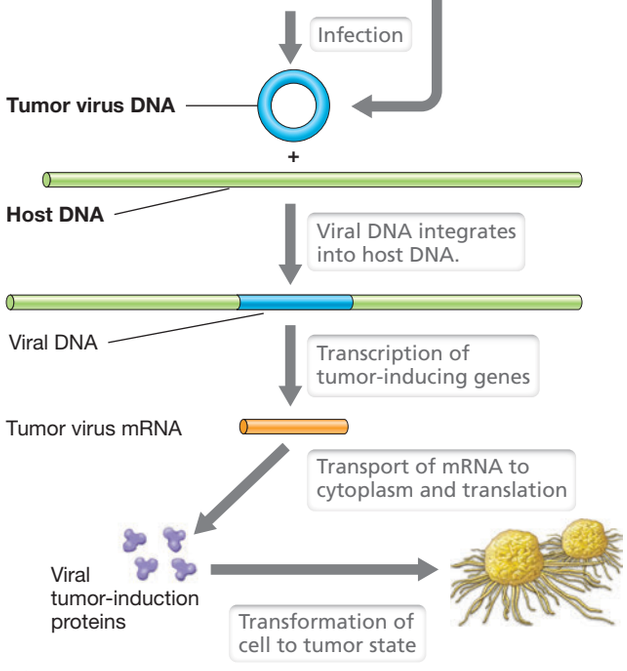


(b)

Figure 11.18 **Polyomaviruses and tumor induction.** *(a)* Transmission electron micrograph of relaxed (nonsupercoiled) circular DNA from a tumor virus. The contour length of each circle is about 1.5 μm. *(b)* Events in cell transformation by a polyomavirus such as SV40. Viral DNA becomes incorporated into the host genome. From there, viral genes encoding cell transformation events are transcribed and transported to the cytoplasm for translation.

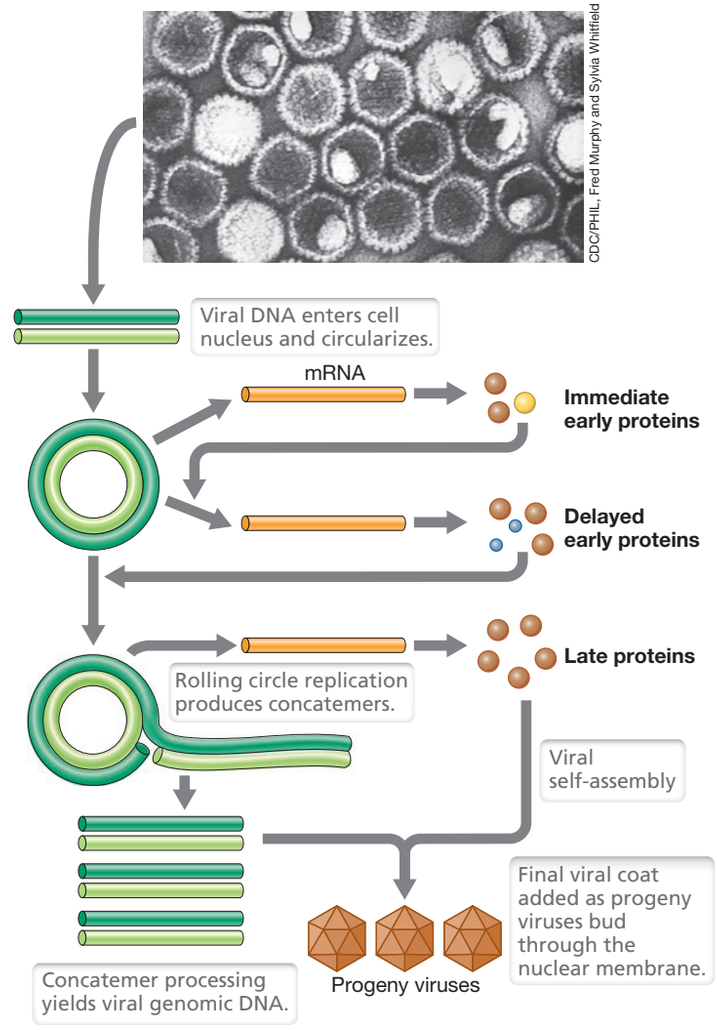


Figure 11.19 **Herpesvirus.** Flow of events in replication of herpes simplex virus starting from a transmission electron micrograph of herpes simplex virus (diameter of a single virion is about 150 nm). Although the viral genome is linear within the virion, it circularizes once inside the host.

pneumonia, retinitis (an eye condition), and certain gastrointestinal disorders, as well as serious disease or even death in immune-compromised individuals.

Herpesviruses can remain latent in the body for long periods of time and become active under conditions of stress or when the immune system is compromised. Herpesvirus virions are enveloped and can have many distinct structural layers over the icosahedral nucleocapsid (**Figure 11.19**). Following viral attachment, the host cytoplasmic membrane fuses with the virus envelope, and this releases the nucleocapsid into the cell. The nucleocapsid is transported to the nucleus, where the viral DNA is uncoated and three classes of mRNA are produced: *immediate early*, *delayed early*, and *late* (Figure 11.19). Immediate early mRNA encodes certain regulatory proteins that stimulate the synthesis of the delayed early proteins. Among the key proteins synthesized during the delayed early stage is a viral-specific DNA polymerase and a DNA-binding protein, both of which are needed for viral DNA replication. As for other viruses, late proteins are primarily viral structural proteins.

Herpesvirus DNA replication takes place in the nucleus. After infection, the herpesvirus genome circularizes and replicates by a rolling circle mechanism (Section 11.3). Long concatemers are formed that become processed into virus-length genomic DNA during the assembly process (Figure 11.19). Viral nucleocapsids are assembled in the nucleus, and the viral envelope is added during budding through the nuclear membrane. Mature herpesvirus virions are subsequently released through the endoplasmic reticulum to the outside of the cell. The assembly of herpesvirus virions thus differs from that of other enveloped viruses, which typically receive their envelope from the cytoplasmic membrane during exit from the cell.

Mastering Microbiology
Art Activity: Figure 11.19 Herpesvirus

Although we have seen a number of unique genomes and replication schemes with DNA viruses, more is in store with RNA viruses, microbes that cause most common human infectious diseases of viral etiology.

Check Your Understanding

- What genomic feature does SV40 share with bacteriophage ϕX174?
- How can the outcome of an SV40 viral infection differ in permissive versus nonpermissive hosts?
- Name two common diseases caused by herpesviruses.

III • RNA Viruses

Viruses that carry an RNA genome cause some of the most notorious diseases of humans and display the most intricate of all viral replication schemes. Because most cellular RNA polymerases are unable to make RNA from an RNA template, RNA viruses carry special enzymes in their virions to accomplish this task.

RNA viruses make up Baltimore classes III–VI (Figure 11.2*b*) and infect both prokaryotic and eukaryotic cells. As in the foregoing sections that dealt with DNA viruses, we organize our coverage of RNA viruses here by genomic characteristics. We also consider Baltimore class VII viruses, which have a *partially* double-stranded DNA genome, in the final section of this part of the chapter.

11.8 Positive-Strand RNA Viruses

Many viruses contain single-stranded RNA genomes of the plus sense and are therefore *positive-strand RNA viruses*. In these viruses, the sequence of the genome and the mRNA are the same (both are of the + complementarity, Figure 11.2*b*). A number of positive-strand animal and bacterial viruses are known, so we restrict our coverage here to just a few well-studied cases. We begin with the tiny bacteriophage MS2.

Bacteriophage MS2

Bacteriophage MS2 is about 25 nm in diameter and has an icosahedral capsid. The virus infects cells of *Escherichia coli* by attaching to the cell's pilus (**Figure 11.20*a***), a structure that normally functions in a form of horizontal gene exchange (conjugation) in bacteria (◀ Section 9.8). How MS2 RNA actually gets inside the *E. coli* cell from the pilus is unknown, but once it has, MS2 replication events begin quickly; the genetic map and major activities of this virus are shown in Figure 11.20*b* and *c*.

The MS2 genome is just 3.5 kb in size and encodes only four proteins, including the maturation protein, coat protein, lysis protein, and one subunit of **RNA replicase**, the enzyme that replicates the viral RNA (Section 11.1). Interestingly, MS2 RNA replicase is a composite protein, with three subunits encoded by the host genome and one subunit by the viral genome. The gene encoding the MS2 lysis protein overlaps that encoding the coat protein and replicase subunit (Figure 11.20*b*). We have seen this phenomenon of *overlapping genes* before (Sections 11.3 and 11.7) as a strategy for making small genomes encode more proteins.

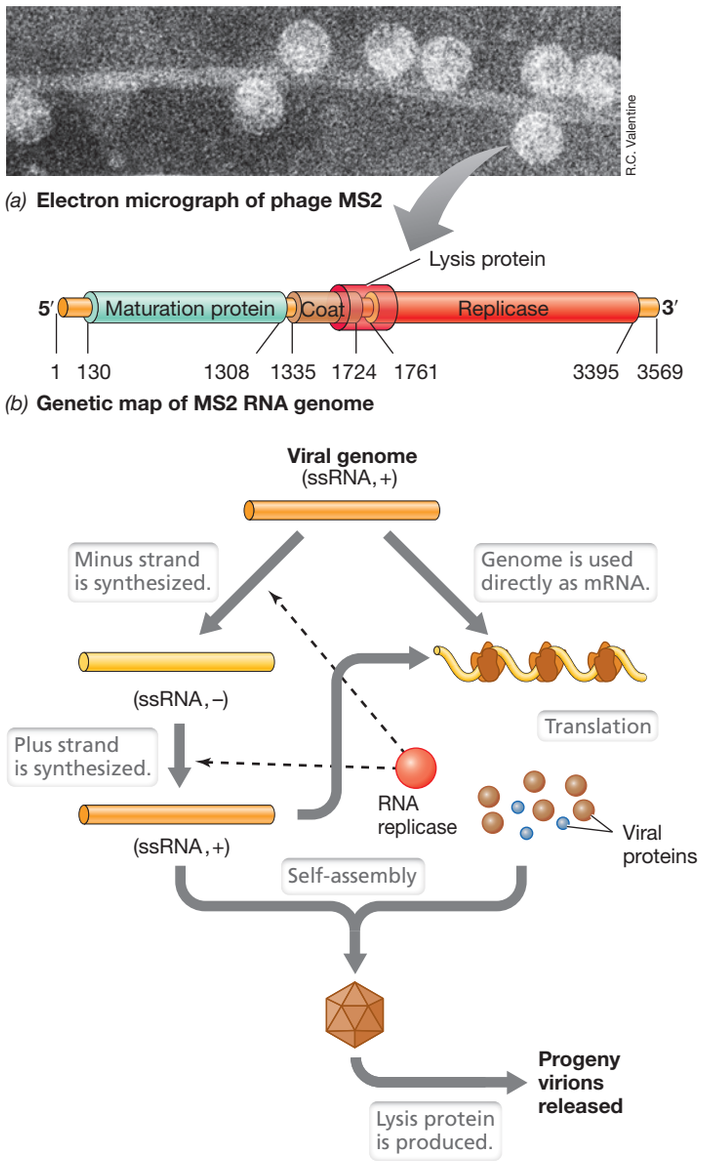


Figure 11.20 A small RNA bacteriophage, MS2. *(a)* Transmission electron micrograph of the pilus of a cell of *Escherichia coli* showing virions of phage MS2 attached. *(b)* Genetic map of MS2. Note how the lysis protein gene overlaps with both the coat protein and replicase genes. The numbers refer to the nucleotide positions on the RNA, the entire genome being 3569 nucleotides in length. *(c)* Flow of events during MS2 replication.

Because the genome of phage MS2 is plus-sense RNA, it is also the mRNA and is translated directly upon entry into the cell by the host RNA polymerase. When RNA replicase is made, it begins synthesis of minus-sense RNA using plus strands as templates. As minus-sense RNA copies accumulate, more plus-sense RNA is made using the minus-sense strands as templates, and some of these are translated for continued synthesis of viral structural proteins.

Phage MS2 regulates synthesis of its proteins by controlling access of host ribosomes to translational start sites on its RNA. MS2 genomic RNA is folded into a complex secondary structure. Of the four AUG translational start sites (◀ Section 6.9) on the MS2 RNA,

the most accessible to the cell's translation machinery is that for the coat protein and replicase. Hence, translation begins at these sites very early following infection. However, as coat protein molecules accumulate, they bind to the RNA around the AUG start site for the replicase protein, effectively turning off synthesis of replicase. Although the gene for the maturation protein is at the 5′ end of the RNA, the extensive folding of the RNA limits access to the maturation protein translational start site, and consequently, only a few copies are synthesized. In this way, all MS2 proteins are made in the relative amounts needed for virus assembly. Ultimately, spontaneous assembly of MS2 virions begins, and the virions are released from the cell as a result of cell lysis.

Poliovirus

Several positive-strand RNA animal viruses cause disease in humans and other animals. These include poliovirus, the rhinoviruses that cause many cases of the common cold, the coronaviruses that cause respiratory syndromes, including severe acute respiratory syndrome (SARS), and the hepatitis A virus. We focus here on poliovirus and coronaviruses, both of which have linear RNA genomes.

Poliovirus is one of the smallest of all viruses with a 30-nm icosahedral structure containing the minimum 60 morphological units (faces) per virion (**Figure 11.21*a*, *b***). At the 5′ terminus of the viral RNA is a protein, called the *VPg protein*, that is attached covalently to the genomic RNA, and at the 3′ terminus is a poly (A) tail (Figure 11.21*c*), a common feature of eukaryotic cell transcripts (◀ Section 6.6). The poliovirus genome (about 7.4 kb) is also the mRNA, and the VPg protein facilitates binding of the RNA to host ribosomes. Translation yields a **polyprotein**, a single protein that self-cleaves into several smaller proteins including virion structural proteins. Other proteins generated from the polyprotein include the VPg protein, an RNA replicase responsible for synthesis of both minus-strand and plus-strand RNA, and a virus-encoded protease, which carries out the polyprotein cleavage (Figure 11.21*c*). This mechanism is called *post-translational cleavage* and is common in many animal viruses as well as animal cells.

Poliovirus replication occurs in the host cell cytoplasm. To initiate infection, the poliovirus virion attaches to a specific receptor on the surface of a sensitive cell and enters the cell. Once inside the cell, the virion is uncoated, and the genomic RNA is attached to ribosomes and translated to yield the polyprotein. Replication of viral RNA by the poliovirus RNA replicase begins shortly after infection. Both the positive and negative strands that are made bind the VPg protein, which also functions as a primer for RNA synthesis following its uridylylation (reaction with uridylic acid; Figure 11.21*c*). Once poliovirus replication begins, host events are inhibited, and about 5 h after infection, cell lysis occurs with the release of new poliovirus virions.

Coronaviruses

Coronaviruses are single-stranded plus RNA viruses that, like poliovirus, replicate in the cytoplasm, but they differ from poliovirus in their larger size and details of replication. Coronaviruses cause respiratory infections in humans and other animals, including about 15% of common colds (▶ Section 31.7 and Figure 32.23*b*).

Coronavirus virions are enveloped and contain club-shaped glycoprotein spikes on their surfaces (**Figure 11.22*a***). These give the virus

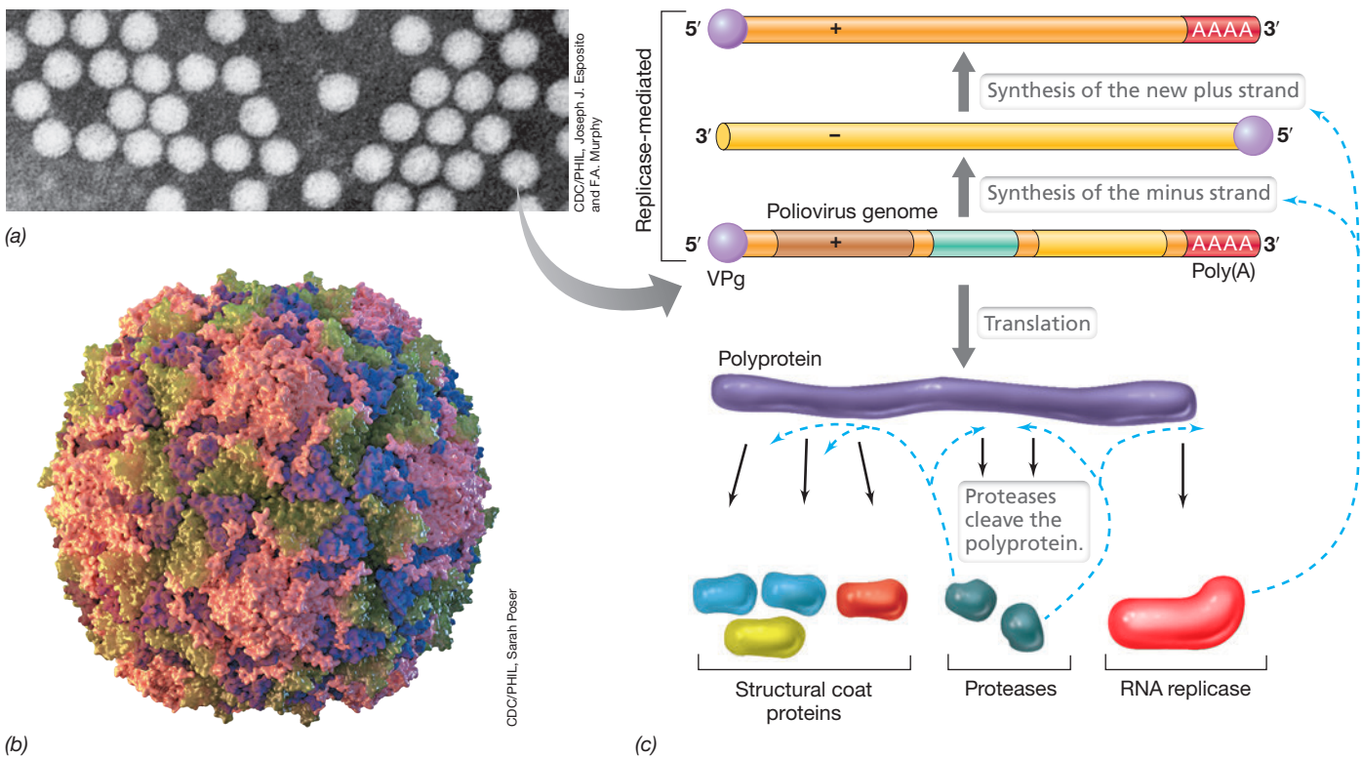


Figure 11.21 Poliovirus. *(a)* Transmission electron micrograph of poliovirus virions; a single virion is about 30 nm in diameter. *(b)* A computer model of a poliovirus virion. The various structural proteins are shown in distinct colors. *(c)* Genomic replication and formation of poliovirus proteins.

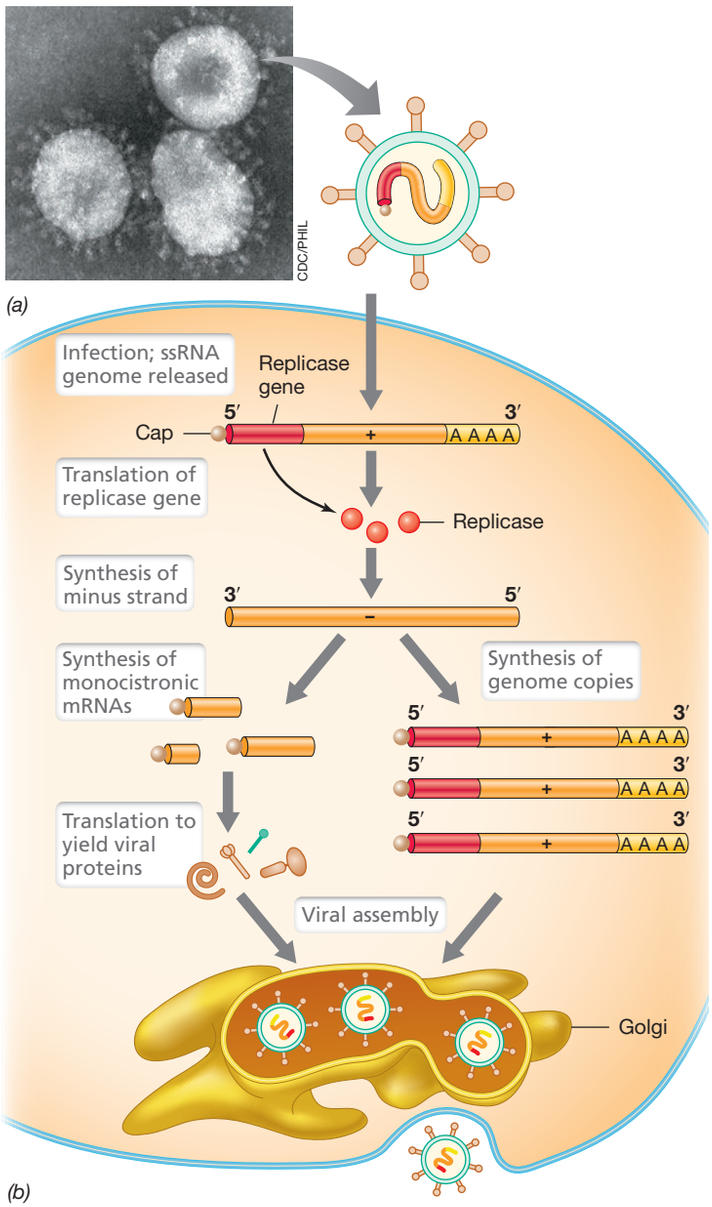


Figure 11.22 **Coronaviruses.** *(a)* Electron micrograph of a coronavirus; a virion is about 150 nm in diameter. *(b)* Steps in coronavirus replication. The mRNA encoding viral proteins is transcribed from the negative strand made by the RNA replicase using the viral genome as a template.

the appearance of having a "crown" (*corona* is Latin for crown). Coronavirus genomes are noteworthy because they are the largest of any known RNA viruses, about 30 kb. Because it is of the plus sense, the coronavirus genome can function directly in the cell as mRNA; however, most coronavirus proteins are not made by translating genomic RNA. Instead, only a portion of the genome is translated, in particular the region encoding RNA replicase (Figure 11.22*b*). The latter then uses the genomic RNA as a template to produce complementary negative strands from which several mRNAs are produced, and these mRNAs are translated to produce coronaviral proteins (Figure 11.22*b*). Full-length genomic RNA is also made from the negative strands. New coronaviral virions are assembled within the Golgi complex, a major secretory organelle in eukaryotic cells (◀ Section 2.15), and the fully assembled virions are released later from the cell surface.

Coronavirus differs from poliovirus in terms of virion and genome size, lack of the VPg protein, and absence of polyprotein formation and cleavage. Nevertheless, their single-stranded plus-sense RNA genomes dictate that many other molecular events must occur in a similar way.

In the next two sections we explore some RNA viruses that have genomes unique in the viral world and that cause animal diseases ranging from mild to deadly.

Check Your Understanding

- How can poliovirus RNA be synthesized in the cytoplasm whereas host RNA must be made in the nucleus?
- What is present in the poliovirus polyprotein?
- How are protein synthesis and genomic replication similar or different in poliovirus and coronavirus?

UNIT 3

11.9 Negative-Strand RNA Animal Viruses

A number of animal viruses have minus-sense RNA genomes (Baltimore class V, Figures 11.2*b* and 11.3). In contrast to the plus-strand viruses just considered, the genomes of these negative-strand RNA viruses are *complementary* in base sequence to the mRNA that is formed. We discuss here two important examples of negative-strand RNA viruses: rabies virus and influenza virus. There are no known negative-strand RNA bacteriophages or archaeal viruses.

Rabies Virus

Rabies virus, which causes the fatal neuroinflammatory disease rabies (▶ Section 32.1), is a rhabdovirus, a name that refers to the characteristic shape of the virion (*rhabdos* is Greek for rod). Rhabdoviruses are commonly bullet-shaped (**Figure 11.23*a***) and have an extensive and complex lipid envelope surrounding the helically symmetrical nucleocapsid. A rhabdovirus virion contains several enzymes that are essential for the infection process, including an RNA replicase. Unlike positive-strand viruses, a rhabdovirus genome cannot be directly translated but must first be transcribed by the replicase. This occurs in the cytoplasm and generates two classes of RNAs. The first is a series of mRNAs encoding each of the viral proteins, and the second is a complementary copy of the entire viral genome; the latter functions as a template for the synthesis of genomic RNA copies (Figure 11.23*b*).

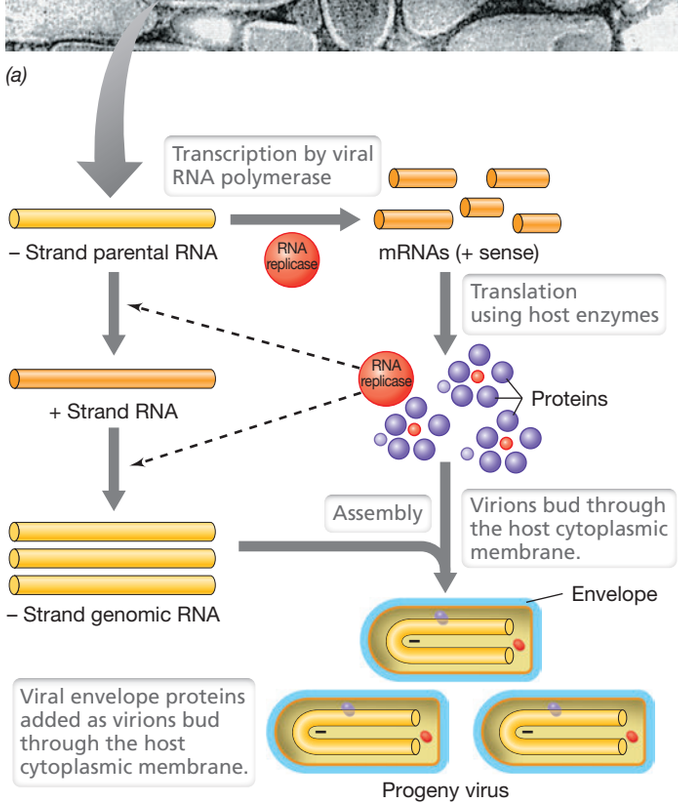
Mastering Microbiology
Art Activity: Figure 11.23*b* Negative-strand RNA virus: rhabdovirus

Assembly of a rhabdovirus virion is a highly orchestrated process. Two different coat proteins are made, nucleocapsid and envelope. The nucleocapsid is formed first by assembly of nucleocapsid protein molecules around the viral RNA genome. The envelope proteins are glycoproteins and they migrate to the cytoplasmic membrane where they are inserted into the membrane. Nucleocapsids then migrate to areas on the cytoplasmic membrane where these virus-specific glycoproteins are embedded and bud through them, becoming coated by the glycoprotein-enriched cytoplasmic membrane in the process. The final result is the release of new virions that can infect neighboring cells.

Influenza Virus

Another group of negative-strand RNA viruses contains the important human pathogen *influenza virus*. Influenza virus has been well studied

(a)

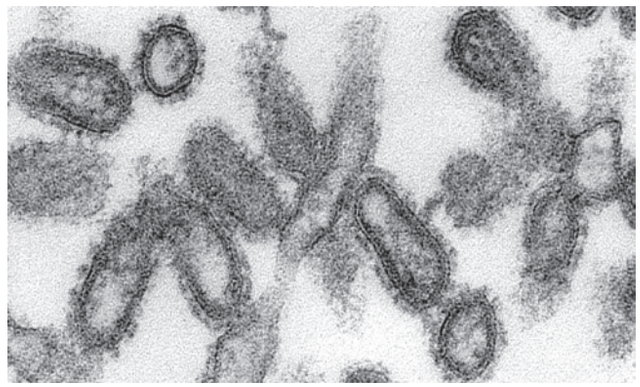


(b)

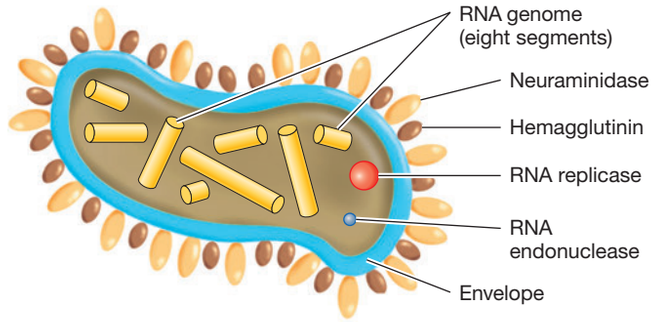
Figure 11.23 Rhabdoviruses. *(a)* Transmission electron micrograph of vesicular stomatitis virus virions. A virion is about 65 nm in diameter. *(b)* Flow of events during replication of a negative-strand RNA virus. Note the importance of the viral-encoded RNA replicase.

over many years, beginning with early work during the 1918 influenza pandemic that killed millions of people worldwide (▶ Sections 30.8 and 31.8). Influenza virus is an enveloped virus in which the viral genome is present in the virion in a number of separate pieces, a condition called a *segmented genome*. In the case of influenza A virus, a common strain, the genome is segmented into eight linear single-stranded molecules ranging in size from 890 to 2341 nucleotides and totaling 13.5 kb. The nucleocapsid of the virus is of helical symmetry, about 6–9 nm in diameter and about 60 nm long, and is embedded in an envelope that has a number of virus-specific proteins as well as lipid derived from the host cytoplasmic membrane. Because of the way influenza virus buds as it leaves the cell, virions do not have a uniform shape and instead are pleomorphic (**Figure 11.24*a***).

Several proteins on the outside of the influenza virion envelope interact with the host cell surface. One of these is *hemagglutinin* (Figure 11.24*b*). Hemagglutinin is highly immunogenic (capable of



(a)



(b)

Figure 11.24 Influenza virus. *(a)* Transmission electron micrograph of thin sections of human influenza virus virions. *(b)* Some of the major components of the influenza virus, including the segmented genome.

stimulating the immune system), and antibodies against it prevent the virus from infecting a cell. This is the mechanism by which immunity to influenza is brought about by immunization (▶ Section 31.8). A second important influenza virus surface protein is the enzyme *neuraminidase* (Figure 11.24*b*). Neuraminidase breaks down sialic acid (a derivative of neuraminic acid) in the host cytoplasmic membrane. Neuraminidase functions primarily in virus assembly, destroying host membrane sialic acid that would otherwise block assembly or become incorporated into the virion. In addition to hemagglutinin and neuraminidase, influenza virions possess two other key enzymes. These include an RNA replicase, which converts the minus-strand genome into a plus strand, and an RNA endonuclease, which cuts the cap from host mRNAs (◀ Section 6.6) and uses them to cap viral mRNAs so they can be translated by the host translational machinery.

After the influenza virion enters the cell, the nucleocapsid separates from the envelope and migrates to the nucleus. Uncoating activates the virus RNA replicase and transcription begins. Ten proteins are encoded by the eight segments of the influenza virus genome; the mRNAs transcribed from six segments each encode a single protein, while the other two segments encode two proteins each. Some of the viral proteins are needed for influenza virus RNA replication, whereas others are structural proteins of the virion. The overall pattern of genomic RNA synthesis resembles that of the rhabdoviruses (Figure 11.23*b*), with full-length positive-strand RNA used as a template for making negative-strand genomic RNA. The complete enveloped virion forms by budding, as for the rhabdoviruses.

The segmented genome of the influenza virus has important practical consequences. Influenza virus exhibits a phenomenon called *antigenic shift* in which segments of the RNA genome from two different strains of the virus infecting the same cell are reassorted. This generates hybrid influenza virions that express unique surface proteins unrecognized by the immune system. Antigenic shift is thought to trigger major outbreaks of influenza because immunity to the new forms of the virus is essentially absent from the population. We discuss antigenic shift, and a related phenomenon called *antigenic drift*, in Section 31.8.

Check Your Understanding

- Why is it essential that negative-strand viruses carry an enzyme in their virions?
- What is a segmented genome?
- In influenza virus, what is antigenic shift?

11.10 Double-Stranded RNA Viruses

Viruses with double-stranded RNA genomes (Baltimore class III, Figure 11.2*b*) infect animals, plants, fungi, and a few bacteria. *Reoviruses* are an important family of animal viruses, and we focus on them here.

Rotavirus is a typical reovirus and is the most common cause of diarrhea in infants 6 to 24 months of age. Other reoviruses cause respiratory infections, and some infect plants. Reovirus virions consist of a nucleocapsid 60–80 nm in diameter, surrounded by a double shell of icosahedral symmetry (**Figure 11.25*a, b***). As we have seen with single-stranded RNA viruses, the virions of double-stranded RNA viruses must carry their own enzyme to synthesize their mRNA and replicate their RNA genomes. Like the influenza virus genome, the reoviral genome is segmented, in this case into 10–12 molecules of linear double-stranded RNA totaling 18 kb.

To initiate infection, a reovirus virion binds to a cellular receptor protein. The attached virus then enters the cell and is transported into lysosomes, where normally it would be destroyed (◀ Section 2.15). However, only the outer coats of the virion are removed by the lysosome, revealing the nucleocapsid; the latter is released into the cytoplasm. This uncoating process activates the viral RNA replicase and initiates virus replication (Figure 11.25*c*).

Reovirus Replication

Reovirus replication occurs exclusively in the host cytoplasm but *within* the nucleocapsid itself (Figure 11.25*c*) because the host has enzymes that recognize double-stranded RNA as foreign and would destroy it. The plus strand of the reoviral genome is inactive as mRNA, and thus the first step in replication is the synthesis of plus-sense mRNA by the viral-encoded RNA replicase, using minus-strand RNA as a template; the nucleotide triphosphates necessary for RNA synthesis are supplied by the host (Figure 11.25*c*). The mRNAs are then capped with a methylated nucleotide (as is typical of eukaryotic mRNAs, ◀ Section 6.6) by viral enzymes and exported from the nucleocapsid into the cytoplasm and translated by host ribosomes.

Most RNAs in the reovirus genome encode a single protein, although in a few cases the protein formed is cleaved to yield the final products. However, one of the reovirus mRNAs encodes two proteins but the

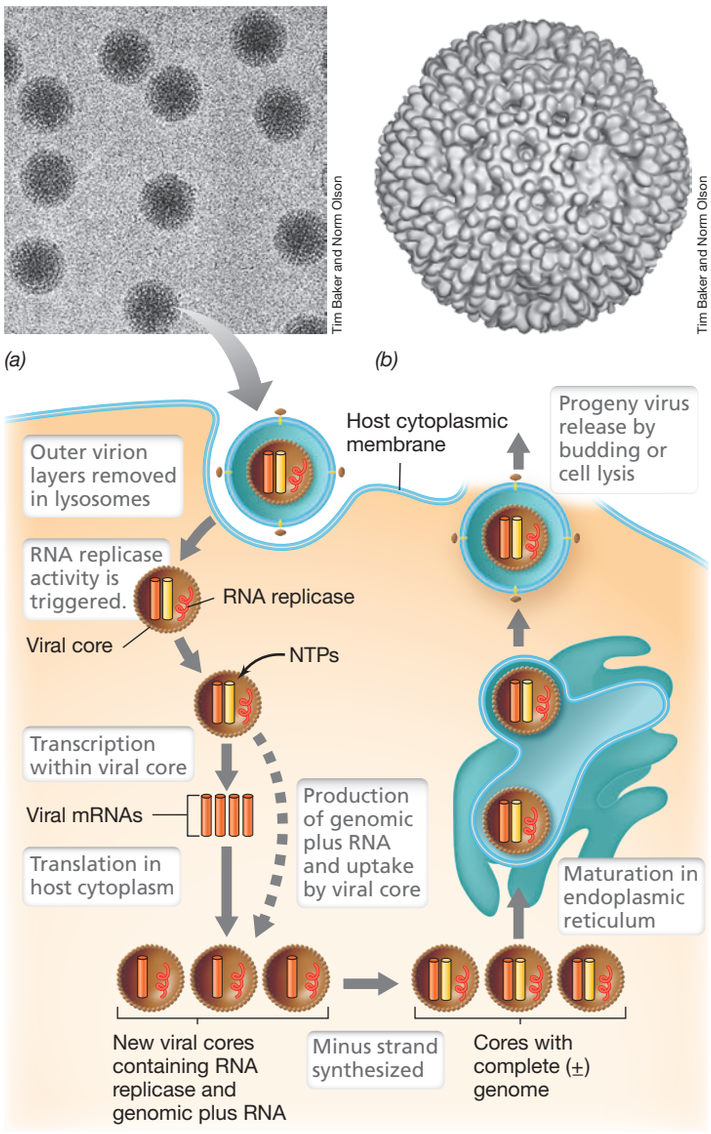


Figure 11.25 The reoviruses. *(a)* Transmission electron micrograph showing reovirus virions (diameter, about 70 nm). *(b)* Three-dimensional computer reconstruction of a reovirus virion calculated from electron micrographs of frozen-hydrated virions. *(c)* The reovirus life cycle. All replication and transcription steps occur inside the nucleocapsids. NTPs, nucleotide triphosphates.

RNA does not have to be processed in order to translate both of these. Instead, a ribosome occasionally "misses" the start codon for the first gene in this mRNA and travels on to the start codon of the second gene to begin translation. When this occurs, the second protein, needed in small amounts, is made but the first protein is not. This "molecular mistake" can be viewed as a primitive form of translational control that ensures that viral proteins are made in their proper amounts.

As viral proteins are formed in the host cytoplasm, they aggregate to form new nucleocapsids, trapping copies of RNA replicase inside as they form (Figure 11.25*c*). Newly formed nucleocapsids then take up the correct complement of genomic (plus-strand) RNA fragments—probably by recognition of specific sequences on each fragment—and as each single-stranded RNA enters a newly formed

nucleocapsid, a double-stranded form is produced from it by RNA replicase. Once genomic synthesis is complete, viral coat proteins are added in the host's endoplasmic reticulum, and the mature reoviral virions are released by budding or cell lysis (Figure 11.25*c*).

Reoviruses and RNA Replication

In addition to the unusual genome structure of reoviruses that forces them to employ special mechanisms to protect their double-stranded RNA genomes from cleavage by host ribonucleases (Figure 11.25*c*), genomic replication in these viruses is also unique and differs in a fundamental way from that of cells and all other viruses.

Because the reovirus RNA genome is double-stranded, one might predict that reovirus replication would parallel that of organisms with double-stranded DNA genomes, but this is not the case. RNA replication in reoviruses is actually a *conservative* process rather than the well-known *semiconservative* process typical of cellular DNA replication and replication in viruses that contain double-stranded DNA genomes (◀ Section 6.3 and Figure 11.2). This is because in the synthesis of reovirus mRNA *only* the minus strand is a template in the infecting nucleocapsids, whereas *only* the plus strand is a template in the synthesis of double-stranded genomic RNA from assimilated plus-strand RNA copies in progeny virions (Figure 11.25*c*). Hence, in addition to their unique double-stranded RNA genomes, reoviruses also display their unusual molecular biology by employing a unique genomic replication mechanism that is neither semiconservative nor rolling circle (Figure 11.7) in nature.

Check Your Understanding

- What does the reovirus genome consist of?
- How does reovirus genome replication resemble that of influenza virus, and how does it differ?
- Why must reoviral replication events occur within the nucleocapsid?

11.11 Viruses That Use Reverse Transcriptase

Two different Baltimore classes of viruses use **reverse transcriptase**, an enzyme that can produce DNA from an RNA template, but they differ in the type of nucleic acid in their genomes; retroviruses have *RNA* genomes while hepadnaviruses have *DNA* genomes (Baltimore classes VI and VII, respectively, Figure 11.2). Besides their unique molecular properties, both classes of viruses include important human pathogens, including HIV (a retrovirus) and hepatitis B (a hepadnavirus).

Retroviruses and Integration into the Host Genome

Retroviruses have enveloped virions that contain two identical copies of the single-stranded plus complementarity RNA genome (Figure 11.2*b* and **Figure 11.26*a***). The prefix *retro* means "backward," and

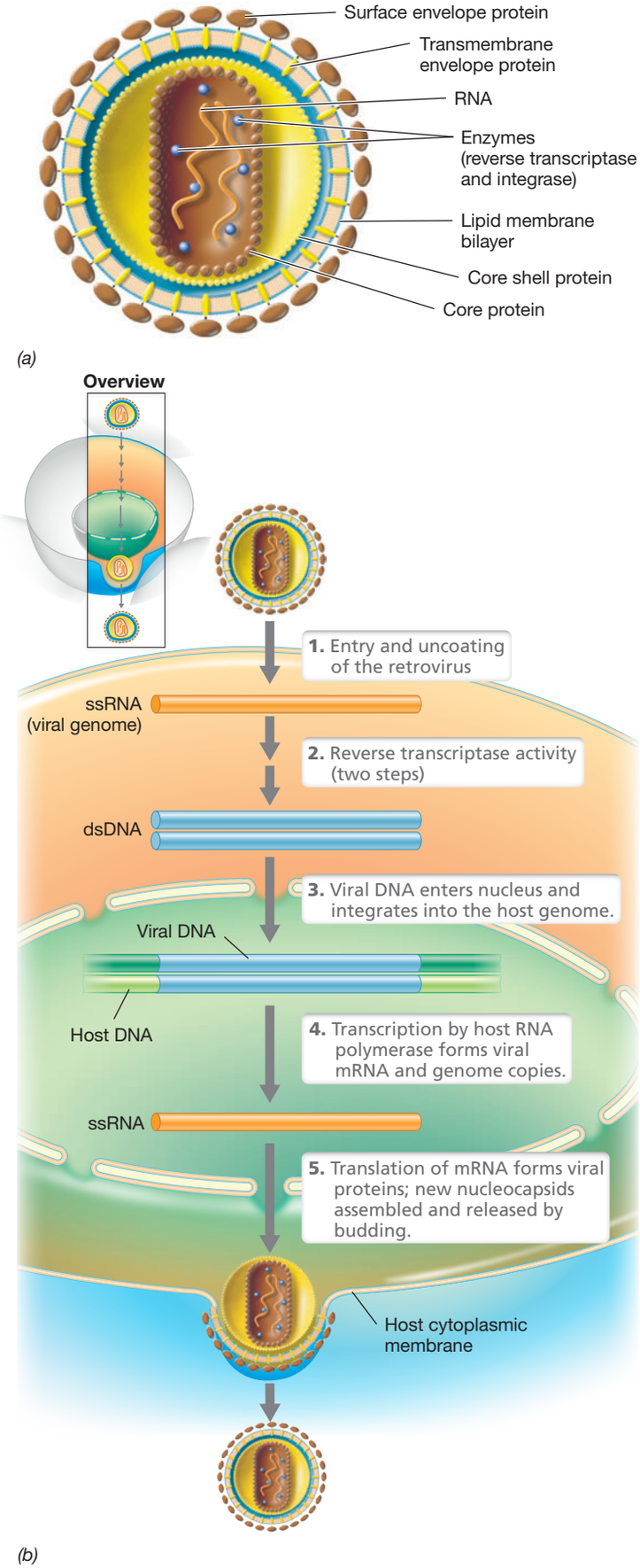


Figure 11.26 Retrovirus structure and replication. *(a)* The virion carries two identical copies of the RNA genome (orange). *(b)* Top, an overview of the infection process. Replication steps: Reverse transcriptase, carried in the virion, makes single-stranded DNA from viral RNA and then double-stranded DNA that integrates into the host genome as a provirus. Transcription and translation of proviral genes leads to the production of new virions that are then released by budding.

the term *retrovirus* refers to the fact that these viruses transfer information from RNA to DNA (in contrast to genetic information flow in cells, which occurs from DNA to RNA). The virion of a retrovirus contains several enzymes, including reverse transcriptase and integrase, and also a specific viral tRNA. Enzymes for retrovirus replication must be carried in the virion because although the retroviral genome is of the plus sense, it is not used directly as mRNA. Figure 11.26*b* illustrates the general scheme for retroviral genome conversion to DNA, integration into the host chromosome, and production of new virions. To begin the process, the genome is converted to DNA by reverse transcriptase inside the nucleocapsid and then the DNA is released to the cytoplasm. The double-stranded DNA is then integrated into the host genome by integrase.

Reverse transcriptase has three enzymatic activities: (1) *reverse transcription* (to synthesize DNA from an RNA template), (2) *ribonuclease activity* (to degrade the RNA strand of an RNA:DNA hybrid), and (3) *DNA polymerase* (to make double-stranded DNA from single-stranded DNA). A more detailed look at steps in reverse transcription is presented in **Figure 11.27**. Reverse transcriptase needs a primer for DNA synthesis and this is the function of the viral tRNA. Using this primer, nucleotides near the 5′ terminus of the RNA are reverse-transcribed into DNA. Once reverse transcription reaches the 5′ end of the RNA, the process stops. To copy the remaining RNA, a different mechanism comes into play. First, terminally redundant RNA sequences at the 5′

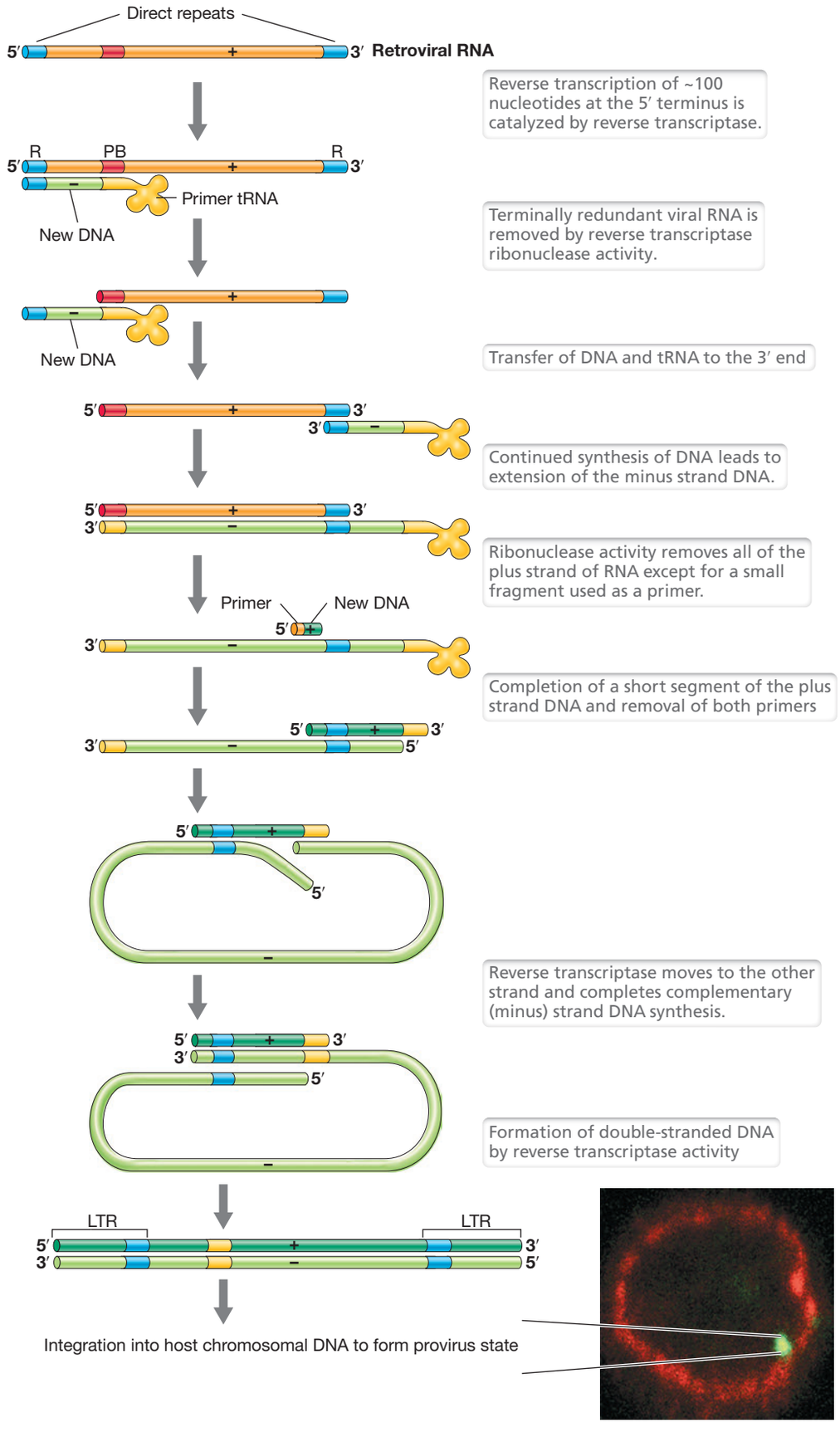


Figure 11.27 Formation of double-stranded DNA from retrovirus single-stranded genomic RNA. The sequences labeled R on the RNA are direct repeats found at either end. The sequence labeled PB is where the primer (tRNA) binds. Note that DNA synthesis yields longer direct repeats on the DNA than were originally on the RNA. These are called long terminal repeats (LTRs). Inset: A special form of fluorescence microscopy allows visualization of HIV genome integration (green) into a chromosome region near the nuclear membrane (red) of the host cell, a CD4 lymphocyte. Image courtesy of Marina Lusic, University Hospital, Heidelberg, Germany.

UNIT 3

Mastering Microbiology
Art Activity: Figure 11.27 Formation of double-stranded DNA from retrovirus single-stranded genomic RNA

end of the molecule are removed by reverse transcriptase. This leads to the formation of a small, single-stranded DNA that is complementary to the RNA segment at the *other end* of the viral RNA. This short, single-stranded piece of DNA then hybridizes with the other end of the viral RNA molecule, where synthesis of DNA begins once again.

Continued reverse transcription leads to the formation of a double-stranded DNA molecule with long terminal repeats, and these assist in integration of the retroviral DNA into the host chromosome by the integrase enzyme carried in the virion (Figure 11.27). For HIV, the chromosomal integration site is not random. Through the use of a special form of fluorescence microscopy, scientists have shown that HIV incorporates into chromosomal loci near the outer shell of the nucleus (photo inset, Figure 11.27). This location is likely favored due to the short life of the viral integrase. Thus the retroviral DNA must be integrated quickly into the host genome upon entry into the nucleus.

Once integrated, retroviral DNA becomes a permanent part of the host chromosome and is considered a *provirus* (◀ Section 5.7); the genes may be expressed or they may remain in a latent state indefinitely. However, if induced, retroviral DNA is transcribed by a cellular RNA polymerase to form RNA transcripts (plus-sense mRNA) that can be either packaged into virions as genomic RNA or translated to yield retroviral proteins.

With retroviruses we thus see a replication scheme that is perhaps the most complex of all known viruses. Despite the complexity of retroviruses, molecular studies of them suggest an ancient origin and possible central importance in the transition from self-replicating RNA life forms to the DNA world of cellular organisms. It is the signature enzyme of retroviruses—reverse transcriptase, the only enzyme known that can make DNA from RNA—that brings retroviruses into this evolutionary limelight, and so it is possible that all cells owe their very existence to this class of viruses.We revisit this topic in Section 13.5.

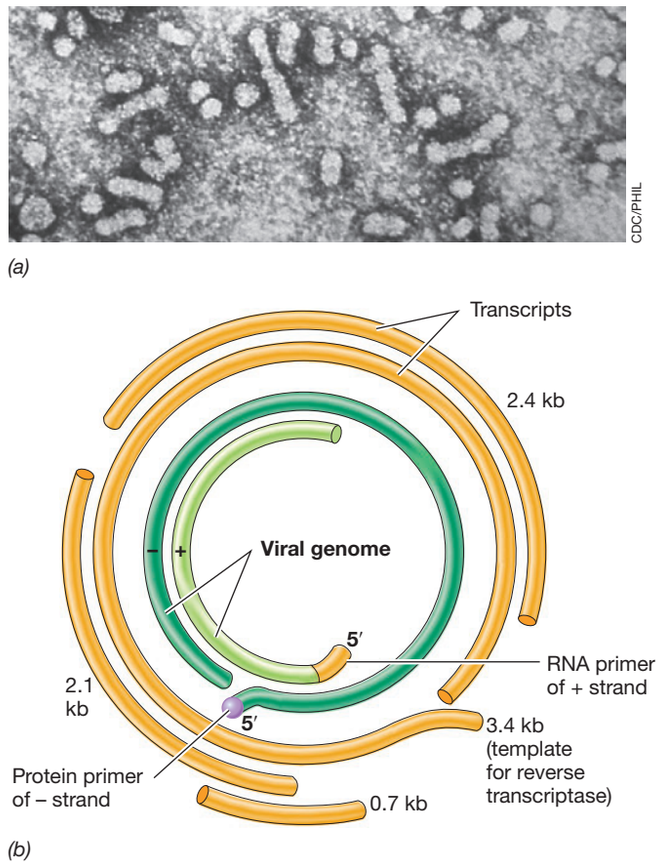


Figure 11.28 Hepadnaviruses. *(a)* Electron micrograph of hepatitis B virions. *(b)* Hepatitis B genome. The partially double-stranded genome is shown in the standard green colors. The sizes of the transcripts (orange) are also shown; all of the genes in the hepatitis B virus overlap. Reverse transcriptase produces the DNA genome from a single genome-length mRNA made by host RNA polymerase.

Hepadnaviruses

In addition to the retroviruses, the second class of unusual viruses that employ the enzyme reverse transcriptase are the **hepadnaviruses**, such as human hepatitis B virus (**Figure 11.28*a***). The tiny DNA genomes of hepadnaviruses are unusual because they are neither single-stranded nor double-stranded; instead, they are *partially* double-stranded. Despite their small size (3–4 kilobase pairs, among the smallest of DNA viruses), the hepadnavirus genomes encode several proteins by containing overlapping genes, a strategy we have seen before in very small viruses (Sections 11.3, 11.7, and 11.8).

In addition to the usual activities of reverse transcriptase we have just considered (Figure 11.27), hepadnaviral reverse transcriptase also functions as a protein primer for synthesis of one of its own DNA strands. In terms of its role in replication events, however, reverse transcriptase plays different roles in retroviral and hepadnaviral genome replication. In hepadnaviruses, the *DNA* genome is replicated through an *RNA* intermediate, whereas in retroviruses, the *RNA* genome is replicated through a *DNA* intermediate (Figures 11.27 and 11.28).

Upon infection, the hepadnavirus nucleocapsid enters the host nucleus where the partial genomic DNA strand is completed by the viral polymerase to form an entire double-stranded molecule. Transcription by host RNA polymerase yields four size classes of viral mRNAs (Figure 11.28*b*), which are subsequently translated to yield the hepadnaviral proteins. The largest of these transcripts is slightly larger than the viral genome and, together with reverse transcriptase, associates with viral proteins in the host cytoplasm to form genomes for new virions. Reverse transcriptase forms single-stranded DNA from this large transcript inside the virion to form the minus-sense strand of the DNA genome and then uses this DNA as a template to form a portion of the plus-sense strand, yielding the partially double-stranded genome characteristic of hepadnaviruses (Figure 11.28*b*). Once mature virions are produced, these associate with membranes in the endoplasmic reticulum and Golgi complex, from which they are exported across the cytoplasmic membrane by budding.

Some viral-like microbes are neither viruses nor cells, and we wrap up this chapter by considering their biology.

Check Your Understanding

- Why are protease inhibitors an effective treatment for human AIDS?
- Contrast the genomes of HIV and hepatitis B virus.
- How does the role of reverse transcriptase in the replication cycles of retroviruses and hepadnaviruses differ?

IV • Subviral Agents

A few noncellular microbes are even more streamlined than viruses; some contain RNA but lack protein while others contain protein but lack nucleic acids of any type. Although biologically minimalist entities, these structures can trigger devastating diseases.

We conclude our genomic tour of the viral world by considering two *subviral* agents: the *viroids* and the *prions*. These are infectious agents that resemble viruses but lack either nucleic acid (prions) or protein (viroids) and are thus not viruses.

11.12 Viroids

Viroids are infectious RNA molecules that lack a protein component. Viroids are small, circular, single-stranded RNA molecules that are the smallest known pathogens. They range in size from 246 to 399 nucleotides and show a considerable degree of sequence homology to each other, suggesting that they have common evolutionary roots. Viroids cause a number of important plant diseases and can have a severe agricultural impact (Figure 11.29). A few well-studied viroids include coconut cadang-cadang viroid (246 nucleotides) and potato spindle tuber viroid (359 nucleotides). No viroids are known that infect animals or microorganisms.

Viroid Structure and Function

The extracellular form of a viroid is naked RNA; there is no protein capsid. Although the viroid RNA is a single-stranded, covalently closed circle, its extensive secondary structure forms a hairpin-shaped double-stranded molecule with closed ends (Figure 11.30). This makes the viroid sufficiently stable to exist outside the host cell and protects the viroid while inside the cell from cellular ribonucleases. Because it lacks a capsid, a viroid does not use a receptor to enter the host cell. Instead, the viroid enters a plant cell through a wound, as from insect or other mechanical damage. Once inside, viroids move from cell to cell via plasmodesmata, which are the thin strands of cytoplasm that link plant cells (Figure 11.31).

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Figure 11.29 Viroids and plant diseases. Photograph of healthy tomato plant (left) and one infected with potato spindle tuber viroid (PSTV) (right). The host range of most viroids is quite restricted. However, PSTV infects tomatoes as well as potatoes, causing growth stunting, a flat top, and premature plant death.

Figure 11.30 Viroid structure. Viroids consist of single-stranded circular RNA that forms a seemingly double-stranded structure by intra-strand base pairing.

Viroid RNAs do not encode proteins and thus the viroid is totally dependent on its host for replication. Plants have several RNA polymerases, one of which has RNA replicase activity, and this is the enzyme that replicates the viroid. The replication mechanism itself resembles the rolling circle mechanism used for genome synthesis by some small viruses (Sections 11.3, 11.4, and 11.7). The result is a large RNA molecule containing many viroid units joined end to end. The viroid has ribozyme activity (catalytic RNA, ▶ Section 13.1) and uses it to self-cleave the large RNA molecule, releasing individual viroids.

Viroid Disease

Viroid-infected plants can be symptomless or show symptoms that range from mild to lethal, depending on the viroid (Figure 11.29). Most disease symptoms are growth related, and it is believed that viroids mimic or in some way interfere with plant small regulatory RNAs. In fact, viroids could themselves be derived from regulatory RNAs that have evolved away from carrying out beneficial roles in the cell to inducing destructive events. Viroids are known to yield small interfering RNAs (siRNAs) as a by-product of replication. It has been proposed that these siRNAs may function by way of an RNA-interference silencing pathway, similar to that of regulatory RNAs in *Bacteria* and *Archaea* (◀ Section 7.12), to suppress the

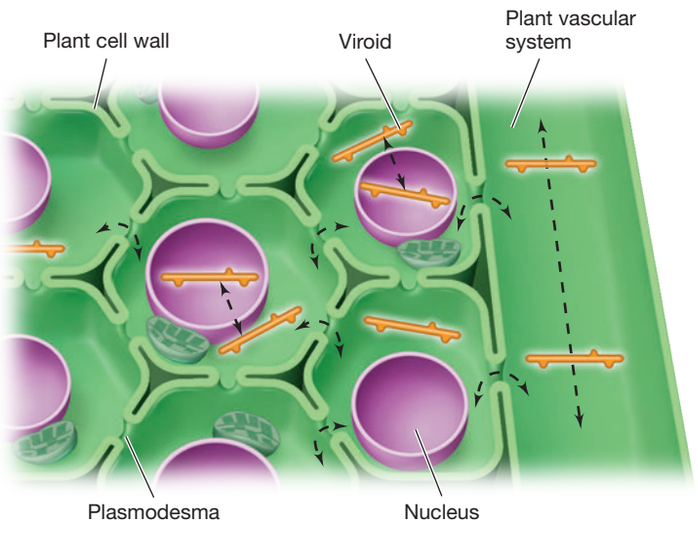


Figure 11.31 Viroid movement inside plants. After entry into a plant cell, viroids (orange) replicate either in the nucleus or the chloroplast. Viroids can move between plant cells via the plasmodesmata (thin threads of cytoplasm that penetrate the cell walls and connect plant cells). On a larger scale, viroids can also move around the plant via the plant vascular system.

expression of plant genes that show some homology to viroid RNA, and in this way induce disease symptoms. This mechanism of regulation is similar to how some bacterial and archaeal regulatory small RNAs target mRNAs for degradation (◀ Figure 7.28*b*).

Check Your Understanding

- If viroids are circular molecules, why are they depicted as hairpins?
- How might viroids cause disease in plants?

11.13 Prions

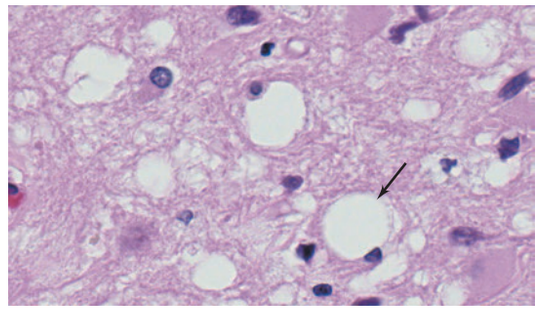
Prions represent the opposite extreme from that of viroids. Prions are infectious agents whose extracellular form consists entirely of protein. That is, *a prion lacks both DNA and RNA*. Prions cause several neurological diseases such as scrapie in sheep, bovine spongiform encephalopathy (BSE or "mad cow disease") in cattle, chronic wasting disease in deer and elk, and kuru and variant Creutzfeldt–Jakob disease in humans by catalyzing protein conformational changes that lead to protein clumping and accumulation. No prion diseases of plants are known, although prions have been detected in yeast. Collectively, animal prion diseases are known as *transmissible spongiform encephalopathies*.

Prion Proteins and the Prion Infectious Cycle

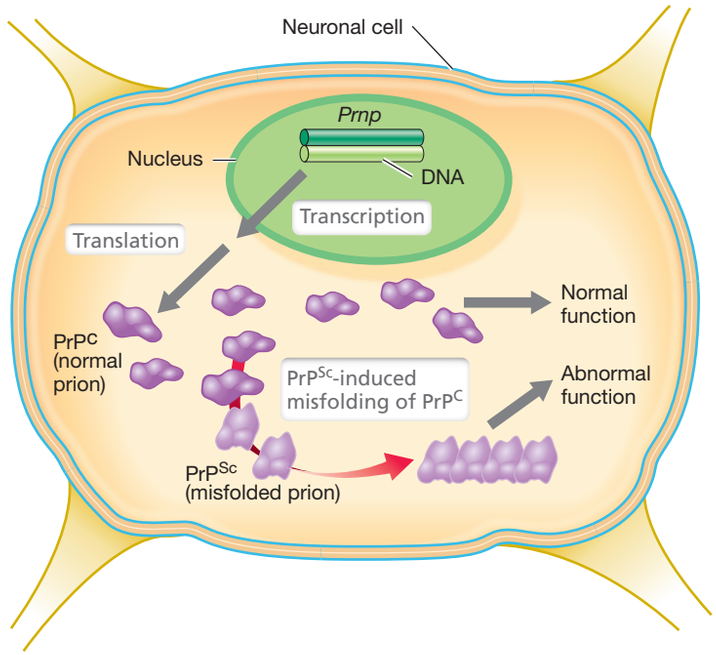
If prions lack nucleic acid, how is prion protein encoded? The answer to this conundrum is that the host cell itself encodes the prion. The host contains a gene, *Prnp* (*Pr*ion *p*rotein), which encodes the native form of the prion, known as PrP^{C} (*P*rion *P*rotein *C*ellular). This is primarily found in the neurons of healthy animals, especially in the brain (**Figure 11.32*a***). The pathogenic form of the prion protein is designated PrP^{Sc} (prion protein *Sc*rapie) because the first prion disease to be discovered was that of scrapie in sheep. PrP^{Sc} is identical in amino acid sequence to PrP^{C} from the same animal species, but it has a different conformation. For example, native prion proteins are largely α-helical, whereas the pathogenic forms contain less α-helix and more β-sheet secondary structure. Prion proteins from different species of mammals are similar but not identical in amino acid sequence, and host range is linked in some way to protein sequence. For example, PrP^{Sc} from BSE-diseased cattle can infect humans, whereas PrP^{Sc} from scrapie-infected sheep apparently cannot.

When the PrP^{Sc} form enters a host cell that is expressing PrP^{C}, it promotes the conversion of PrP^{C} into the pathogenic form (Figure 11.32*b*). That is, the pathogenic prion "replicates" by converting preexisting native prions into the pathogenic form. As the pathogenic prions accumulate and aggregate, they form insoluble crystalline fibers referred to as amyloids in neural cells; this leads to disease symptoms including the destruction of brain and other nervous tissue (Figure 11.32*a*). PrP^{C} functions in the cell as a cytoplasmic membrane glycoprotein, and it has been shown that membrane attachment of pathogenic prions is necessary before disease symptoms commence. Mutant versions of PrP^{Sc} that can no longer attach to nerve cell cytoplasmic membranes still aggregate but no longer cause disease.

Besides the transmissible spongiform encephalopathies, amyloids are also associated with debilitating human diseases such as Alzheimer's, Huntington's, Parkinson's, and type 2 diabetes. Whether any or all of these are truly prion diseases is unclear. However, protein aggregation has been linked to these diseases, so the possibility remains that they are manifestations of prion infection.



(a)



(b)

Figure 11.32 Prions. *(a)* Section through brain tissue of a human with variant Creutzfeldt–Jakob disease. Note the spongy nature of the tissue (clearings, arrow) where neural tissue has been lost. *(b)* Mechanism of prion misfolding. Neuronal cells produce the native form of the prion protein. The pathogenic form catalyzes the refolding of native prions into the pathogenic form. The pathogenic form is protease resistant, insoluble, and forms aggregates in neural cells. This eventually leads to destruction of neural tissues (see part *a*) and neurological symptoms. About 70% of those diagnosed with variant Creutzfeldt–Jakob disease die within a year.

Nonpathogenic Prions

Certain fungi have proteins that fit the prion definition of an inherited self-perpetuating change in protein structure, although these proteins do not cause noticeable disease. Instead they adapt the fungal cells to survive dynamic environmental conditions by conferring traits such as altered nutrient utilization, antibiotic resistance, and biofilm formation. In yeast, for example, the [URE3] prion is a protein that regulates the transcription of genes encoding certain nitrogen metabolism functions. The normal (soluble) form of this

protein functions to repress genes encoding proteins that metabolize certain nitrogen sources. However, when the [URE3] prion accumulates, it forms insoluble aggregates and when this occurs, the normally repressed genes are derepressed and the nitrogen sources are metabolized.

Humans also have beneficial prions. MAVS is a human protein that is part of our innate immune system and has been shown to convert to a self-perpetuating prion-like form in cells that become infected with a virus. The aggregation of MAVS protein triggers the production of immune modulators called *interferons* (▶ Section 26.10). These proteins trigger the recruitment of phagocytic cells such as macrophages (cells that ingest and destroy pathogens, foreign particles, and cell debris) and other immune factors to the viral-infected cell, resulting in its destruction. While the conversion of MAVS to a prion-like form ultimately kills the cell, it prevents the virus from hijacking the cell's molecular machinery, which would allow the virus to replicate and lyse the host cell to infect adjacent cells.

The viral and subviral worlds have revealed an almost limitless variation on the molecular principles that govern information flow in cells. We turn our attention in the following chapter to how scientists have exploited some of these unusual molecular principles (along with the enormous repertoire of genetic tools available from cells) to create genetically modified or even totally synthetic organisms for the benefit of mankind.

Check Your Understanding

- On what basis can prions be differentiated from all other infectious agents?
- What is the difference between the native and pathogenic forms of the prion protein?
- How does a prion differ from a viroid? How does a prion differ from a virus?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Viral Genomes and Classification

11.1 Viral genomes can be single-stranded or double-stranded DNA or RNA and vary from a few to hundreds of kilobases in size. Viral mRNA is always of the plus configuration by definition, but single-stranded genomes can be of the plus or minus configuration. Viruses with RNA genomes must either carry an RNA replicase in their virions or encode this enzyme in their genomes in order to synthesize RNA from an RNA template.

Q Describe the classes of viruses based on their genomic characteristics. For each class, describe how viral mRNA is made and how the viral genome is replicated.

11.2 Besides classification based on genomic characteristics, viruses can also be assigned to orders, families, genera, and species based on taxonomy. Taxonomic classifications are made on a combination of host range, structural morphology, replication cycle, nucleic acid sequence, and pathogenicity. Phylogenetic trees illustrating viral relationships can also be constructed using genes conserved within a group of viruses.

Q What trait associated with archaeal viruses is likely responsible for the high percentage of viruses assigned to unclassified orders?

II • DNA Viruses

11.3 Single-stranded DNA viruses contain DNA of the plus configuration, and a double-stranded replicative form is necessary for transcription and genome replication. The genome of the virus ϕX174 is so small that some of its genes overlap, and the genome replicates by a rolling circle mechanism. Some related viruses, such as M13, have filamentous virions that are released from the host cell without lysis.

Q Describe how the genome of bacteriophage ϕX174 is transcribed and translated.

11.4 Bacteriophage T4 contains a double-stranded DNA genome that is both circularly permuted and terminally redundant. T4 encodes its own DNA polymerase and several other replication proteins, but it utilizes the host RNA polymerase to express early, middle, and late viral genes. Bacteriophage T7 contains a double-stranded DNA genome that encodes both early genes, transcribed by the host RNA polymerase, and late genes, transcribed by a virus-encoded RNA polymerase. Replication of the T7 genome employs T7 DNA polymerase and involves terminal repeats and concatemers. Bacteriophage lambda possesses a linear double-stranded DNA genome that circularizes at the *cos* sites once injected into its host. Depending on cellular conditions, lambda can either integrate into the host chromosome and be maintained as a prophage through lysogeny or enter the lytic cycle and be replicated by rolling circle replication.

Q Why can it be said that transcription of the bacteriophage T4 genome requires one enzyme, while the T7 genome requires two enzymes? How does lambda get incorporated into a host cell's chromosome?

11.5 Several double-stranded DNA viruses infect cells of *Archaea*, most of which inhabit extreme environments. Many of these genomes are circular, in contrast to the linear double-stranded DNA genomes of bacteriophages. Although many head-and-tail viruses are known, other archaeal viruses have an unusual spindle-shaped morphology.

Q How is a spindle-shaped double-stranded DNA archaeal virus infecting *Acidianus* capable of surviving in an extremely hot and acidic environment?

11.6 Pox viruses are large, double-stranded DNA viruses that replicate entirely in the cytoplasm and are responsible for several human diseases, including smallpox. Adenoviruses are double-stranded DNA viruses whose genome replication employs protein primers and a mechanism that occurs without lagging-strand synthesis.

Q Of all the double-stranded DNA animal viruses, pox viruses stand out concerning one unique aspect of their DNA replication process. What is this unique aspect, and how can this be accomplished without special DNA replication enzymes being packaged in the virion?

11.7 Some double-stranded DNA viruses cause cancer in humans. SV40 is such a tumor virus and has a tiny genome containing overlapping genes. The activity of certain SV40 genes can trigger cell transformation (tumor induction). Some herpesviruses also cause cancer, but most cause various human infectious diseases. Herpesviruses can maintain themselves in a latent state in the host indefinitely, initiating viral replication periodically.

Q How does the way herpesviruses obtain their envelope differ from other enveloped viruses?

III • RNA Viruses

11.8 In single-stranded plus-sense RNA viruses, the genome is also the mRNA, and a negative strand is synthesized to produce more mRNA and genome copies. The tiny bacteriophage MS2 contains only four genes, one of which encodes a subunit of its RNA replicase. In poliovirus, the viral RNA is translated directly, producing a polyprotein that is cleaved into several small viral proteins. Coronaviruses are large RNA viruses that resemble poliovirus in some but not all of their replication features.

Q What is the function of the VPg protein of poliovirus, and how can coronaviruses replicate without a VPg protein?

11.9 In negative-strand viruses, the virus RNA is not mRNA but must first be copied to form mRNA by RNA replicase present in the virion. The positive strand is the template for production of genome copies. Important pathogenic negative-strand viruses include rabies virus and influenza virus.

Q Rabies virus and poliovirus both have single-stranded RNA genomes, but only in poliovirus can the genome be translated directly. Explain.

11.10 Reoviruses contain segmented linear double-stranded RNA genomes. Like negative-strand RNA viruses, reoviruses contain an RNA-dependent RNA polymerase within the virion. Newly forming virions take up plus-strand RNA that is the template for the complementary strand produced in the new virions.

Q Compare the reovirus genome to those of influenza virus and bacteriophage MS2. Why do reovirus replication events have to happen in the nucleocapsid?

11.11 Some viruses employ reverse transcriptase, including retroviruses (HIV) and hepadnaviruses (hepatitis B). Retroviruses have single-stranded RNA genomes and use reverse transcriptase to make a DNA copy. Hepadnaviruses contain partially double-stranded DNA genomes and use reverse transcriptase to make a single strand of genomic DNA from a full-length complementary strand of RNA.

Q Why do both hepadnaviruses and retroviruses require reverse transcriptase when their genomes are double-stranded DNA and single-stranded RNA, respectively?

IV • Subviral Agents

11.12 Viroids are circular single-stranded RNA molecules that do not encode proteins and are dependent on host-encoded enzymes for replication. Unlike viruses, viroid RNA is not enclosed within a capsid, and all known viroids are plant pathogens.

Q What are the similarities and differences between viruses and viroids?

11.13 Prions are made of protein but have no nucleic acid of any kind. Prions exist in two conformations, the native cellular form and the pathogenic form, which takes on a different protein structure. The pathogenic form "replicates" itself by converting native prion proteins, encoded by the host cell, into the pathogenic conformation.

Q What are the similarities and differences between prions and viruses?

APPLICATION QUESTIONS

1. Not all proteins are made from the RNA genome of bacteriophage MS2 in the same amounts. Can you explain why? One of the proteins functions very much like a repressor, but it functions at the translational level. Which protein is it and how does it function?
2. Replication of both strands of DNA in adenoviruses occurs in a continuous (leading) fashion. How can this happen without violating the rule that DNA synthesis always occurs in a $5' \rightarrow 3'$ direction?
3. Imagine that you are a researcher at a pharmaceutical company charged with developing new drugs against human RNA viral pathogens. Describe at least two types of drugs you might pursue, what class of virus they would affect, and why you feel that the drugs would not harm the patient.
4. Reoviruses contain genomes that are unique in all of biology. Why? Why can't reovirus replication occur in the host cytoplasm? Contrast reovirus genomic replication events with those of a cell. Why can it be said that reovirus genome replication is not semiconservative even though the reovirus genome consists of complementary strands?

CHAPTER GLOSSARY

Concatemer a genomic nucleic acid molecule formed from the recombination of several genomic units

Hepadnavirus a virus whose DNA genome replicates by way of an RNA intermediate

Negative strand a nucleic acid strand that has the opposite sense to (is complementary to) the mRNA

Overlapping genes two or more genes in which part or all of one gene is embedded in another gene

Polyprotein a large protein expressed from a single gene and subsequently cleaved to form several individual proteins

Positive strand a nucleic acid strand that has the same sense as the mRNA

Prion an infectious protein whose extracellular form lacks nucleic acid

Replicative form a double-stranded molecule that is an intermediate in the replication of viruses with single-stranded genomes

Retrovirus a virus whose RNA genome is replicated via a DNA intermediate

Reverse transcriptase the retroviral enzyme that produces DNA from an RNA template

Reverse transcription the process of copying genetic information found in RNA into DNA

RNA replicase an enzyme that can produce RNA from an RNA template

Rolling circle replication a mechanism, used by some plasmids and viruses, of replicating circular DNA, which starts by nicking and unrolling one strand. For a single-stranded genome, this is preceded by using the still-circular strand as a template for DNA synthesis; for a double-stranded genome, the unrolled strand is used as a template for DNA synthesis

Viroid an infectious RNA whose extracellular form lacks protein

12 Biotechnology and Synthetic Biology

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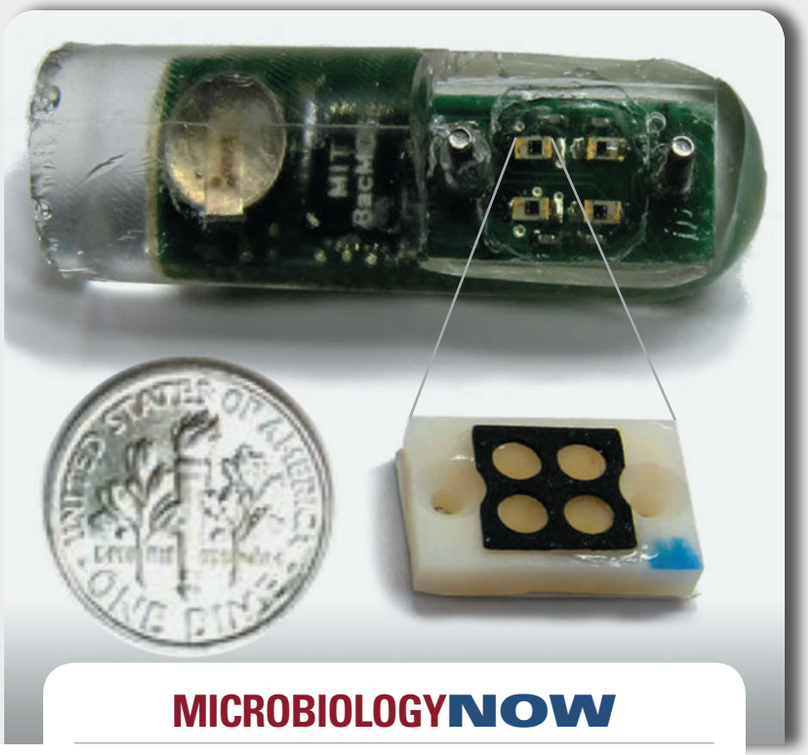


MICROBIOLOGY**NOW**

An Ingestible Biosensor: Using Bacteria to Monitor Gastrointestinal Health

By constantly monitoring and responding to molecules in their environment, microbes possess a remarkable ability to sense and adapt to a range of conditions. Synthetic biologists have exploited this resiliency, along with genetic tractability, to develop microbially driven biosensors for detecting specific stimuli. Now the technology behind these biosensors has been modified to detect markers of human disease.

At the top of the image shown here is a tiny ingestible biosensor that is able to monitor gastrointestinal bleeding (the coin below indicates the scale). The device detects the presence of blood through the response of *Escherichia coli* cells that have been genetically modified to express luminescence genes when exposed to heme, a component released from lysed red blood cells. The square image in the bottom right of the photo shows four quadrants of the device's semipermeable membrane that confines millions of the engineered bacteria. Once loaded with bacteria, the membrane is attached to the capsule such that the bacteria are both protected and exposed to surrounding molecules as the device travels through the digestive tract.

As the engineered bacteria encounter heme, they modify their gene expression to emit light. Photo transmitters located under the membrane then communicate this response to a microprocessor that ultimately transmits the signal wirelessly to a cell phone. While this biosensor has been successfully used to detect the presence of blood in the stomachs of swine, engineers are working to further miniaturize the electronics. Small as it is, the biosensor's current size (~3.8 cm long) is difficult for humans to swallow.

With current medical procedures for analyzing upper gastrointestinal health requiring expensive and unpleasant procedures, such as endoscopy, the exciting fields of biotechnology and synthetic biology promise future noninvasive and real-time monitoring with as little as a biosensor capsule and a cell phone!

Source: Mimee, M., et al. 2018. An ingestible bacterial-electronic system to monitor gastrointestinal health. *Science 360*: 915.

Industrial microbiology uses microbes on a large scale to produce products such as enzymes, foods, and beverages. These microbes are typically not genetically modified. Instead, naturally overproducing strains are selected from wild-type strains and used for industrial purposes. In contrast, biotechnology uses genetically modified microorganisms to produce high-value products that the organisms do not naturally produce. In this chapter we discuss the basic techniques of genetic engineering that underlie biotechnology, in particular those used to clone, alter, and express genes efficiently in host organisms. We also explore how genetic engineering and biotechnology can be used for industrial, medical, and agricultural applications (Figure 12.1) and introduce the exciting new field of **synthetic biology**. The latter is an emerging science that assembles bits and pieces of DNA—aptly called *biobricks*—into entirely new chromosomes or other genetic elements that can modify microbes or even macroorganisms in ways that benefit humans (Figure 12.1).

I • Tools of the Genetic Engineer

DNA from any source can be manipulated in the laboratory in unprecedented ways using the powerful tools of PCR, restriction enzymes, molecular cloning and recombineering, nucleic acid hybridization, and a host of gene expression systems.

Performing genetics *in vivo* (in living organisms) has many limitations that can be overcome by manipulating DNA *in vitro* (in a test tube). **Genetic engineering** refers to the use of in vitro techniques to alter genes in the laboratory. Such altered genes may be reinserted into the original source organism or into some other host organism. Expression of a gene from one organism in a different host organism that does not normally possess the gene is called **heterologous expression**.

Genetic engineering requires that specific DNA be isolated, purified, and further manipulated. We begin by considering some of the basic tools of the genetic engineer, including amplification of DNA, the separation of nucleic acids by electrophoresis, nucleic acid hybridization, and molecular cloning. We also describe methods for expressing foreign genes in bacteria and targeted mutagenesis.

12.1 Manipulating DNA: PCR and Nucleic Acid Hybridization

The first objective of genetic engineering is to isolate copies of specific genes in pure form, and the key method for doing so is the **polymerase chain reaction (PCR)** (Figure 12.2). Simply put, the polymerase chain reaction is DNA replication in vitro, as segments of target DNA are multiplied by up to a billionfold in the process of *amplification*. During each round of amplification, the amount of DNA *doubles*, leading to an exponential increase in the target DNA. Using an automated PCR machine called a *thermocycler*, a large amount of amplified DNA can be produced from only a few molecules of target DNA. In some cases it is desirable to quantify the initial amount of target DNA, and a variation on PCR called *quantitative PCR* (*qPCR*) is used for this purpose (▶ Section 29.8). A second variation on the original PCR technique allows for amplification of RNA (following its conversion to DNA, as discussed later in this section).

Mastering Microbiology
Art Activity: Figure 12.1 The polymerase chain reaction (PCR)

UNIT 3

PCR and Polymerases

To synthesize DNA, PCR requires DNA polymerase, the enzyme that naturally copies DNA molecules (◀ Section 6.3), and artificially synthesized oligonucleotide primers (Section 12.4) made of DNA (rather than the RNA primers used by cells to replicate DNA). PCR does not actually copy whole DNA molecules but amplifies stretches of up to a few thousand base pairs (the *target*) from within a larger DNA molecule (the *template*) during the following steps (Figure 12.2):

1. Template DNA is heated to denature it (that is, to separate the two strands), and then two DNA oligonucleotide primers complementary to sequences flanking the target DNA on each strand are added in excess. This ensures that most template strands anneal to a primer, and not to each other, as the mixture cools (Figure 12.2*a*).
2. DNA polymerase then extends the primers using the original DNA as the template (Figure 12.2*b*).
3. After an appropriate incubation period, the mixture is heated again to separate the strands, but now the target gene is present

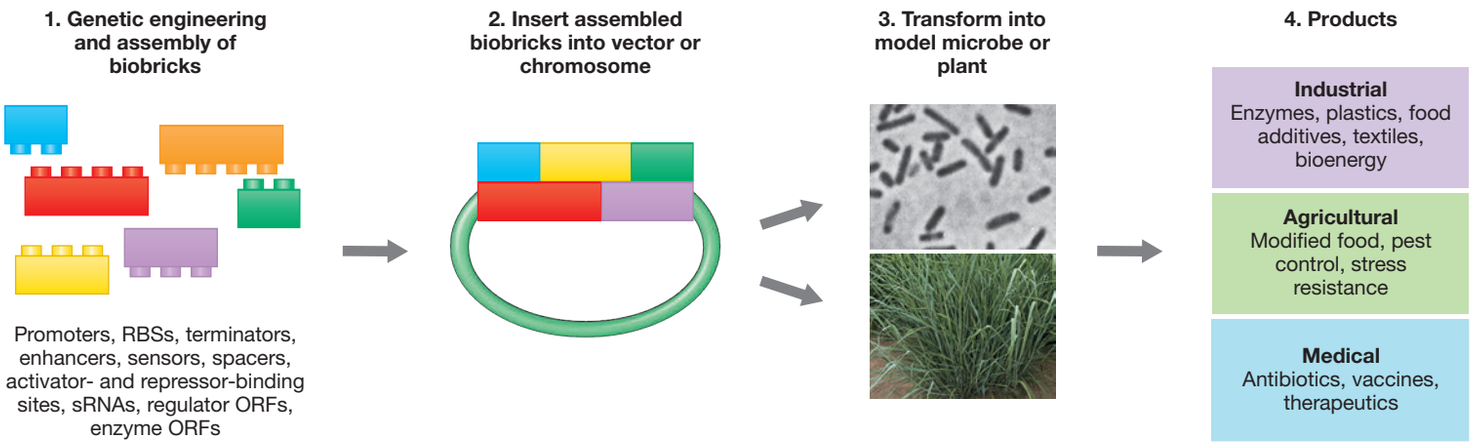
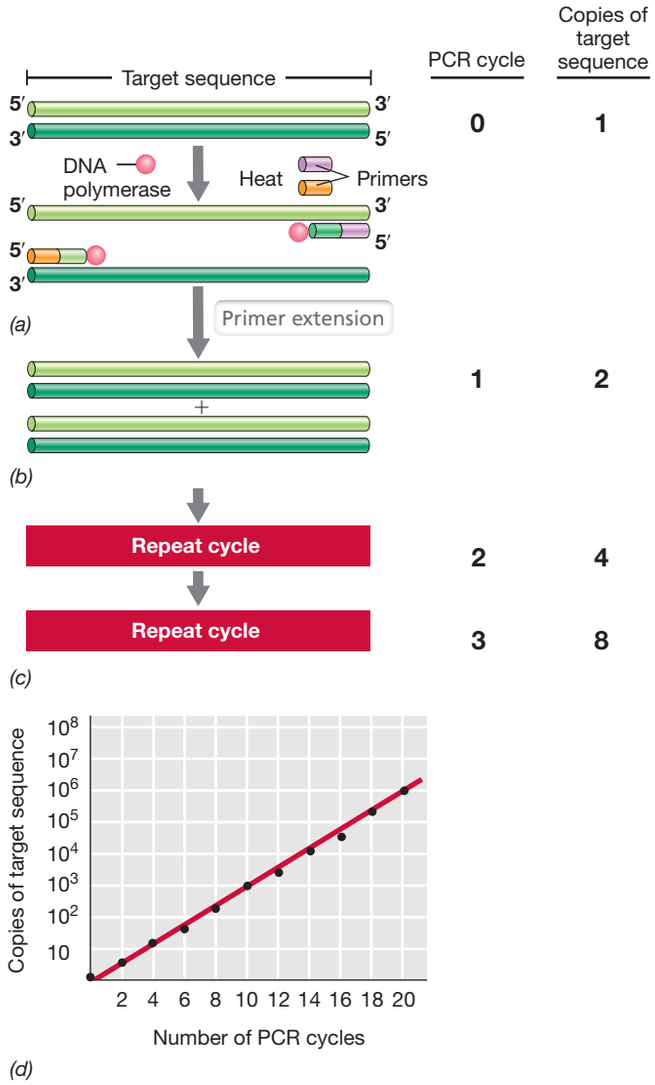


Figure 12.1 Genetic engineering and biobrick assembly. Molecular cloning techniques are used to modify organisms for the production of high-value products. RBS, ribosome-binding site; sRNAs, small RNAs; ORFs, open reading frames.



Mastering Microbiology
Art Activity: Figure 12.2 Reverse transcription PCR

Figure 12.2 The polymerase chain reaction (PCR). The PCR amplifies specific DNA sequences. *(a)* Target DNA is heated to separate the strands, and a large excess of two oligonucleotide primers, one complementary to each strand, is added along with DNA polymerase. *(b)* Following primer annealing, primer extension yields a copy of the original double-stranded DNA. *(c)* Two additional PCR cycles yield four and eight copies, respectively, of the original DNA sequence. *(d)* Effect of running 20 PCR cycles on a DNA preparation originally containing 1 copy of a target gene. Note that the plot is semilogarithmic.

in twice the original amount. The mixture is then cooled to allow the primers to hybridize with complementary regions of newly synthesized and original DNA, and the process is repeated (Figure 12.2*c*). In practice, 20–30 cycles are typically run, yielding a 10^6-fold to 10^9-fold increase in the target sequence (Figure 12.2*d*).

Because high temperatures are used to denature the double-stranded copies of DNA in vitro, use of a thermostable DNA polymerase is critical. *Taq polymerase*, a DNA polymerase isolated from the thermophilic hot spring bacterium *Thermus aquaticus* (▶ Section 16.20), is stable to 95°C and thus is unaffected by the denaturation step employed in the PCR (Figure 12.2). A DNA polymerase from *Pyrococcus furiosus*, a hyperthermophilic species of *Archaea* with a growth temperature optimum of 100°C (▶ Section 17.4), is called *Pfu polymerase* and is even more thermostable than *Taq* polymerase. Moreover, unlike *Taq* polymerase, *Pfu* polymerase has proofreading activity (◀ Section 6.4), making it especially useful when high accuracy is crucial. To supply the demand for thermostable DNA polymerases in biotechnology, the genes encoding these enzymes have been cloned into *Escherichia coli*, allowing the enzymes to be produced commercially in large quantities.

PCR Applications and RT-PCR

PCR is extremely valuable for obtaining DNA for gene cloning or for sequencing purposes because the gene or genes of interest can easily be amplified if flanking sequences are known. PCR is also used routinely in comparative or phylogenetic studies to amplify genes from various sources. In these cases the primers are made commercially to base-pair with regions of the gene that are conserved in sequence across a wide variety of organisms. Because small ribosomal subunit (SSU) rRNA—a molecule used for phylogenetic analyses—has both highly conserved and highly variable regions (▶ Section 13.11 and Figure 13.24), primers specific for the SSU rRNA gene from different taxonomic groups can be used to survey habitats for their microbial communities (▶ Section 19.6). Also, because it is so sensitive, PCR can be used to amplify even very small quantities of DNA. For example, PCR has been used to amplify DNA from sources as varied as mummified human remains, fossilized plants and animals, and even single microbial cells (◀ Section 10.11). PCR is also a common tool of medical diagnostics in clinical microbiology laboratories (▶ Section 29.8) and is widely used in forensic science to link crime scene evidence such as blood, semen, or tissue samples to a specific suspect. In clinical laboratories, especially, time is of the essence, and the faster an infectious disease is diagnosed, the sooner an effective treatment can be chosen and administered.

An important extension of the standard PCR procedure is *reverse transcription PCR* (RT-PCR), used to make *DNA* from an *mRNA* template (**Figure 12.3**). This procedure can be used to detect if a gene is expressed or to produce an intron-free eukaryotic gene for expression in bacteria (Section 12.3). RT-PCR uses the retroviral enzyme *reverse transcriptase* to convert RNA into **complementary DNA (cDNA)** (◀ Sections 11.1 and 11.11). Figure 12.3 illustrates how reverse transcriptase makes a single strand of cDNA using RNA as a template. To make cDNA, a primer complementary to the 3′ end of the target RNA is used by the enzyme reverse transcriptase to initiate DNA synthesis. If the template is eukaryotic mRNA, a primer complementary to the poly(A) tail (◀ Section 6.6) of the mRNA can be used. The activity of reverse transcriptase results in a hybrid nucleic acid molecule containing both DNA and RNA. RNase H, a ribonuclease specific for the hybrid molecule, hydrolyzes the RNA, leaving the single-stranded cDNA as template for standard PCR using an additional 5′ primer complementary to the 3′ end of the cDNA. Note that some commercial reverse transcriptase enzymes used for RT-PCR also possess an RNase H domain.

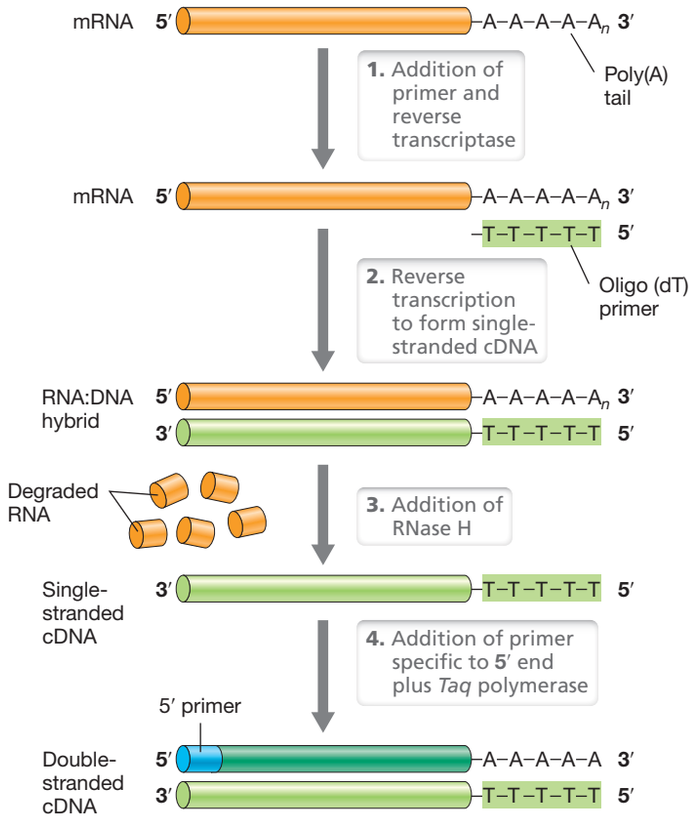


Figure 12.3 Reverse transcription PCR. Steps in the synthesis of complementary DNA (cDNA) from a eukaryotic mRNA. Reverse transcriptase synthesizes a hybrid molecule containing both RNA and DNA using the mRNA as a template and oligo(dT) primer as a substrate. Next, the enzyme RNase H hydrolyzes the RNA portion of the hybrid molecule, yielding a single-stranded molecule of complementary DNA (cDNA). Following the addition of a 5′ primer complementary to the 3′ end of the cDNA, *Taq* polymerase produces a double-stranded cDNA.

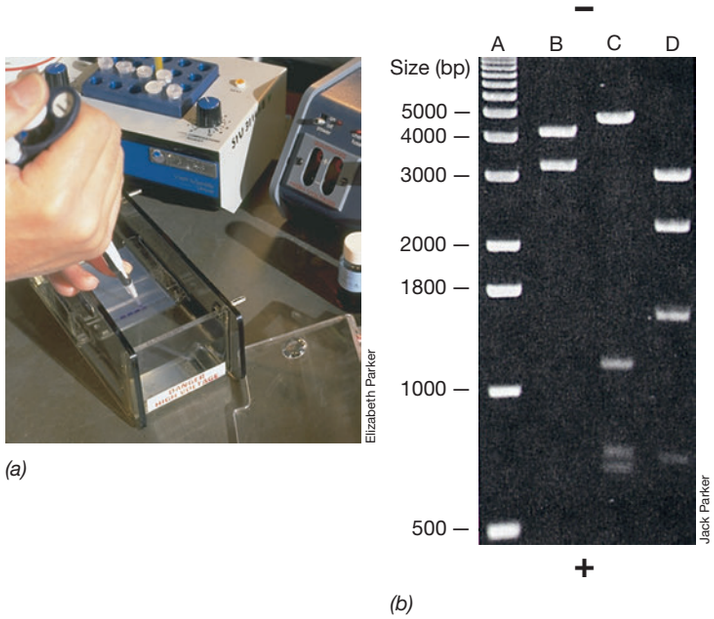


Figure 12.4 Agarose gel electrophoresis of DNA. *(a)* DNA samples are loaded into wells in a submerged agarose gel. *(b)* A photograph of a stained agarose gel. The DNA was loaded into wells toward the top of the gel (negative pole) as shown, and the positive electrode is at the bottom. The standard sample in lane A (DNA ladder) has fragments of known size that may be used to determine the sizes of the fragments in the other lanes. Bands stain less intensely at the bottom of the gel because the fragments are smaller, and thus there is less DNA to stain.

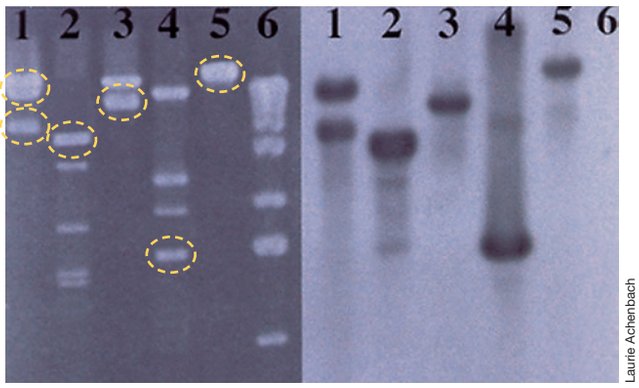
Gel Electrophoresis and Nucleic Acid Hybridization

To verify that amplification of a nucleic acid was successful and for other nucleic acid manipulation steps, DNA or RNA fragments can be separated from each other by **gel electrophoresis**, a technique that employs an agarose gel to separate nucleic acid fragments based on differences in their size and charge (Figure 12.4*a*). When an electrical current is applied, nucleic acids move through the gel toward the positive electrode because of their negatively charged phosphate groups, and small molecules migrate more rapidly than large molecules. After the gel has been run for a time sufficient to separate the molecules, the gel is stained with a dye that binds to nucleic acids and makes them fluoresce, such as *ethidium bromide* (Figure 12.4*b*). To determine the size of the DNA or RNA of interest, the migration is compared to a standard sample consisting of nucleic acid fragments of known sizes, called a *ladder*. DNA fragments can then be purified from gels and used for a variety of purposes, such as cloning or hybridization.

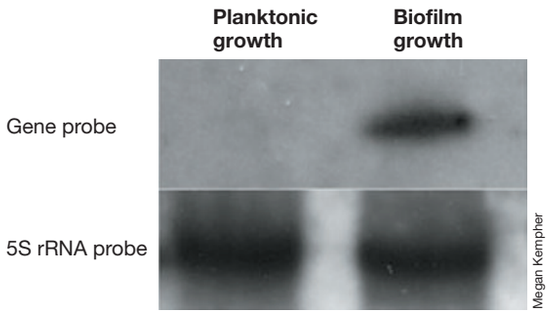
When DNA is denatured, the single strands can be used to form hybrid double-stranded molecules with other single-stranded DNA (or RNA) molecules by complementary base pairing (◀ Section 6.1) in a process called *nucleic acid hybridization*, or **hybridization** for short. Hybridization is widely used in detecting, characterizing, and identifying segments of DNA and RNA. Single-stranded nucleic acids whose identity is already known and that are used in hybridization are called **nucleic acid probes**, or simply *probes*. To allow detection, probes are made radioactive or are labeled with chemicals that are colored or yield fluorescent products (▶ Section 19.5), and by varying the hybridization conditions, the "stringency" of the hybridization can be adjusted such that complementary base pairing is somewhat flexible or, alternatively, must be nearly exact.

Hybridization is useful for finding related sequences in different genomes or other genetic elements and to determine if a gene is expressed into an RNA transcript. In *Southern blotting*, probes of known sequence are hybridized to target DNA fragments that have been separated by gel electrophoresis. The hybridization procedure in which DNA is the target sequence in the gel, and RNA or DNA is the probe, is called a **Southern blot**. By contrast, a **Northern blot** uses RNA as the target sequence and DNA or RNA as the probe to detect gene expression. In both techniques, the nucleic acid fragments must be in a single-stranded form and are transferred to a synthetic membrane. The membrane is then exposed to the labeled probe. If the probe is complementary to any of the fragments, hybrids form, and the probe attaches to the membrane at the locations of the complementary fragments. Figure 12.5 shows how a Southern blot can be used to identify fragments of DNA containing sequences that hybridize to the probe and how the intensity of a signal on a Northern blot gives a rough estimate of mRNA abundance from the target gene.

Nucleic acid hybridization has many other uses. Hybridization is the basis of the fluorescence in situ hybridization (FISH) technique (▶ Section 19.5) (Figure 12.6), in which fluorescent probes



(a) **Southern blot**



(b) **Northern blot**

Figure 12.5 Nucleic acid hybridization. *(a)* Southern blotting. (Left panel) Purified molecules of DNA from several different plasmids were treated with restriction enzymes and then subjected to agarose gel electrophoresis. (Right panel) Blot of the DNA gel shown to the left. After blotting, DNA in the gel was hybridized to a radioactive probe. The positions of the bands were visualized by X-ray autoradiography. Note that only some of the DNA fragments (circled in yellow in the left panel) have sequences complementary to the labeled probe. Lane 6 contained DNA used as a size marker, and none of its bands hybridized to the probe. *(b)* Northern blotting. (Top panel) Hybridization and detection of a radioactive gene-specific probe to a blot of total RNA. The probe only bound to RNA from biofilm-grown cells, indicating that the target gene is not expressed during planktonic (suspended) growth. (Bottom panel) Hybridization and detection of a radioactive probe corresponding to the 5S rRNA to the same blot. The signal intensity indicates that equal amounts of RNA from each sample were loaded into the gel.

are used to target specific DNA (or RNA) sequences in cells. This approach allows the identification of pathogens in clinical samples or bacteria of interest in environmental samples. For example, Figure 12.6 demonstrates the simultaneous use of eight different oligonucleotide probes in combinations to distinguish between 28 different strains of *E. coli* whose SSU (small subunit) rRNA sequences varied only slightly from strain to strain. The variations in color give a visual indication of the specificity and power of nucleic acid probes. Hybridization is also important in various "omics," in particular transcriptomics and metatranscriptomics, where genome-wide gene expression can be monitored in pure cultures and natural populations, respectively, using microarray technology (◀ Section 10.8).

In the next two sections we consider the important processes of gene cloning and the expression of cloned genes, respectively. If either of these events fail, the desired outcome of the genetic engineering will be in doubt.

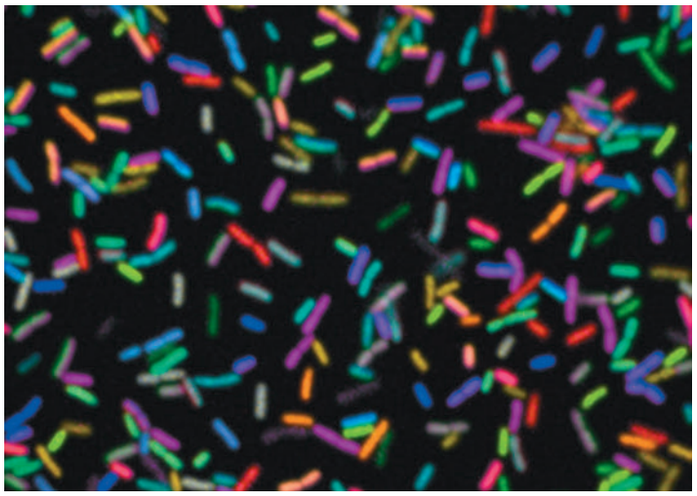


Figure 12.6 Fluorescence spectral image of 28 differently labeled strains of *Escherichia coli*. Cells were labeled with combinations of fluorophore-conjugated oligonucleotides that are complementary to *E. coli* 16S rRNA. (▶ Figure 13.24).

Check Your Understanding

- Why is a primer needed at each end of the DNA segment being amplified by PCR?
- How does RT-PCR differ from traditional PCR?
- What are some applications of nucleic acid hybridization in molecular biology?

12.2 Molecular Cloning

The movement of desired genes from their original source to a small and manipulable genetic element (the **vector**) is called **molecular cloning**. Molecular cloning results in **recombinant DNA**, a molecule containing DNA from different sources. Once cloned, the gene(s) of interest can be manipulated, and when the recombinant vector is placed in an appropriate host, the cloned DNA is replicated, providing the foundation for much of genetic engineering.

An Overview of Gene Cloning and Restriction Enzymes

Following isolation of the source DNA, the major steps in gene cloning are (1) inserting the DNA into a cloning vector (**Figure 12.7**), and (2) inserting the vector into a host. The source DNA can be a gene or genes amplified by the polymerase chain reaction (Section 12.1), DNA synthesized from an RNA template by reverse transcriptase (Section 12.1), or even completely synthetic DNA made in vitro (Section 12.4). Cloning vectors are small, independently replicating genetic elements that can both carry and replicate cloned DNA segments (see Figure 12.10). Cloning vectors are typically designed to allow for easy insertion of foreign DNA. One way to insert foreign DNA into a vector is by using a *restriction site* (Figures 12.7 and 12.8). Restriction endonucleases, or **restriction enzymes** for short, recognize specific base sequences (restriction sites) within DNA and cut the phosphodiester backbone, resulting in double-stranded breaks (**Figure 12.8**). The recognition sequences—which are unique to each restriction enzyme—are typically inverted repeats and are called *palindromes*.

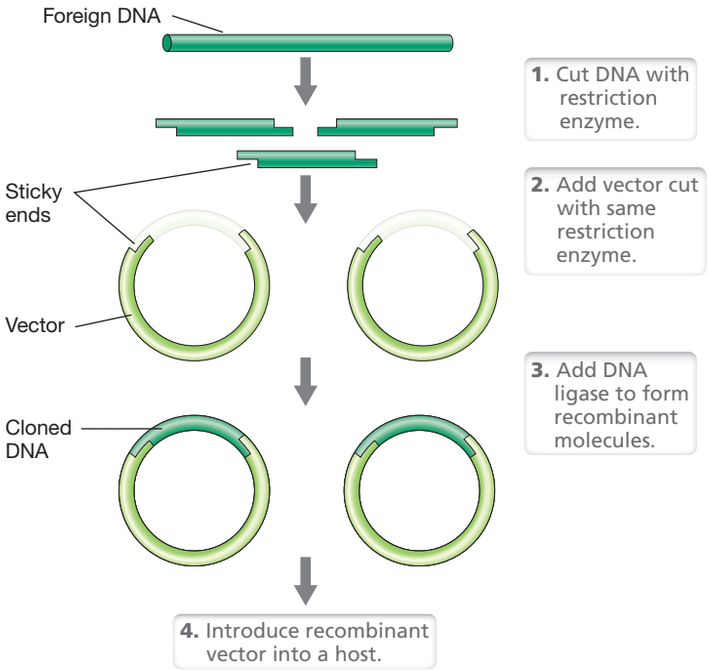


Figure 12.7 Major steps in gene cloning using restriction enzymes. By cutting the foreign DNA and the vector DNA with the same restriction enzyme, complementary sticky ends are generated that allow foreign DNA to be inserted into the vector.

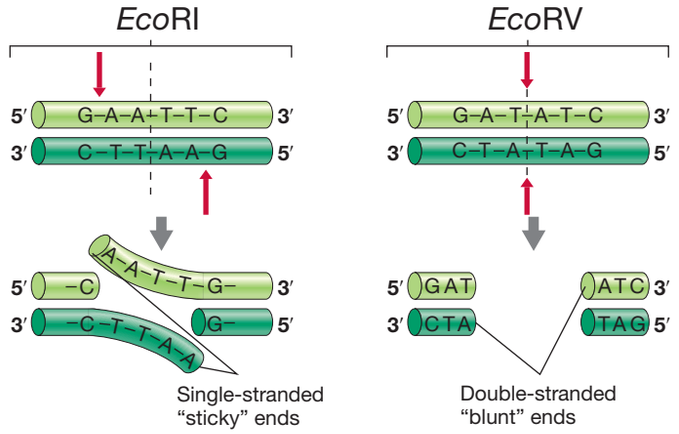


Figure 12.8 Restriction and modification of DNA. Sequences of DNA recognized by the restriction endonucleases *Eco*RI and *Eco*RV. The red arrows indicate the bonds cleaved by the enzyme, and the dashed line indicates the axis of symmetry of the sequence. After cutting DNA with these restriction enzymes, note the single-stranded "sticky" ends generated by *Eco*RI versus the "blunt" ends generated by *Eco*RV.

UNIT 3

Restriction enzymes with different sequence specificities are widespread among *Bacteria*, where they help protect cells from attack by viral DNA (◀ Section 9.12). The cell is protected from its own restriction enzyme(s) by chemical modification (typically by methylation) of one of the bases in any potential restriction sites that exist in its genome. The restriction enzyme *Eco*RI makes staggered cuts, leaving short, single-stranded overhangs called "sticky" ends at the termini of the two fragments. Other restriction enzymes such as *Eco*RV cut both strands of the DNA directly opposite each other, resulting in blunt ends (Figure 12.8). If the source DNA and the vector are both cut with the *same* restriction enzyme that yields complementary sticky ends, the two molecules can be joined (annealed) using *DNA ligase*, an enzyme that covalently links the strands of the vector and the source DNA. If the source DNA is PCR generated, DNA ligase is used to join the amplified DNA to specialized vectors (see Figure 12.11*a*, *b*).

In the final step of gene cloning, recombinant DNA molecules are introduced into suitable host organisms where they can replicate. But in practice, this often yields a mixture of *recombinant* constructs, where only some of the cells contain the desired cloned gene. To identify a host colony containing the correct recombinant DNA, one can select host cells expressing a vector-encoded marker such as antibiotic resistance. Colonies can then be screened for recombinant vectors by looking for the inactivation of a vector gene due to insertion of foreign DNA (see Figures 12.10 and 12.11*a*, *b*).

Recombineering

While cloning with restriction enzymes has been the standard of molecular biology (Figure 12.7), its biggest limitation is that modifications can only be made at restriction enzyme sites. To avoid this requirement, specialized strains of *Escherichia coli* have been designed to allow for gene cloning using homologous recombination (◀ Section 9.5). Through the use of *recombineering* (*recombi*nation-mediated genetic eng*ineering*), foreign DNA can be inserted into vectors (or the chromosome) by designing the insert DNA to possess short regions (30–50 bases) of sequence homology to the target DNA molecule and activating recombinase enzymes. These recombinase enzymes cleave, exchange, and rejoin strands of DNA between areas of homology. Thus, if the ends of a DNA insert are constructed through PCR to share homology with a vector at the target insertion position, recombinase enzymes will "flip" the DNA insert into the vector (**Figure 12.9**).

Recombineering was originally developed in the yeast *Saccharomyces cerevisiae*; this microbe, widely used in genetic engineering

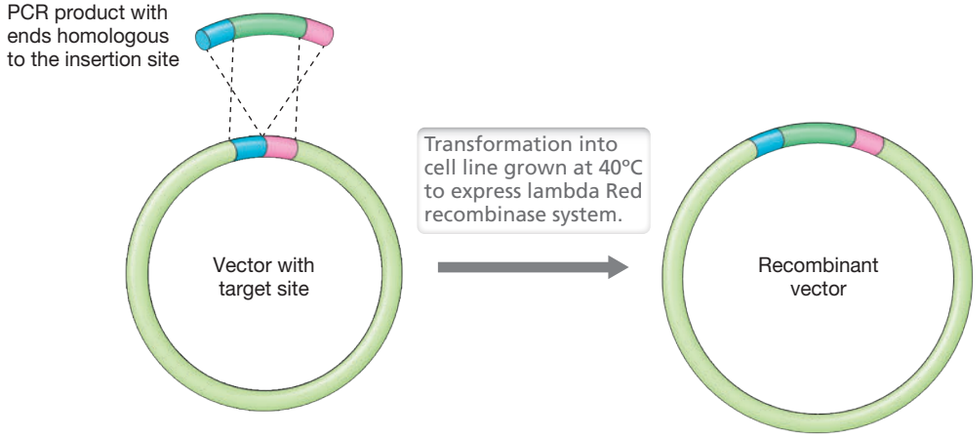


Figure 12.9 Recombineering. Enzymes from the lambda Red recombinase system coupled with sequence homology can be used to insert foreign DNA into target sites of vectors and chromosomes. The sequence homology can be generated by PCR and primer design.

(see Figure 12.12), possesses native recombinase enzymes that can recombine DNA regions of sequence homology as short as 20 bases (see Figure 12.40). While homologous recombination does occur naturally in *E. coli*, the efficiency is very low with short regions of homology. Thus, the engineered strains for cloning express the bacteriophage lambda Red recombinase system. The lambda Red (identified from *re*combination *d*efective phage mutants) system is a mutant form of the system used by the bacteriophage to insert its genome into the *E. coli* chromosome during lysogeny (◀Section 11.4). The genes encoding this modified recombinase system are located on either a plasmid or the chromosome of the engineered strain, and their transcription is controlled by a repressor protein (Chapter 7) that is sensitive to temperature (Figure 12.9). Thus, the recombinase enzymes are only expressed and active when the temperature is shifted to 40°C. This allows a molecular biologist to control the time point when the recombinases are produced to recombine the foreign DNA in the vector (or chromosome) and helps avoid unwanted recombination events.

Recombineering has revolutionized cloning in *E. coli* because compatible restriction enzyme sites in the vector and the DNA to be cloned are not needed. PCR products or synthesized DNA fragments (Section 12.3) can be recombined into a target site as long as they possess at least 30 base pairs of homology to the desired insertion site. Because the Red recombinase system can also handle single-stranded DNA molecules, oligonucleotide primers can also be synthesized with homology to the target site. Thus, recombineering can be used not only to clone foreign genes into *E. coli*, but also to generate point mutations and gene fusions (see Figure 12.18). After recombineering, recombinant DNA molecules are selected in the same way as gene cloning with restriction enzymes (see Figures 12.10 and 12.11).

Cloning Vectors

Several types of cloning vectors exist, including viruses, cosmids (plasmids with a lambda phage *cos* site, ◀Figure 11.11), and artificial chromosomes, and their use is dependent on the size of the DNA fragment to be cloned and the host in which the vector will be inserted. Plasmids are widely used cloning vectors that often exist as multiple copies in bacterial cells. The plasmid pUC19 (**Figure 12.10**) is a standard cloning plasmid that contains an ampicillin resistance gene for selection and a blue–white color-screening system to select for recombinants. It also contains a short segment of artificial DNA containing cut sites for many different restriction enzymes. This segment, called a *multiple cloning site* (*MCS*), is inserted into the *lacZ* gene encoding the lactose-degrading enzyme β-galactosidase (◀ Section 7.8 and Figure 7.23). The presence of the short MCS does not inactivate *lacZ*, and cut sites for restriction enzymes present in the MCS are absent from the rest of the vector.

The use of pUC19 in gene cloning is shown in Figure 12.10. A suitable restriction enzyme with a cut site within the MCS is chosen, and both the vector and the foreign DNA to be cloned are cut with this enzyme. The vector is linearized, and segments of the foreign DNA are inserted into the open cut site and ligated into position with the enzyme DNA ligase. This insertion disrupts the *lacZ* gene—a phenomenon called *insertional inactivation*—and is used to detect the presence of foreign DNA within the vector or recombinant vector. After DNA ligation, the resulting plasmids are transformed (◀ Section 9.6) into cells of *Escherichia coli* and the cells plated on media containing both ampicillin

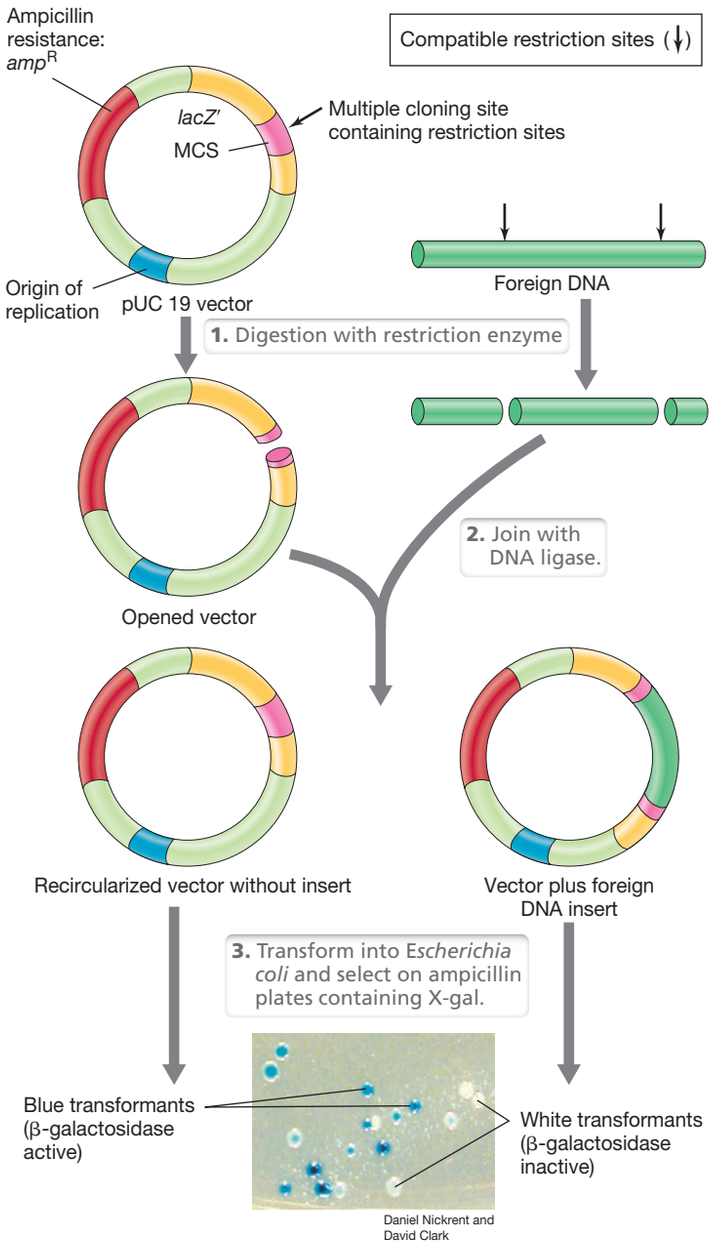


Figure 12.10 Cloning into the plasmid vector pUC19. Essential features include an ampicillin resistance marker and the multiple cloning site (MCS) with multiple restriction enzyme cut sites. The cloning vector and foreign DNA are cut with compatible restriction enzymes at positions indicated by the arrows. Insertion of DNA within the MCS inactivates β-galactosidase, allowing blue–white screening for the presence of the insert. The photo on the bottom shows colonies of *Escherichia coli* on an X-gal plate. The enzyme β-galactosidase can cleave the normally colorless X-gal to form a blue product.

(to select for cells containing the plasmid) and a lactose analog called *X-gal* (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), to detect β-galactosidase activity. X-gal, which is colorless, can be cleaved by β-galactosidase to generate a blue product. Thus, cells containing the vector *without* cloned DNA form blue colonies (that is, β-galactosidase is active), whereas cells containing the vector *with* an insert of cloned DNA do not form β-galactosidase and are therefore white and are the focus of further analyses.

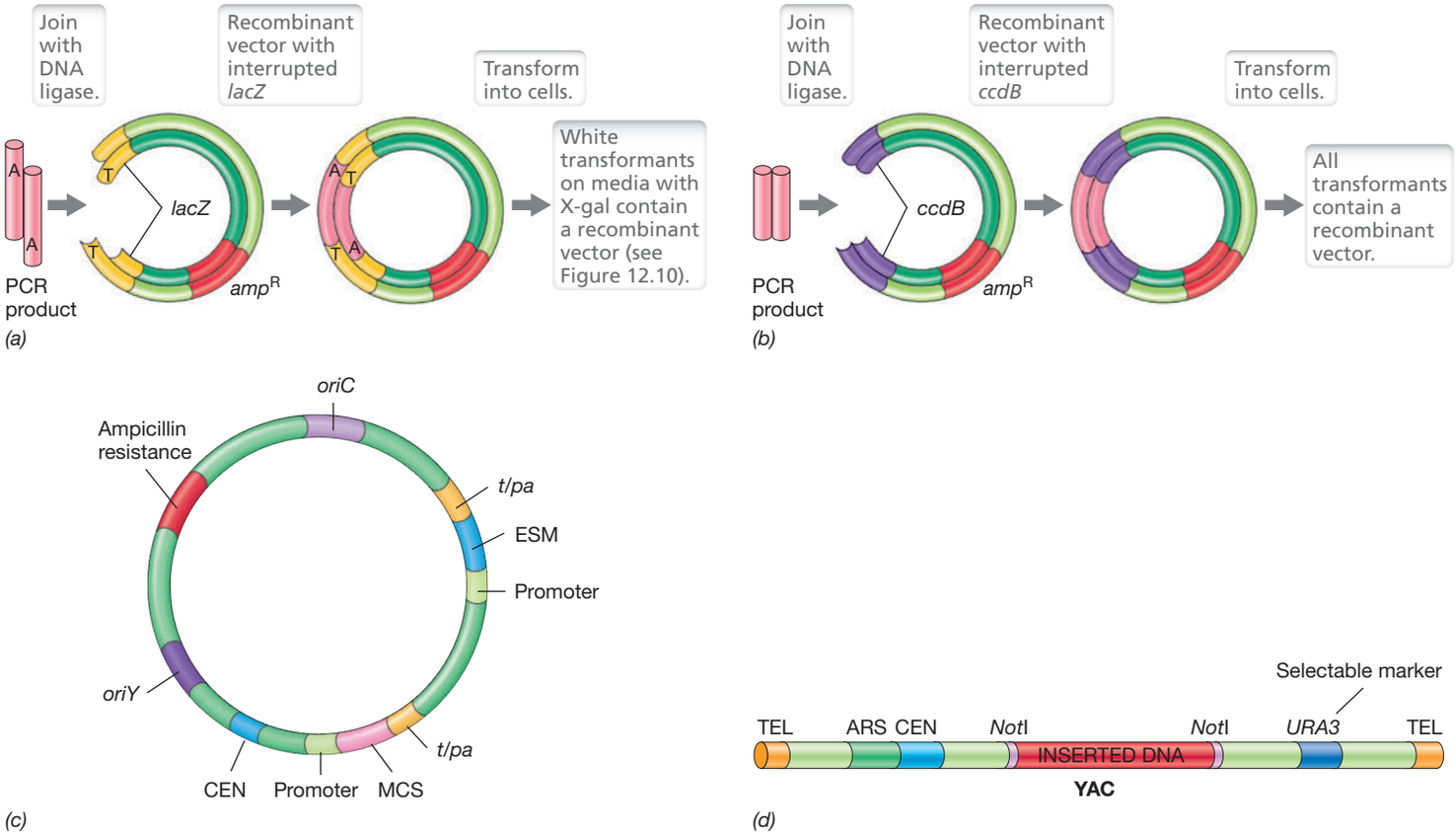


Figure 12.11 Specialized vectors. *(a)* PCR vector with overhangs and *lacZ*. The linearized cloning vector contains overhanging thymidine residues that base-pair with the adenosine residues present on the 3′ ends of *Taq*-polymerase-generated PCR. Ligation of the two pieces of DNA yields a circular plasmid containing an interrupted *lacZ* and thus white colonies on plates containing X-gal (see Figure 12.10). *(b)* PCR vector with blunt ends and *ccdB*. Ligation of a blunt PCR product generated by *Pfu* polymerase yields a circular plasmid containing an interrupted *ccdB*, and thus all transformants that survive contain a recombinant plasmid. *(c)* Genetic map of a shuttle vector used in yeast. The vector contains components that allow it to shuttle between *Escherichia coli* and yeast and be selected in each organism: *oriC*, origin of replication in *E. coli*; *oriY*, origin of replication in yeast; MCS, multiple cloning site; ESM, eukaryotic selectable marker; CEN, yeast centromere sequence; promoter; *t/pa*, transcription termination/polyadenylation signals. Ampicillin resistance allows for selection of the vector in *E. coli*. Arrows indicate the direction of transcription. *(d)* A yeast artificial chromosome (YAC) containing inserted DNA. The foreign DNA was cloned into the vector at a *Not*I restriction site. The telomeres are labeled TEL and the centromere CEN. The origin of replication is labeled ARS (for *a*utonomous *r*eplication *s*equence). The *URA3* gene is used for selection. The host into which the clone is transformed has a mutation in *URA3* and requires uracil for growth (Ura^-). Host cells containing this YAC become Ura^+. The diagram is not to scale; vector DNA is only 10 kbp whereas cloned DNA can be up to 800 kbp.

Plasmids developed specifically for cloning DNA products synthesized by the polymerase chain reaction (PCR; Section 12.1) have also been designed (**Figure 12.11*a*, *b***). The enzymatic activity of *Taq* polymerase adds a template-independent adenosine residue to the 3′ ends of its products. Linearized vectors are commercially available that contain overhanging thymidine residues that allow for base pairing with the *Taq* PCR product and subsequent ligation using DNA ligase. Ligation of the PCR product results in a circular vector with an interrupted *lacZ* gene for selection of a transformant (◀ Section 9.6) with a recombinant vector (Figure 12.11*a*).

Because *Pfu* DNA polymerase does *not* add template-independent adenosine residues, separate linear vectors with blunt ends are available for cloning. These vectors typically rely on insertional inactivation of the *ccdB* gene to select against vectors that rejoin without an insert (Figure 12.11*b*). CcdB is the toxin component of a toxin–antitoxin module (◀ Section 8.12) that inhibits the enzyme DNA gyrase, and when CcdB is expressed, it prevents DNA replication. Thus, if the vector is not recombinant, the resulting transformed *E. coli* cells will die from expressing CcdB. This is a strong selection tool because all the resulting colonies from the transformation should contain recombinant vectors (Figure 12.11*b*).

For manipulating genes in *E. coli* and subsequent cloning into other organisms, **shuttle vectors** are used. These vectors can replicate and be stably maintained in two distinct organisms, such as *E. coli* and yeast or *E. coli* and mammalian cells. The importance of a shuttle vector is that DNA cloned in one organism can be replicated in a second host without having to modify the vector. Thus, the vectors must contain replication, transcription, and selection features for each host, and this is shown for a shuttle vector used in yeast in Figure 12.11*c*. However, genes can also be cloned directly into the yeast *Saccharomyces cerevisiae* using **yeast artificial chromosomes (YACs)** (Figure 12.11*d*). YACs are linear vectors that replicate in yeast like normal chromosomes but have sites where very large fragments of DNA can be inserted. To function like

normal eukaryotic chromosomes, YACs have an origin of DNA replication, telomeres for replicating DNA at the ends of the chromosome, and a centromere for segregation during mitosis. YACs also contain a cloning site and a gene for selection following transformation into the host (Figure 12.11*d*).

Hosts for Cloning Vectors

The most useful hosts for cloning are nonpathogenic microorganisms that are easy to grow and transform with engineered DNA. They must also be genetically stable in culture and have the appropriate enzymes to allow replication of the vector. It is also helpful if considerable background information on the host and a wealth of tools for its genetic manipulation exist. Hosts that meet these conditions include the bacteria *E. coli* and *Bacillus subtilis*, and the yeast *S. cerevisiae* (**Figure 12.12**). Complete genome sequences are available for all of these organisms, and they are widely used as cloning hosts.

Although *E. coli* is found in the human intestine and some wild-type strains are potentially harmful (▶ Section 33.11), several modified *E. coli* strains have been developed specifically for cloning purposes. However, if cloned gene *expression* is desired, the outer membrane of this gram-negative bacterium (◀ Section 2.4) can hinder protein secretion. This problem can be overcome using the gram-positive bacterium *B. subtilis* as a cloning host (Figure 12.12). Cloning of DNA from eukaryotic sources into eukaryotic rather than prokaryotic cells is often done since eukaryotic hosts already possess the complex RNA and post-translational processing systems required for the production of eukaryotic proteins (◀ Section 6.6). Because it is easy to grow and manipulate, the workhorse for cloning in eukaryotic cells is the yeast *S. cerevisiae.* However, some cloning applications require the use of plant tissues, insect cell lines, or cultured mammalian cells. Regardless of host type, it is necessary to get cloned DNA into the host. While chemical transformation and electroporation (◀ Section 9.6) are often used to introduce DNA into cells of *Bacteria* and *Archaea*, methods such as transfection (see Figure 12.21), microinjection, and electroporation can be used for eukaryotic cells.

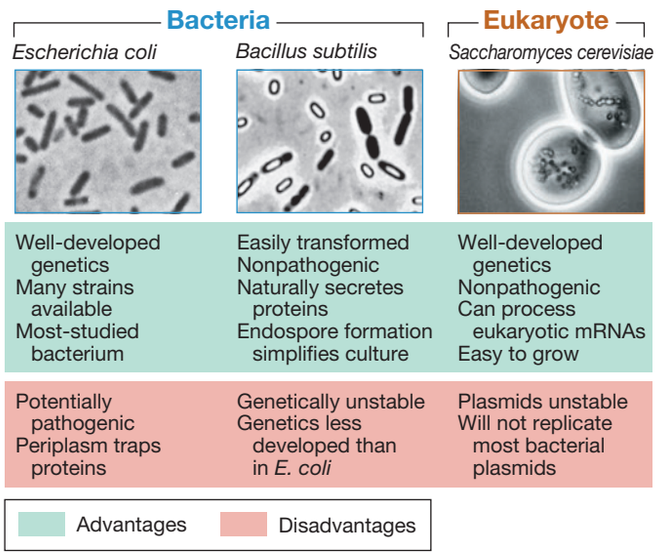


Figure 12.12 Hosts for molecular cloning. A summary of the advantages and disadvantages of some common cloning hosts.

Check Your Understanding

- What is the purpose of molecular cloning?
- What do the terms multiple cloning site, insertional inactivation, and recombineering mean?
- When would it be beneficial to use a eukaryotic host for molecular cloning?

12.3 Expressing Foreign Genes in *Bacteria*

Once genes are cloned, they can be transcribed and translated (expressed) to produce their encoded proteins. Obstacles to the expression of genes from mammalian or other eukaryotic sources in bacteria include the following: (1) The genes must be placed under control of a bacterial promoter; (2) any introns (◀ Section 6.6) must be removed; (3) codon usage (codon bias, ◀ Section 6.9) may require edits to gene sequences; and (4) many eukaryotic proteins require host modification after translation to yield the active form and bacteria cannot perform most such modifications. Here we consider solutions to these challenges.

Transcription and Translation of Cloned Genes Using Expression Vectors

Expression vectors are designed to allow the experimenter to control the expression of cloned genes. However, the native promoter of a cloned gene may work poorly in a new host, and the overproduction of foreign proteins may also damage the host cell. Therefore, it is important to regulate the expression of cloned genes. Typically, the regulation is at the transcriptional level, and in practice, high levels of transcription require strong promoters that bind RNA polymerase efficiently (◀ Section 6.5). An example of this is the use of the bacteriophage T7 promoter and T7 RNA polymerase to regulate gene expression. When T7 infects *Escherichia coli*, it encodes its own RNA polymerase that recognizes only T7 promoters (◀ Section 11.4). In T7 expression vectors, cloned genes are placed under control of the T7 promoter. To achieve this, the gene for T7 RNA polymerase must also be present in the cell under the control of an easily regulated system, such as *lac* (◀ Section 7.3; **Figure 12.13**). This is usually done by integrating the gene for T7 RNA polymerase with a *lac* promoter into the chromosome of a specialized host strain.

The BL21 series of *E. coli* host strains are especially designed to work with the pET series of T7 expression vectors (Figure 12.13). The cloned genes are expressed shortly after T7 RNA polymerase transcription has been switched on by a *lac* inducer, such as the chemical IPTG (◀ Section 7.3). Because it recognizes only T7 promoters, the T7 RNA polymerase transcribes only the cloned genes. The T7 RNA polymerase is so highly active that it uses most of the RNA precursors, thereby limiting transcription to the cloned genes. Consequently, host genes that require host RNA polymerase are for the most part not transcribed and thus the cells stop growing; translation in such cells then yields primarily the protein of interest. The T7 control system is thus very effective for generating large amounts of a specific protein.

Expression vectors must also be designed to ensure that the mRNA produced is efficiently translated. To synthesize protein from an mRNA molecule, it is essential for the ribosomes to bind at the correct site and begin reading in the correct frame. In bacteria this is

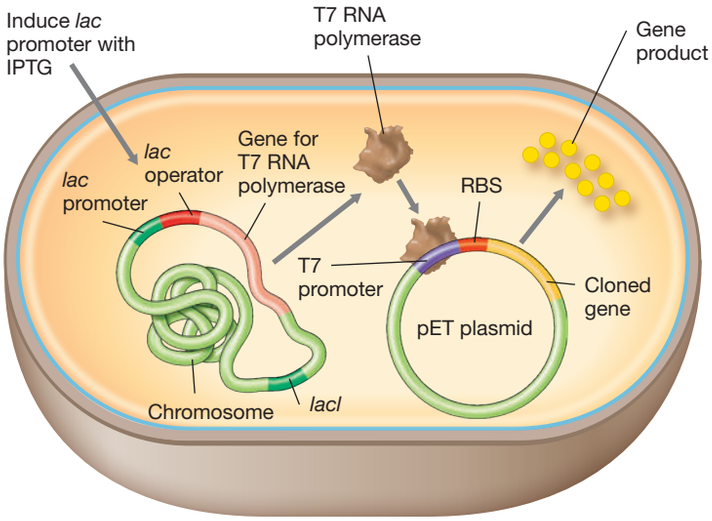


Figure 12.13 The T7 expression system. The gene for T7 RNA polymerase is in a gene fusion under control of the *lac* promoter and is inserted into the chromosome of a special host strain of *Escherichia coli* (BL21). Addition of IPTG induces the *lac* promoter, causing expression of T7 RNA polymerase. This transcribes the cloned gene, which is under control of the T7 promoter and is carried by the pET plasmid. RBS, ribosome-binding site.

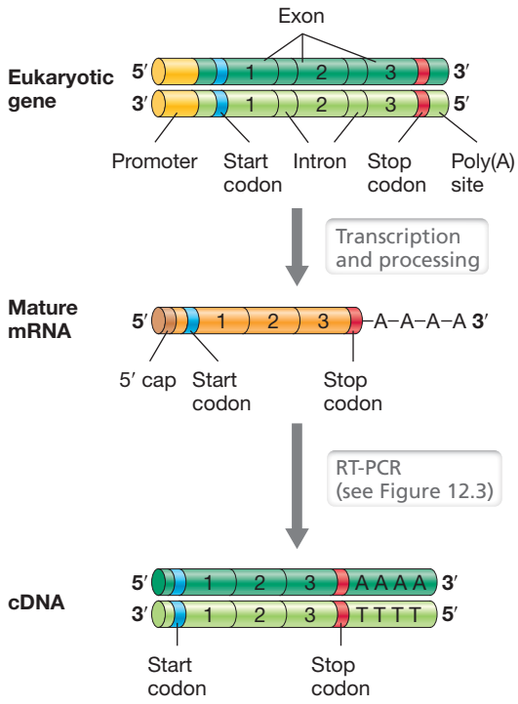


Figure 12.14 Complementary DNA (cDNA). Steps illustrating the synthesis of an intron-lacking cDNA corresponding to a eukaryotic gene generated by reverse transcription PCR (RT-PCR; see Figure 12.3).

accomplished by having a ribosome-binding site (RBS, ◀ Section 6.9) and a nearby start codon on the mRNA. Bacterial RBSs are not found in eukaryotic genes and must be engineered into the vector if high levels of expression of the eukaryotic gene are to be obtained.

Other adjustments to a cloned gene may be necessary to ensure high-efficiency translation. For example, *codon usage* can be an obstacle. Codon usage is related to the concentration of the appropriate tRNA in the cell (◀ Section 10.2 and Table 10.3). Because of the redundancy of the genetic code, more than one tRNA exists for most amino acids (◀ Section 6.9). Therefore, if a cloned gene has a codon usage pattern distinct from that of its expression host, it will probably be translated inefficiently in that host. Site-directed mutagenesis (Section 12.4) can be used to change selected codons in the gene, making it more amenable to the codon usage pattern of the host.

Cloning the Gene via mRNA or Artificial Synthesis

If a cloned gene contains introns, as eukaryotic genes typically do (◀ Section 6.6), the correct protein product will not be made in a bacterial host unless modifications are made. This can be done via mRNA. In a typical mammalian cell, less than 5% of the total RNA is mRNA. However, eukaryotic mRNA is unique because of the poly(A) tails found at the 3′ end (◀ Section 6.6), and this makes it easy to isolate, even though it is of low abundance. If a cell extract is passed over a chromatographic column containing strands of poly(T) linked to a cellulose support, most of the mRNA separates from other RNAs by sticking to the support by specific pairing of As and Ts. The RNA is then released from the column by a low-salt buffer, which gives a preparation greatly enriched in mRNA.

Once mRNA has been isolated, the genetic information is converted into complementary DNA (cDNA) by RT-PCR as was illustrated in Figure 12.3. This double-stranded cDNA contains the coding sequence but lacks introns (**Figure 12.14**), and thus it can be inserted into a plasmid or other vector for cloning in bacteria. However, because the cDNA contains only coding sequences, it lacks a promoter and other upstream regulatory sequences necessary for expression. Thus expression vectors containing bacterial promoters and ribosome-binding sites (RBS) are used to obtain high-level expression of genes cloned in this way (see Figure 12.15).

For small proteins it is possible to artificially synthesize the entire gene (Section 12.4). Many mammalian proteins such as high-value peptide hormones are made by protease cleavage of large precursor molecules. Thus, in order to produce a short peptide such as insulin in its active form, construction and cloning of an artificial gene that encodes just the final hormone rather than the larger precursor protein from which it was derived may have several advantages. Constructed genes are naturally free of introns and thus the mRNA does not need processing. Also, promoters and other regulatory sequences can be inserted into the gene upstream of the coding sequences, and codon bias (◀ Sections 6.9 and 10.2) can be adjusted to best suit the expression host.

Protein Stability and Purification

The synthesis of a protein in a new host may spawn additional problems. For example, some proteins are susceptible to degradation by protease enzymes and others may be toxic to their host. Also, when proteins are massively overproduced, they sometimes aggregate into insoluble inclusions. Although inclusions are relatively easy to purify, the protein they contain is often difficult to solubilize and may be partially denatured. Protein purification can be simplified if the target protein is made as a *fusion protein* along with a carrier protein encoded by the vector. To do this, the

two genes are fused to yield a single coding sequence. A short segment that is recognized and cleaved by a commercially available protease is included between them. After transcription and translation, a single protein is made that is purified by methods designed for the carrier protein. The fusion protein is then cleaved by the protease to release the target protein from the carrier protein. Fusion proteins simplify purification of the target protein because a carrier protein is chosen that will not form inclusions and is easy to purify.

Several vectors are available to generate fusion proteins, and **Figure 12.15** shows an example of a fusion vector that is also an expression vector. In this example, the carrier protein is the *E. coli* maltose-binding protein (encoded by *malE*, Figure 12.15), a protein that is easily purified by methods based on its high affinity for maltose. Once purified, the two portions of the fusion protein are separated by protease or chemical treatment. One other advantage of making a fusion protein is that the carrier protein can be chosen to contain the bacterial *signal sequence*, a peptide rich in hydrophobic amino acids that enables transport of the protein across the cytoplasmic membrane (◀ Section 6.12). This makes possible a bacterial expression system that not only *makes* and *secretes* mammalian proteins, but also allows for the heterologously expressed protein to be separated from all of the other proteins secreted by the cell using binding resins specific for the maltose-binding protein. Thus, carrier proteins can be used to save time, money, and effort in obtaining a desired product.

In the next two sections we will see how some of the DNA manipulations we have already considered can be used to generate mutants of interest and how some genes can be exploited as signals of gene expression.

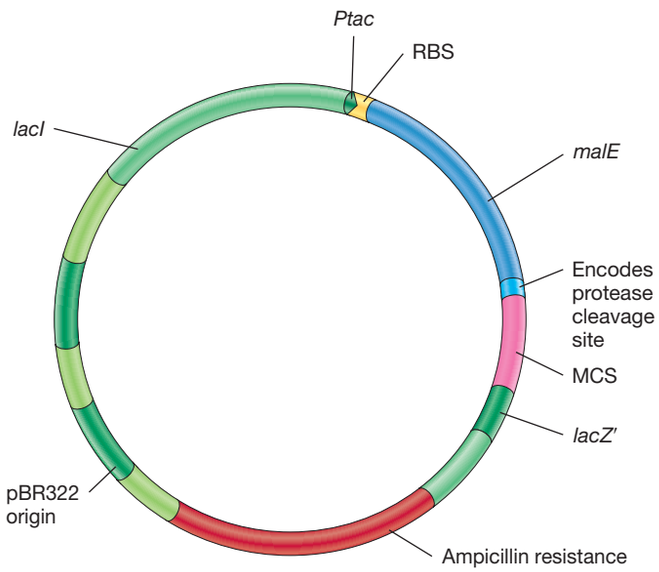


Figure 12.15 An expression vector for gene fusions. The gene to be cloned is inserted into the multiple cloning site (MCS) to be in frame with the *malE* gene, which encodes maltose-binding protein. The insertion inactivates the gene for the alpha fragment of *lacZ*, which encodes β-galactosidase. The fused gene is under control of the hybrid *tac* promoter (*Ptac*) and an *Escherichia coli* ribosome-binding site (RBS). The plasmid also contains the *lacI* gene, which encodes the *lac* repressor. Therefore, an inducer must be added to turn on the *tac* promoter. The plasmid contains a gene conferring ampicillin resistance on its host.

Check Your Understanding

- How can the bacteriophage T7 promoter be used to control expression of a eukaryotic gene in *Escherichia coli*?
- What major advantage does cloning mammalian genes from mRNA or using synthetic genes have over PCR amplification and cloning of the native gene?
- How is a fusion protein made?

12.4 Molecular Methods for Mutagenesis

Conventional mutagens introduce mutations *at random* in the DNA of the intact organism (◀ Section 9.4). In contrast, **site-directed mutagenesis** uses synthetic DNA plus DNA cloning techniques to introduce mutations into genes *at precisely determined sites*. In addition to changing one or just a few bases, mutations may also be engineered by inserting large segments of DNA at precisely determined locations.

Site-Directed Mutagenesis

Site-directed mutagenesis is now a fairly straightforward process due to the ease in obtaining 12- to 40-base DNA oligonucleotides of precise sequence through chemical synthesis; primers or probes for use in the polymerase chain reaction (PCR) and hybridization are obtained in the same manner (Section 12.1). In fact, DNA oligonucleotides over 100 bases long can be obtained. Thus, PCR can be used to obtain a gene with a specific mutation depending on the position of the desired change. If the target gene is part of the *Escherichia coli* chromosome or has already been cloned into a vector, a PCR product (or even an oligonucleotide) containing the desired mutation can be exchanged through recombineering using an appropriate strain of *E. coli* (Figure 12.9 and **Figure 12.16*a***). This scheme allows any base pair in a specific gene to be changed. Progeny bacteria are then screened through PCR and subsequent Sanger sequencing of the product to detect those carrying the mutation (◀ Figure 10.4). When the mutated gene is expressed, a protein with an altered amino acid sequence will be produced. Site-directed mutagenesis can thus be used to manipulate proteins to test the functional importance of specific amino acids at specific sites in the protein.

Site-directed mutagenesis has many applications. The technique has been widely used by enzymologists to change a specific amino acid in the active site of an enzyme to see how the modified enzyme compares with the wild-type enzyme. In such experiments, the vector encoding the mutant enzyme is inserted into a mutant host strain unable to make the original enzyme. Consequently, the activity measured is due to the mutant version of the enzyme alone. Using in vitro mutagenesis, enzymologists can link virtually any aspect of an enzyme's activity—catalysis, resistance, susceptibility to chemical or physical agents, interactions with other proteins—to specific amino acids in the enzyme. In genetic engineering, site-directed mutagenesis has been used to improve the properties of specific proteins, and we discuss some examples in Section 12.6.

Cassette Mutagenesis and Gene Disruption

To make more than a few base-pair changes or replace entire sections of a gene of interest, synthetic fragments called **DNA cassettes** (or cartridges) can be used to mutate DNA in a process known as

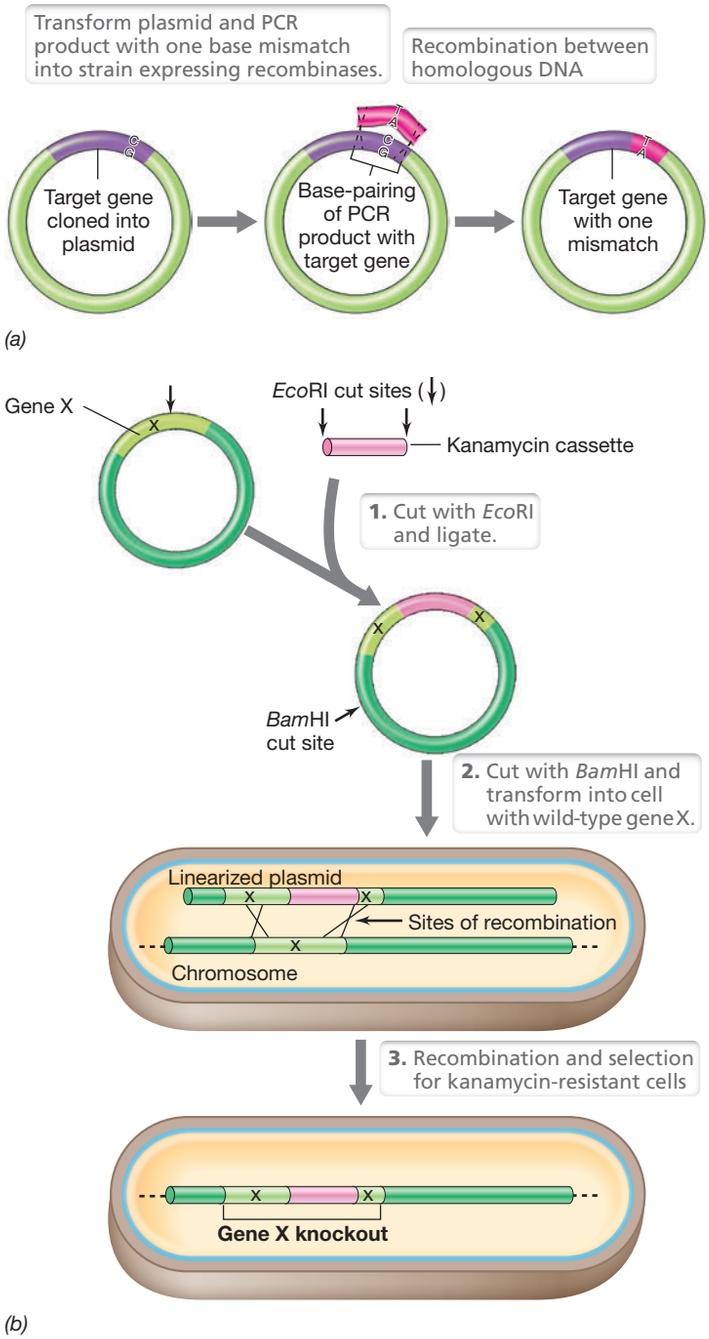


Figure 12.16 Site-directed mutagenesis and gene disruption by cassette mutagenesis. *(a)* Site-directed mutagenesis. A short stretch of DNA containing a single base change is inserted into a target gene such that the target gene product is altered in a known way at a known position in the polypeptide. *(b)* Gene disruption by a cassette insertion. (1) A cloned wild-type copy of gene X, carried on a plasmid, and a kanamycin cassette are cut with *Eco*RI, mixed, and ligated. (2) The resulting plasmid contains the kanamycin cassette as an insertion mutation within gene X. This new plasmid is cut with *Bam*HI and transformed into a cell. (3) The transformed cell contains the linearized plasmid with a disrupted gene X and its own chromosome with a wild-type copy of the gene. In some cells, homologous recombination occurs between the wild-type and mutant forms of gene X. Cells that can grow in the presence of kanamycin have only a single, disrupted copy of gene X. Site-directed mutagenesis is widely used to create "knockout" mutants to identify essential genes (◀ Section 10.4).

cassette mutagenesis. These cassettes can be synthesized using the polymerase chain reaction (PCR) or by direct DNA synthesis, the cost of which is now a minor laboratory expense. Once the appropriate cassette has been obtained, it can then replace sections of the DNA of interest using restriction sites or recombineering (Section 12.2) to facilitate the manipulations. Cassettes used to replace sections of genes are typically the same size as the wild-type DNA fragments they replace.

Another type of cassette mutagenesis is called **gene disruption**. In this technique, cassettes are inserted into a gene, thus disrupting the coding sequence (Figure 12.16*b*). Cassettes used for making insertion mutations can be almost any size and can even carry an entire gene. To facilitate selection, cassettes that encode antibiotic resistance are commonly used. For example, a DNA cassette containing a gene conferring kanamycin resistance is inserted at a restriction site in a cloned gene. The vector carrying the disrupted gene is then converted from a circular to a linear form by cutting it with a different restriction enzyme. Finally, the linear DNA is transformed into the host and kanamycin resistance is selected. The linear plasmid cannot replicate, and so resistant cells arise mostly by homologous recombination (◀ Section 9.5) between the mutated gene on the plasmid and the wild-type gene on the chromosome (Figure 12.16*b*). Because the gene disruption cassette is designed to contain regions of homology with the target gene that are longer than 50 base pairs, special recombinase systems used for recombineering are not needed (Section 12.2).

When a cassette is inserted, the cells not only gain antibiotic resistance but also *lose the function of the gene* into which the cassette is inserted. Such mutations are called *knockout mutations* and are widely used in biology. Knockouts are similar to insertion mutations made by transposons (◀ Section 9.11), but here the experimenter chooses which gene will be mutated. Knockout mutations in haploid organisms yield viable cells only if the disrupted gene is nonessential. Thus, gene knockouts are commonly used for determining whether a gene of interest is essential (◀ Section 10.4).

Check Your Understanding

- How can site-directed mutagenesis be useful to enzymologists?
- What is used to alter more than a few base pairs in a gene of interest?
- What are knockout mutations?

12.5 Reporter Genes and Gene Fusions

DNA manipulation has revolutionized the study of gene regulation, and *gene fusions* have been a major tool for studying regulatory events. We briefly mentioned gene fusions in our discussion of the utility of fusion proteins for stabilizing and purifying cloned gene products in Section 12.3. In a reporter gene fusion, a coding sequence from one source (the *reporter*) is fused to a regulatory region from another source to form a hybrid gene. Regulation of gene expression is then studied by assaying for the product of the reporter as a function of different conditions sensed by the regulator.

Reporter Genes

The key property of a **reporter gene** is that it encodes a protein that is easy to detect and assay. Reporter genes are used for a variety of purposes. They may be used to report the presence or absence of a particular genetic element (such as a plasmid) or DNA inserted within a vector. They can also be fused to other genes or to the promoter of other genes so that gene expression can be studied (◀ Figure 8.1).

The first widely used reporter gene was *lacZ* from *Escherichia coli*, a gene that encodes the enzyme *β-galactosidase*, required for lactose catabolism (◀ Section 7.3). Cells expressing β-galactosidase can be detected easily by the color of their colonies on indicator plates that contain the artificial substrate X-gal (Section 12.2); X-gal is cleaved by β-galactosidase to yield a blue color (see Figure 12.10).

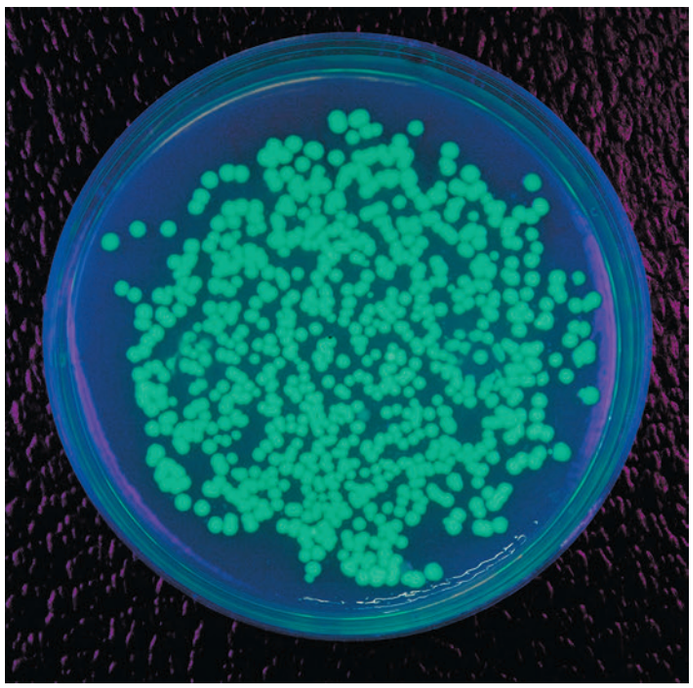
The **green fluorescent protein (GFP)** is widely used as a reporter (**Figure 12.17**). Although the gene encoding GFP was originally cloned from the jellyfish *Aequorea victoria*, GFP can be expressed in both prokaryotic and eukaryotic cells (Figure 12.17) because it is stable and causes little or no disruption of host cell metabolism. If expression of a cloned gene is linked to the expression of GFP, the latter signals (reports) that the cloned gene has also been expressed (Figure 12.17). Since the discovery of the GFP, many similar but differently colored fluorescent proteins have been developed as reporters (◀ Section 8.1).

Gene Fusions

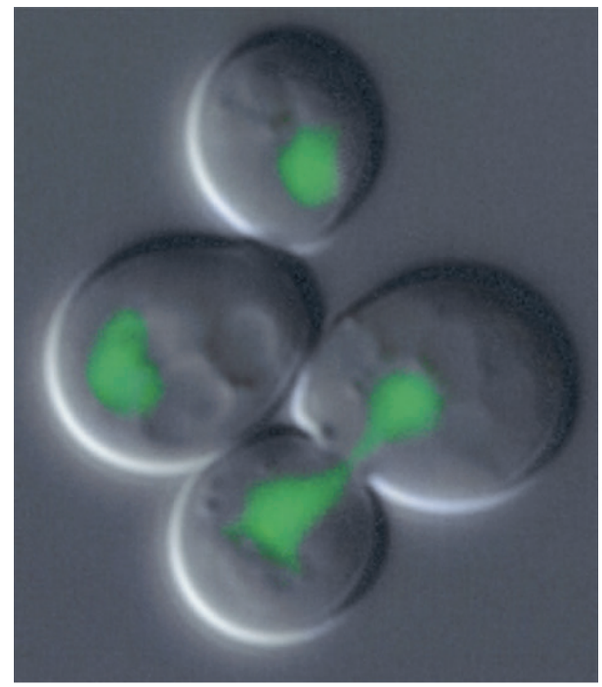
Gene fusions are genetic constructs that consist of segments from two different genes. If the promoter that controls a coding sequence is removed, the coding sequence can be fused to a different regulator to place the gene under the control of a different promoter. Alternatively, the promoter region can be fused to a gene whose product is easy to assay. There are two different types of gene fusions. In **operon fusions**, a coding sequence that retains its own translational start site and signals is fused to the transcriptional signals of another gene. In **protein fusions**, genes that encode two different proteins are fused together so that they share the same transcriptional and translational start and stop signals. Following translation, protein fusions yield a single hybrid polypeptide (Section 12.3).

Gene fusions are often used in studying gene regulation, especially if measuring the levels of the natural gene product is difficult, expensive, or time consuming. The regulatory region of the gene of interest is fused to the coding sequence for a reporter gene, such as that for β-galactosidase or GFP. The reporter is then made under the conditions that would trigger expression of the target gene (**Figure 12.18**). The expression of the reporter is assayed under a variety of conditions to determine how the gene of interest is regulated (Chapter 7). *Transcriptional control* is assayed by fusing the transcriptional start signals of the gene of interest to a reporter gene, whereas *translational control* is assayed by fusing translational start signals of a gene of interest to a reporter gene under the control of a known promoter.

Gene fusions may also be used to test for the effects of regulatory genes. Mutations that affect regulatory genes are introduced into cells carrying gene fusions, and expression is measured and compared to cells lacking the regulatory mutations. This allows the rapid screening of multiple regulatory genes that are suspected of controlling the target gene. Besides the use of fusions to monitor for the



(a)



(b)

Jason A. Kahana and Pamela A. Silver

Figure 12.17 Green fluorescent protein (GFP). GFP can be used as a general tag or as a specific tag for protein localization. *(a)* Using prokaryotic cells, a plate shows uniformly green bacterial colonies because several genes were fused with the gene encoding GFP. *(b)* Using a eukaryotic example, the gene encoding the DNA-binding protein Pho2 from the yeast *Saccharomyces cerevisiae* was fused to the gene encoding GFP. The recombinant gene was transformed into budding yeast cells that expressed the fluorescent fusion protein only in the nucleus. Budding cell division (▶ Section 18.10 and Figures 18.27 and 18.28) is in progress in the two cells on the lower right.

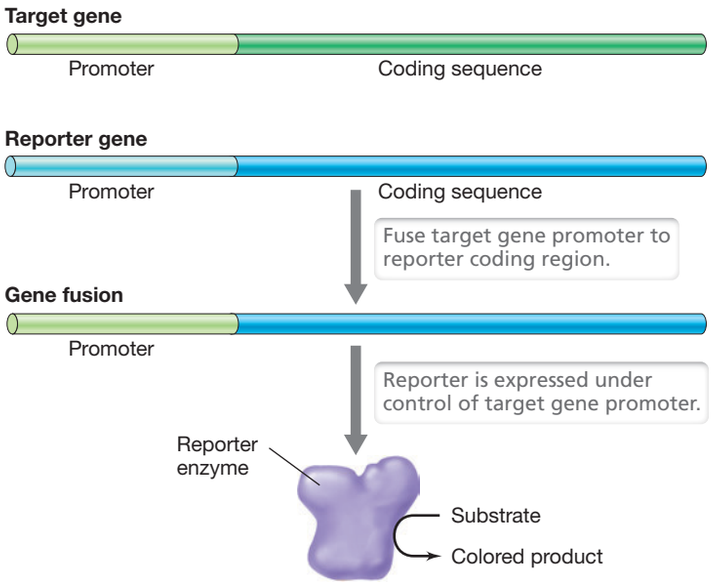


Figure 12.18 Construction and use of gene fusions. The promoter of the target gene is fused to the reporter coding sequence. Consequently, the reporter gene is expressed under those conditions where the target gene would normally be expressed. The reporter shown here is an enzyme (such as β-galactosidase) that converts a substrate to a colored product that is easy to detect. This approach greatly facilitates the investigation of regulatory mechanisms.

presence or expression of a gene, fusions can also be used to join proteins that are easily purified to proteins of interest to aid in purification of the latter (Section 12.3).

We now move on from considering how genes can be manipulated to the application of gene technologies for the synthesis of valuable commercial products by genetically engineered organisms.

Check Your Understanding

- What is a reporter gene? The product of which reporter gene yields a green color?
- Why are gene fusions useful in studying gene regulation?

II • Making Products from Genetically Engineered Microbes: Biotechnology

Microbes can be altered by the genetic engineer to manufacture products such as therapeutic human proteins, vaccines, and biofuels, and genetic tools obtained from microbes can be used to create genetically novel transgenic plants and animals.

Genetic engineering can transform microorganisms into tiny factories for the production of valuable products including fuels, chemicals, drugs, and human hormones, such as insulin. This is the science of **biotechnology**. Up to this point we have only considered the techniques used for manipulating, cloning, and expressing DNA. We now consider how these techniques are applied in biotechnology to produce valuable proteins and genetically modified plants, animals, vaccines, and metabolic pathways.

12.6 Somatotropin and Other Mammalian Proteins

One of the most economically profitable areas of biotechnology has been the production of human proteins. Many mammalian proteins have high pharmaceutical value but are typically present in very low amounts in normal tissue, and it is therefore extremely costly to purify them. Even if the protein can be produced in cell culture, this is much more expensive and difficult than growing microbial cultures that produce the protein in high yield. Therefore, the biotechnology industry has developed genetically engineered microorganisms to produce many different mammalian proteins.

Somatotropin

Although insulin was the first human protein to be produced by bacteria, the genetic engineering required was complicated because insulin consists of two short polypeptides held together by disulfide bonds. A more straightforward example is *human somatotropin* (growth hormone), which consists of a single polypeptide encoded by a single gene; a deficiency of somatotropin in the body results in hereditary dwarfism. Because the human somatotropin gene has been successfully cloned and expressed in bacteria, children showing stunted growth can be treated with *recombinant human somatotropin* to correct this. However, some forms of dwarfism are caused by a lack of the somatotropin receptor, and in such cases, administration of somatotropin has no effect.

The human somatotropin gene was cloned as complementary DNA (cDNA) from mRNA as described in Section 12.3 (see Figure 12.19). The cDNA was then expressed in a bacterial expression vector. The main problem with producing relatively short polypeptide hormones such as somatotropin is their susceptibility to protease digestion, but this problem was overcome by using bacterial host strains lacking key protease enzymes. Today recombinant human growth hormone taken by injection is marketed under several brand names in the United States and has successfully treated thousands of children afflicted with any of several different syndromes that result in short stature. Recombinant somatotropin has also been used to treat some cases of tissue atrophy in adults. However, use in adults is not a common practice, and growth hormone is banned by the International Olympic Committee and by some professional sports leagues for its alleged performance-enhancing capabilities.

Recombinant bovine somatotropin (rBST) is used in the dairy industry (**Figure 12.19**). Injection of rBST into cows does not make them grow larger but instead stimulates milk production. This is because somatotropin has two binding sites; one is the somatotropin receptor that stimulates growth while the other is the prolactin receptor that promotes milk production. Thus, cows treated with rBST produce more milk. However, when human somatotropin is used to treat short stature conditions, it is desirable to avoid any side effects from the hormone's prolactin activity. To alleviate this problem, site-directed mutagenesis (Section 12.4) of the human

Mastering Microbiology
Art Activity: Figure 12.19 Cloning and expression of bovine somatotropin

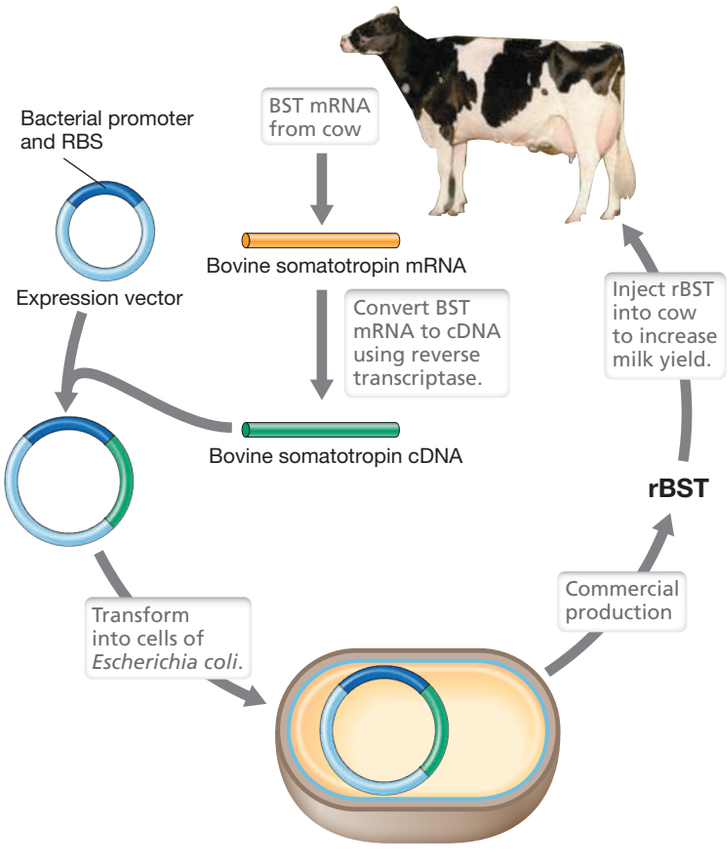


Figure 12.19 Cloning and expression of bovine somatotropin. The mRNA for bovine somatotropin (BST) is obtained from a cow, and the mRNA is converted to cDNA by reverse transcriptase. The cDNA version of the somatotropin gene is then cloned into a bacterial expression vector that has a bacterial promoter and ribosome-binding site (RBS). The construct is transformed into cells of *Escherichia coli*, and recombinant bovine somatotropin (rBST) is produced. Milk production increases in cows treated with rBST.

somatotropin gene was used to alter those amino acids of somatotropin that bind to the prolactin receptor, thus ensuring that the hormone would only target growth. As this example shows, it is possible not only to make genuine human hormones but also to alter their specificity and activity to make them better pharmaceuticals.

Other Mammalian Proteins

Many other mammalian proteins are produced today by genetic engineering (Table 12.1). These include, in particular, an assortment of hormones and proteins for blood clotting and other blood processes. For example, *tissue plasminogen activator* (TPA) is a protein that dissolves blood clots in the bloodstream that may form in the final stages of the healing process. TPA is primarily used in heart patients or others suffering from poor circulation to prevent the development of clots that can be life-threatening. Heart disease is a leading cause of death in many developed countries, especially in the United States, so microbially produced TPA is in high demand.

In contrast to TPA, the blood clotting factors VII, VIII, and IX are critically important for the *formation* of blood clots. Hemophiliacs suffer from a deficiency of one or more clotting factors and can therefore be treated with microbially produced clotting factors. In the past hemophiliacs have been treated with clotting factor extracts from pooled human blood, some of which was contaminated with viruses such as HIV and hepatitis C, putting hemophiliacs at high risk for contracting AIDS, hepatitis, or liver cancer. Recombinant clotting factors have eliminated this problem.

Some mammalian proteins made by genetic engineering are enzymes rather than hormones (Table 12.1). For instance, *human DNase I* is used to treat the buildup of DNA-containing mucus in the lungs of patients with cystic fibrosis. The mucus forms because cystic fibrosis is often accompanied by life-threatening lung infections by the bacterium *Pseudomonas aeruginosa*. The bacterial cells form biofilms (◀ Section 8.10 and ▶ Section 20.4) within the lungs that make drug treatment difficult. DNA is released when the bacteria lyse, and this fuels mucus formation, making it difficult to breathe. DNase digests the DNA and greatly decreases the viscosity of the mucus.

TABLE 12.1 A few human medical products made by genetic engineering

Product	Function
Blood proteins	
Erythropoietin	Treats certain types of anemia
Factors VII, VIII, IX	Promotes blood clotting
Tissue plasminogen activator (TPA)	Dissolves blood clots
Urokinase	Promotes blood clotting
Human hormones	
Epidermal growth factor	Wound healing
Follicle-stimulating hormone	Treatment of reproductive disorders
Insulin	Treatment of diabetes
Nerve growth factor	Treatment of degenerative neurological disorders and stroke
Relaxin	Facilitates childbirth
Somatotropin (growth hormone)	Treatment of some growth abnormalities
Immune modulators	
α-Interferon	Antiviral, antitumor agent
β-Interferon	Treatment of multiple sclerosis
Colony-stimulating factor	Treatment of infections and cancer
Interleukin-2	Treatment of certain cancers
Lysozyme	Anti-inflammatory
Tumor necrosis factor	Antitumor agent, potential treatment of arthritis
Replacement enzymes	
β-Glucocerebrosidase	Treatment of Gaucher disease, an inherited neurological disease
Therapeutic enzymes	
Human DNase I	Treatment of cystic fibrosis
Alginate lyase	Treatment of cystic fibrosis

Check Your Understanding

- What is the advantage of using genetic engineering to make insulin?
- What are the major problems when manufacturing proteins in bacteria?
- Explain how an enzyme can be useful in treating a bacterial infection, such as that which occurs with cystic fibrosis.

12.7 Transgenic Organisms in Agriculture and Aquaculture

Genetic improvement of plants and animals by traditional selection and breeding has a long history, but recombinant DNA technology has led to revolutionary changes. Although the genetic engineering of higher organisms is not truly microbiology, much of the DNA manipulation leading up to the genetically engineered plant or animal is carried out using bacteria and the techniques we have learned thus far. Hence, we consider the genetic manipulation of plants and animals here with a focus on the microbiology that supported it.

Because genetically engineered plants or animals contain a gene from another organism—called a *transgene*—they are **transgenic organisms**. The public knows these as **genetically modified organisms (GMOs)**. Strictly speaking, the term *genetically modified* refers to genetically engineered organisms whether or not they contain foreign DNA. In this section we discuss how foreign genes are inserted into plant and fish genomes and how transgenic organisms may be used.

The Ti Plasmid and Transgenic Plants

While recombinant DNA can be transformed into plant cells by electroporation or transfection, the **Ti plasmid** from the gram-negative bacterium *Agrobacterium tumefaciens*, a plant pathogen, can be used to transfer DNA directly into the cells of certain plants. This plasmid is responsible for *A. tumefaciens* virulence and encodes genes that mobilize DNA for transfer to the plant, which as a result contracts crown gall disease (▶ Section 23.6). The segment of the Ti plasmid DNA that is actually transferred to the plant is called **T-DNA**. The sequences at the ends of the T-DNA are essential for transfer, and the foreign DNA to be transferred must reside between these ends.

One common Ti-vector system that has been used for the transfer of genes to plants is a two-plasmid system called a *binary vector*, which consists of a cloning vector plus a helper plasmid. The cloning vector is a shuttle vector (Section 12.2) that contains the two ends of the T-DNA flanking a multiple cloning site, two origins of replication so that it can replicate in both *Escherichia coli* (the host for cloning) and *A. tumefaciens*, and two antibiotic resistance markers, one for selection in plants and the other for selection in bacteria. The foreign DNA is inserted into the vector, which is transformed into *E. coli* and then moved to *A. tumefaciens* by conjugation (**Figure 12.20**).

This cloning vector lacks the genes needed to transfer T-DNA to a plant. However, when placed in an *A. tumefaciens* cell that contains a suitable helper plasmid, the T-DNA can be transferred to a plant. The "disarmed" helper plasmid, called *D-Ti*, contains the virulence (*vir*) region of the Ti plasmid but lacks the T-DNA. It also lacks the genes that initiate disease but supplies all the functions needed to transfer the T-DNA from the cloning vector. The cloned DNA and the kanamycin resistance marker of the vector are mobilized by D-Ti and transferred into a plant cell where they enter the nucleus (Figure 12.20*d*). Following integration into a plant chromosome, the foreign DNA can be expressed and confer new properties on the plant.

A number of transgenic plants have been produced using the Ti plasmid of *A. tumefaciens*. The Ti system works well with broadleaf plants (dicots), including crops such as tomato, potato, tobacco, soybean, alfalfa, and cotton. It has also been used to produce transgenic trees, such as walnut and apple. The Ti system does not work with plants from the grass family (monocots, including the important crop plant corn), but other methods of introducing DNA, such as transfection by microprojectile bombardment with a particle gun (**Figure 12.21**), have been used successfully for them.

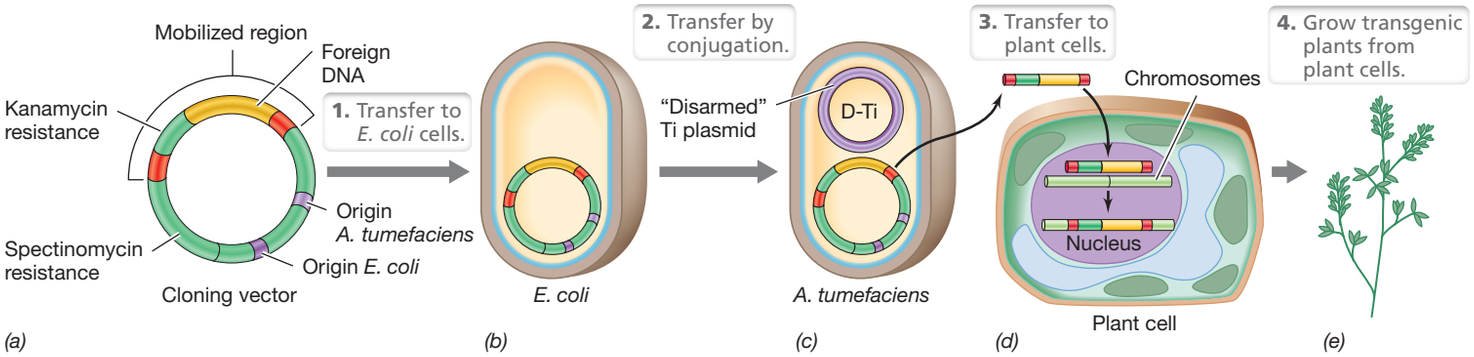


Figure 12.20 Production of transgenic plants using a binary vector system in *Agrobacterium tumefaciens*. *(a)* Plant cloning vector containing ends of T-DNA (red), foreign DNA, origins of replication, and resistance markers. *(b)* The vector is transformed into cells of *Escherichia coli* for cloning and then *(c)* transferred to *A. tumefaciens* by conjugation. The resident Ti plasmid (D-Ti) has been genetically engineered to remove key pathogenesis genes. *(d)* D-Ti can still mobilize the T-DNA region of the vector for transfer to plant cells grown in tissue culture. *(e)* From the recombinant plant cell, a whole plant can be grown. (Details of Ti plasmid transfer from bacterium to plant are shown in Figure 23.26.)

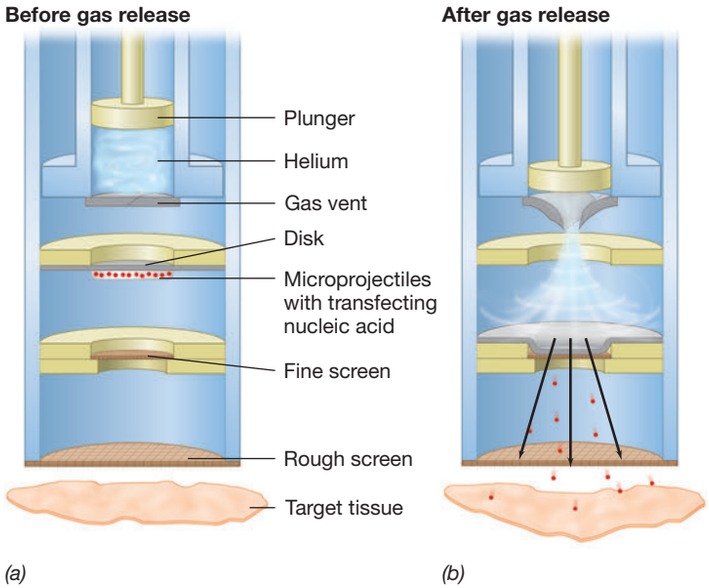


Figure 12.21 DNA gun for transfection of eukaryotic cells. The inner workings of the gun show how metal pellets coated with nucleic acids (microprojectiles) are propelled at target cells. *(a)* Before firing and *(b)* after firing. A shock wave due to gas release propels the disk carrying the microprojectiles against the fine screen. The microprojectiles continue on into the target tissue.

Herbicide- and Insect-Resistant Plants

Major areas targeted for genetic improvement in plants include herbicide, insect, and microbial disease resistance, as well as improved product quality. The main genetically modified (GM) crops today are soybeans, corn, cotton, and canola. Almost all the GM soybeans and canola planted in the United States are herbicide resistant, whereas the corn and cotton are herbicide resistant or insect resistant, or both.

Herbicide resistance is genetically engineered into a crop plant to protect it from herbicides applied to kill weeds. Many herbicides inhibit a key plant enzyme or protein necessary for growth. For example, the herbicide *glyphosate* (Roundup™, made by Monsanto) kills plants by inhibiting an enzyme necessary for making aromatic amino acids. Some bacteria contain an equivalent enzyme and are also killed by glyphosate. However, mutant bacteria were selected that were resistant to glyphosate and contained a resistant form of the enzyme. The gene encoding this resistant enzyme from *A. tumefaciens* was cloned, modified for expression in plants, and transferred (Figure 12.20) into important crop plants, such as soybeans. When sprayed with glyphosate, plants containing the bacterial gene are not killed (**Figure 12.22**). Thus glyphosate can be used to kill weeds that compete for water and nutrients with the growing crop plants. Herbicide-resistant soybeans are now widely planted in the United States, including plants resistant to both glyphosate and a second widely used herbicide, dicamba.

Transgenic plants resistant to damage by certain insects have also been produced by genetic engineering (**Figure 12.23**). One widely used approach is based on introducing genes encoding the toxic proteins of the gram-positive, endospore-forming bacterium *Bacillus thuringiensis* into plants. As it sporulates, *B. thuringiensis* produces a crystalline protein called *Bt toxin* (▶ Section 16.8) that is toxic to

Figure 12.22 Transgenic plants: herbicide resistance. The photograph shows a portion of a field of soybeans that has been treated with Roundup™, a glyphosate-based herbicide manufactured by Monsanto Company (St. Louis, Missouri, USA). The remnants of plants on the right are normal soybeans; the plants on the left have been genetically engineered to be glyphosate resistant.

moth and butterfly larvae. Many variants of Bt toxin exist that are specific for different insects. Certain strains of *B. thuringiensis* produce additional proteins toxic to beetle and fly larvae and mosquitoes.

The Bt transgene is normally inserted directly into the plant genome. For example, a natural Bt toxin gene was cloned into a plasmid vector under control of a chloroplast ribosomal RNA promoter and then transfected into tobacco plant chloroplasts by microprojectile bombardment (Figure 12.21). This yielded transgenic plants that expressed Bt toxin at levels that were extremely toxic to larvae of several insect species (Figure 12.23). Binding Bt triggers a change in the toxin's conformation that disrupts the insect digestive system, causing death. Bt toxin is harmless to mammals (including humans) because any toxin ingested is destroyed in the stomach and the specific Bt receptors in the insect intestine are absent from the intestines of other groups of organisms.

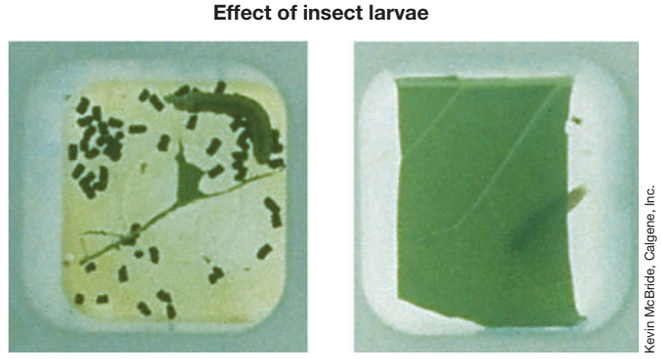


Figure 12.23 Transgenic plants: insect resistance. The results of an assay to determine the effect of beet armyworm larvae on tobacco leaves. *(a)* Leaf from a wild-type plant. *(b)* Leaf from a transgenic plant expresses Bt toxin in its chloroplasts and the toxin kills the larvae.

Despite the toxicity and availability of numerous Bt variants, insect resistance to the toxin is emerging. This is especially problematic with corn rootworms. To combat resistance issues, plant engineers are looking at other soil microbes for the production of different types of insecticidal proteins. This has resulted in the discovery of a small insecticidal peptide from the gram-negative soil-dwelling *Pseudomonas chlororaphis*. Transfer of the gene encoding the peptide into corn using an *Agrobacterium*-mediated system (Figure 12.20) resulted in protection from the rootworm. While the mechanism by which the *P. chlororaphis* peptide kills rootworms is not completely understood, the peptide kills Bt-resistant rootworms but does not affect other common insects such as bees and other pollinators. Thus, a new weapon is now available to specifically combat corn rootworm and may help to retard the development of resistance in this potentially devastating crop pest.

Transgenic Fish

Many foreign genes have been incorporated and expressed in laboratory research animals and in commercially important animals. The genetic engineering uses microinjection to deliver cloned genes to fertilized eggs; genetic recombination then incorporates the foreign DNA into the genomes of the eggs. More recently, farm animals and fish have been genetically modified to improve yields.

An interesting practical example of a transgenic animal is the *AquAdvantage* salmon developed by AquaBounty Technologies (**Figure 12.24**). These transgenic salmon do not grow to be larger than normal salmon but simply reach market size much faster—18 months versus 3 years. The gene for growth hormone in native salmon is activated by light. Consequently, salmon grow rapidly only during the summer months. In the genetically engineered salmon, the promoter for the growth hormone gene was replaced with the promoter from another fish that grows at a more or less constant rate all year round. The result was salmon that make growth hormone continuously and thus grow faster. Such transgenic salmon can be grown commercially in aquaculture operations and harvested more quickly than with non-GMO farm-raised salmon.

In 1995 AquaBounty applied to the U.S. Food and Drug Administration (FDA) for approval to distribute the fast-growing salmon. After two decades of debate regarding the potential risks of consuming genetically modified fish, approval for its distribution occurred in 2015. However, in response to remaining concerns regarding the sale of the modified salmon, the United States enacted a law at the end of 2015 that prohibited its sale until labeling could be agreed upon. However, in early 2019, the FDA removed the final hurdle to the sale of GMO salmon in the United States, and it can now be sold (marketed as AquaAdvantage® salmon and containing a label indicating that it is a bioenginered product).

Figure 12.24 Fast-growing transgenic salmon. The AquAdvantage™ salmon (top) was engineered by AquaBounty Technologies (Maynard, Massachusetts, USA). The transgenic and control fish are both 18 months old but weigh 4.5 kg and 1.2 kg, respectively.

Check Your Understanding

- What is a transgenic plant?
- Give an example of a genetically modified plant and describe how its modification benefits agriculture.
- How have transgenic salmon been engineered to reach market size faster?

12.8 Engineered Vaccines and Therapeutic Agents

Genetic engineering is used to manufacture certain vaccines and medical therapeutic agents. Of interest to human health and biotechnology are the two types of relationships microbes form within the human body. While some microbes form beneficial relationships within humans by aiding digestion, producing nutrients, and "educating" the immune system (▶ Section 24.2), other microbes cause disease through their ability to infiltrate cells and release virulence factors such as toxins and destructive enzymes. With this in mind, scientists have explored the use of both types of microbial communities for making novel vaccines and for delivering drugs or toxic agents to specific cell types. We consider both of these potential medical miracles here.

Recombinant Vaccines, Vaccinia Virus, and Subunit Vaccines

Vaccines are substances that elicit immunity to a disease when injected into an animal (▶ Section 28.3). Genetic engineering can modify a pathogen by deleting genes from its genome that encode virulence factors (▶ Section 25.5) while retaining those whose products elicit an immune response. This yields a recombinant and infective vaccine that is considered **attenuated** because it is less virulent than the original strain (▶ Section 25.3 and Figure 25.10). Conversely, one can add genes from a pathogenic virus to the genome of a relatively harmless virus, called a *carrier virus*. Such vaccines are called **vector vaccines** and induce immunity to the pathogenic virus. Indeed, one can even combine the two approaches by disarming one pathogen and adding back to it immunity-inducing genes from a second pathogen. This yields a **polyvalent vaccine**, a vaccine that immunizes against two different diseases at the same time.

Vaccinia virus (◀ Section 11.6) is widely used to prepare recombinant vaccines for human use; however, cloning into vaccinia requires a selective marker, which is provided by the gene encoding the enzyme thymidine kinase. Vaccinia virus contains a gene encoding thymidine kinase, an enzyme that converts thymidine into thymidine triphosphate. However, this enzyme also converts the base analog 5-bromodeoxyuridine (BrdU) into a nucleotide that is incorporated into DNA, causing a lethal reaction. Thus, cells that express either host- or virus-encoded thymidine kinase are killed when treated with BrdU.

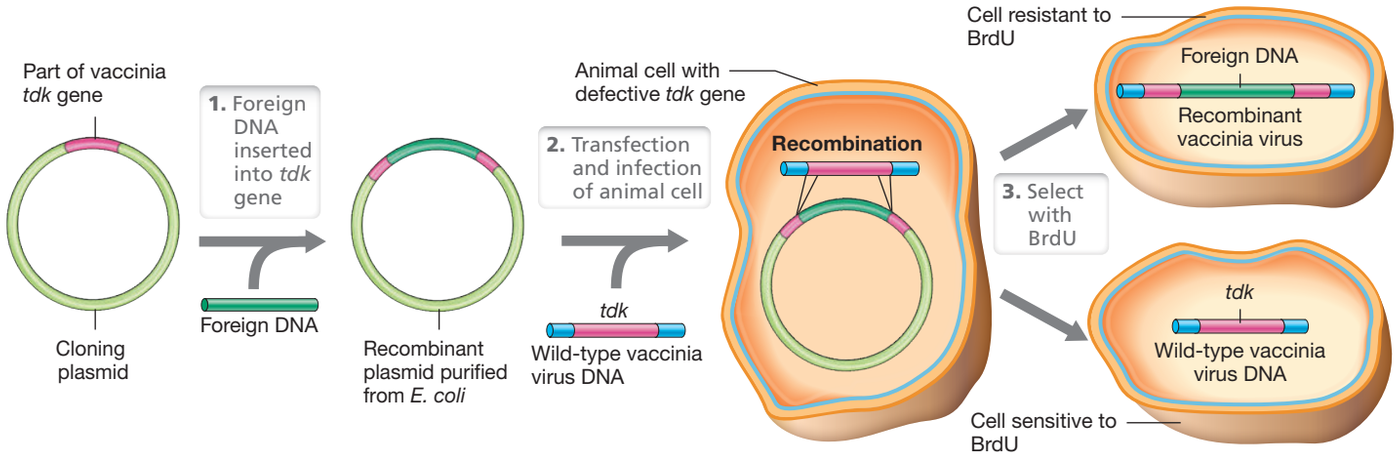


Figure 12.25 Production of recombinant vaccinia virus. Foreign DNA is inserted into a short segment of the thymidine kinase gene (*tdk*) from vaccinia virus carried on a plasmid. Following replication of this plasmid in *Escherichia coli*, both the recombinant plasmid and wild-type vaccinia virus are put into the same animal host cell to promote recombination. The animal cells are treated with 5-bromodeoxyuridine (BrdU), which kills only cells with an active thymidine kinase. Only recombinant vaccinia viruses whose *tdk* gene is inactivated by insertion of foreign DNA survive.

Genes to be put into the vaccinia virus genome are first inserted into an *Escherichia coli* plasmid that contains part of the vaccinia thymidine kinase (*tdk*) gene (**Figure 12.25**). The foreign DNA is inserted into the *tdk* gene, which is therefore disrupted. This recombinant plasmid is then transformed into animal cells whose own *tdk* genes have been inactivated. These cells are also infected with wild-type vaccinia virus. The two versions of the *tdk* gene—one on the plasmid and the other on the virus—then recombine. Some viruses gain a disrupted *tdk* gene plus its foreign insert (Figure 12.25). Cells infected by wild-type vaccinia virus (with an active thymidine kinase) are killed by BrdU. By contrast, cells infected by *recombinant* vaccinia virus (with a disrupted *tdk* gene) grow long enough to yield a new generation of virus particles (Figure 12.25). This protocol thus selects for viruses whose *tdk* gene contains a cloned insert of foreign DNA. Vaccinia viruses can also be engineered to carry genes from multiple viruses, forming polyvalent vaccines. Currently, several vaccinia vector vaccines have been developed and licensed for veterinary use, including one for rabies, while many other vaccinia vaccines are at the clinical trial stage.

Subunit vaccines, vaccines that contain only a specific protein or two from a pathogen, are also produced by recombinant means. For a pathogenic virus, the gene encoding its coat or capsid protein is often the best vaccine candidate because capsid proteins are typically highly immunogenic (◀ Section 5.1 and ▶ Section 28.3). Subunit vaccines are popular because large amounts of immunogenic proteins are produced that can be administered at high dosage without the risk that exists with attenuated or killed-cell vaccines that may inadvertently contain viable pathogen cells or viruses. However, some subunit vaccines, such as that prepared against a surface protein of human hepatitis B (▶ Section 31.11), require that the immunogenic proteins be glycosylated by the host before they are immunologically active. To solve this problem, the subunit hepatitis B vaccine was produced in a eukaryotic host (yeast), which generated the glycosylated and immunologically active form of the vaccine.

Commensal *Bacteria* and Therapeutic Delivery

While new therapeutic agents to treat different diseases are constantly being developed, *delivery* to the target tissues or cells can be problematic. This is especially true for drugs that are rapidly degraded in the bloodstream or by the acidity of the stomach. To alleviate these problems, members of the microbiome that form positive relationships in the body—such as the gut normal flora—have been exploited to produce and release drugs while inside a host (**Figure 12.26**). Because of their commensal lifestyle, these microbes do not illicit an immune response and are tolerated well by the body. For therapeutic delivery of *anticancer* drugs, members of the microbiome that flourish in anaerobic niches such as the intestinal tract and in tumors are of particular interest. Tumors become anaerobic because of rapid cell proliferation, which results in the tumor rapidly outgrowing its blood supply. Dying tumor cells also release nutrients that attract members of the microbiome. Through genetic engineering, some of these bacteria that are naturally tolerated by the body and colonize tumors have been harnessed to not only deliver therapeutic substances specifically to diseased or cancerous cells (Figure 12.26*a*), but also to release tumor-specific antigens within circulating immune cells to facilitate the immune system's targeting of tumors (see below and Figure 12.26*b*).

An exciting example of the targeted delivery of a *beneficial* drug is the use of engineered commensal bacteria to convert intestinal cells into glucose-responsive insulin-secreting cells. In type 1 (juvenile) diabetes mellitus, the immune system destroys insulin-producing beta cells in the pancreas (▶ Section 28.1). Studies on how to generate new beta cells are under way; meanwhile, different studies have focused on ways to convert other cell types into insulin producers. From these studies, a therapeutic molecule known as the full-length glucagon-like peptide 1 (GLP1) has been shown in a rat diabetes model to be able to reprogram intestinal epithelial cells to respond to glucose and produce insulin. However, GLP1 has a short half-life in the bloodstream and the target epithelial cells are

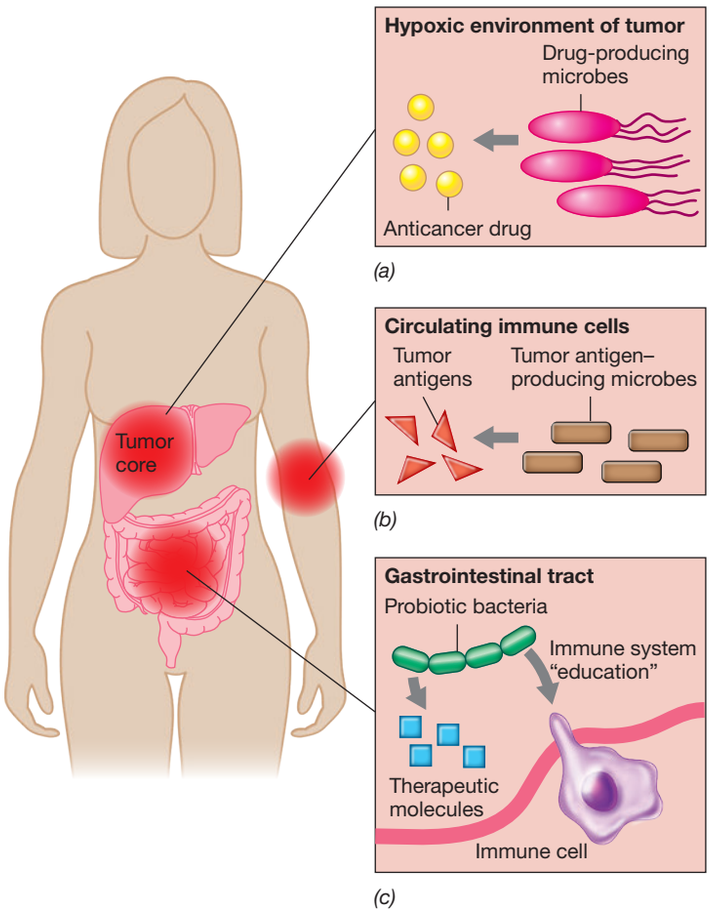


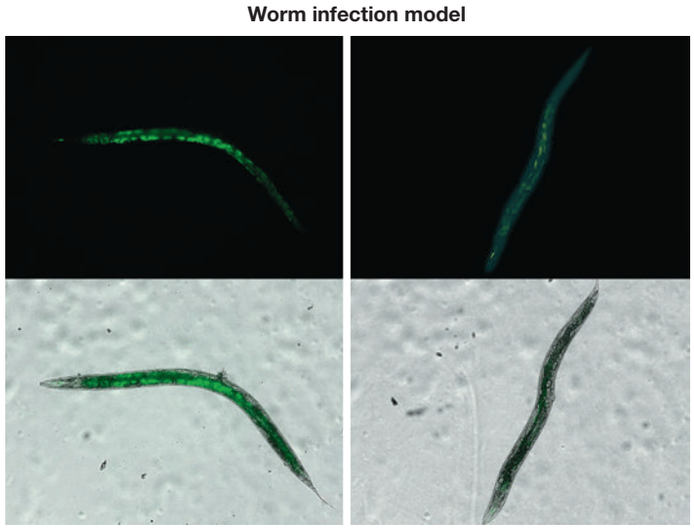
Figure 12.26 Strategies for bacterial delivery of therapeutic agents. *(a)* Pathogens that grow in the low-oxygen environment of a tumor can be attenuated and genetically modified to release drugs directly inside tumors. *(b)* Pathogens that elicit a strong immune response can be attenuated and engineered to release antigens normally found on tumor cells. This antigen release stimulates antibody production and immune system destruction of tumors. *(c)* Probiotic strains normally found in the healthy gut can be engineered to release therapeutic molecules to treat various diseases as well as help train the immune system to tolerate beneficial microbes.

naturally shed from the intestine over the course of days. Thus, to deliver GLP1 to intestinal epithelial cells on a continuous basis, genetic engineers modified a strain of *Lactobacillus*, a gram-positive bacterium that can propagate in the intestine, to constitutively secrete GLP1 in the gut (Figure 12.26*c*). While this work has only been done in an experimental animal, it offers promise for the future of treating diabetes in humans.

Probiotics are live commensal microorganisms administered to a human to benefit health (▶ Section 24.12), and engineered probiotics have been used to sense and kill biofilm-forming strains of *Pseudomonas aeruginosa*. The ability to sense *P. aeruginosa* is based on its production of the quorum-sensing inducer molecule *N*-acyl homoserine lactone (AHL; ◀ Section 7.7). A probiotic strain of *Escherichia coli* has been modified not only to sense *P. aeruginosa*, but also to release a cocktail of therapeutic substances in response to AHL levels. These include a dispersin molecule to destroy *P. aeruginosa* biofilms, as well as bacteriocin (◀ Section 6.2) and lysis proteins to specifically kill *P. aeruginosa* cells. **Figure 12.27** illustrates the success of this engineered probiotic in a worm experimental model infected with *P. aeruginosa* producing green fluorescent protein (GFP, Section 12.5 and Figure 12.17). While *P. aeruginosa* can successfully colonize the host worm in the presence of a wild-type strain of *E. coli* (Figure 12.27*a*), the pathogen cannot colonize the host worm in the presence of an engineered strain of *E. coli* releasing therapeutic molecules in response to *P. aeruginosa* AHL levels (Figure 12.27*b*). A major goal of such research is to develop new tools to fight the human genetic disease cystic fibrosis, a disease in which *P. aeruginosa* biofilms in the lungs are a life-threatening complication.

Pathogens as Engineered Anticancer Therapies

While many cancers are treatable with radiation and chemotherapy, how to specifically target the immune system, drugs, or radiation to tumor cells has been a long-standing problem, and biotechnology may have a solution. *Listeria monocytogenes* is a pathogenic bacterium that causes listeriosis, a serious foodborne illness (▶ Section 33.13 and Figures 33.14 and 33.15). *L. monocytogenes* grows within human cells, which allows wild-type strains to evade the immune system. By contrast, attenuated strains of *L. monocytogenes* can be attacked by the immune system. These traits have been harnessed to engineer recombinant *L. monocytogenes* strains that express antigens found on tumor cells. The presence of these tumor-associated antigens triggers the immune system to produce tumor-specific antibodies



(a) ***Pseudomonas aeruginosa* + *Escherichia coli*** *(b)* ***Pseudomonas aeruginosa* + engineered *Escherichia coli***

Figure 12.27 Probiotic *Escherichia coli* engineered to kill *Pseudomonas*. Infection of the worm *Caenorhabditis elegans* with biofilm-forming *P. aeruginosa* expressing the green fluorescent protein (GFP) and treatment with an engineered strain of *E. coli*. *(a)* Active *P. aeruginosa* infection (visualized by GFP production) in the presence of a probiotic (attenuated) strain of *E. coli* (top, fluorescent micrograph; bottom, phase-contrast micrograph). *(b)* Limited *P. aeruginosa* survival occurs in response to the addition of the engineered version of the *E. coli* probiotic strain. The engineered strain was genetically modified to release therapeutic molecules that disrupt biofilms and kill *P. aeruginosa* cells when a quorum-sensing molecule produced by *Pseudomonas* is detected. Image adapted from Hwang, I.Y., Koh, E., Wong, A., March, J.C., Bentley, W.E., Lee, Y.S., and Chang, M.W. 2017. *Nat. Commun. 8*: 15028.

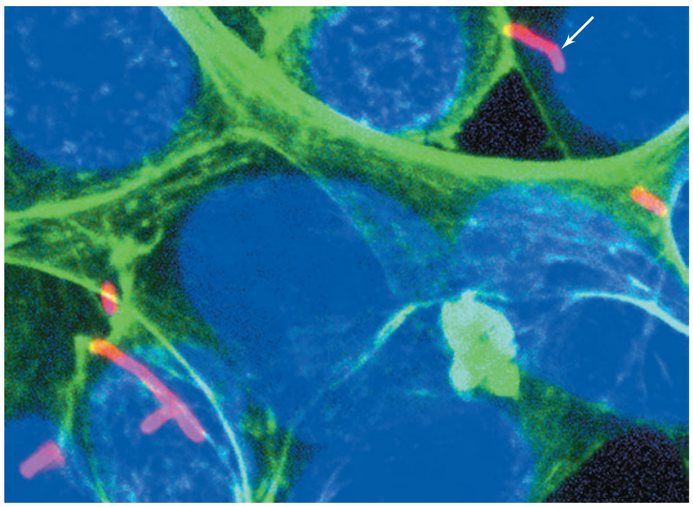


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Figure 12.28 Therapeutic *Listeria*. *L. monocytogenes* cells (pink) linked to the radionuclide ^{188}rhenium enter and multiply inside mouse pancreatic tumor cells (blue, cells; green, extracellular matrix) that have spread from the primary tumor. Radiation from the ^{188}rhenium slowly kills the tumor cells.

Mastering Microbiology
Art Activity: Figure 12.26 Engineered anthrax toxin

(Figure 12.26*b*), and these ultimately lead to destruction of not only the bacterium but also the tumor. An example of such a tumor-targeting bacterial vaccine is an engineered strain of *L. monocytogenes* that expresses antigens specific to tumors caused by the human papillomavirus (HPV, ▶ Section 31.14).

While attenuated *L. monocytogenes* strains in healthy cells are cleared by the immune system, clearance does not occur in tumor cells. This observation hinted that weakened strains of *L. monocytogenes* might also be turned into anticancer vehicles to deliver toxic drugs or radioisotopes specifically to tumor cells. This was accomplished by coupling the radionuclide ^{188}rhenium to a recombinant strain of *L. monocytogenes*. In experiments with mice, this therapeutic strain infected and multiplied in pancreatic tumor cells (**Figure 12.28**) without harming normal pancreatic cells. In Section 12.11 we will pick up on this theme again and describe how synthetic biology has been used not only to attenuate a pathogen to persist in tumor cells, but also to contain a synthetic biological circuit for the cyclical delivery of an antitumor drug to minimize drug toxicity to the rest of the body (see Figure 12.39).

Specific Delivery of Antibodies as an Anticancer Therapy

Another mechanism for treating cancer is by using antibodies, proteins produced by the immune system to attack foreign substances (▶ Section 27.3). It has been found that the binding of antibodies to specific targets inside cancer cells can trigger the host's immune system to kill the cancer cell. However, antibodies do not freely enter cells and thus a transport mechanism has been genetically engineered using a toxin produced by *Bacillus anthracis*, the bacterium that causes anthrax (▶ Sections 30.9 and 32.8) (**Figure 12.29**). Anthrax toxin contains three components: edema factor, lethal factor, and protective antigen; the latter is essential for carrying the toxic edema and lethal factors into the cell.

Using genetic engineering, scientists have modified the *B. anthracis* protective antigen to carry a *synthetic anticancer antibody* instead of the toxic edema and lethal factors. Injection of the modified and now harmless toxin results in the protective antigen recognizing and binding to a receptor on the outside of the cancer cell (Figure 12.29). The protective antigen:antibody complex is then taken up into the cancer cell through the formation of an endosome. Once the antibody is released into the cytoplasm, it specifically binds a protein essential to tumor viability. This binding then triggers the cell's immune system, which recognizes the antibody:cellular protein complex as foreign and kills the cell (Figure 12.29). Any antigen:antibody complex incorporated by normal cells is harmless because both the toxic portions of the toxin and the specific cancer proteins targeted by the antibody are absent from normal cells.

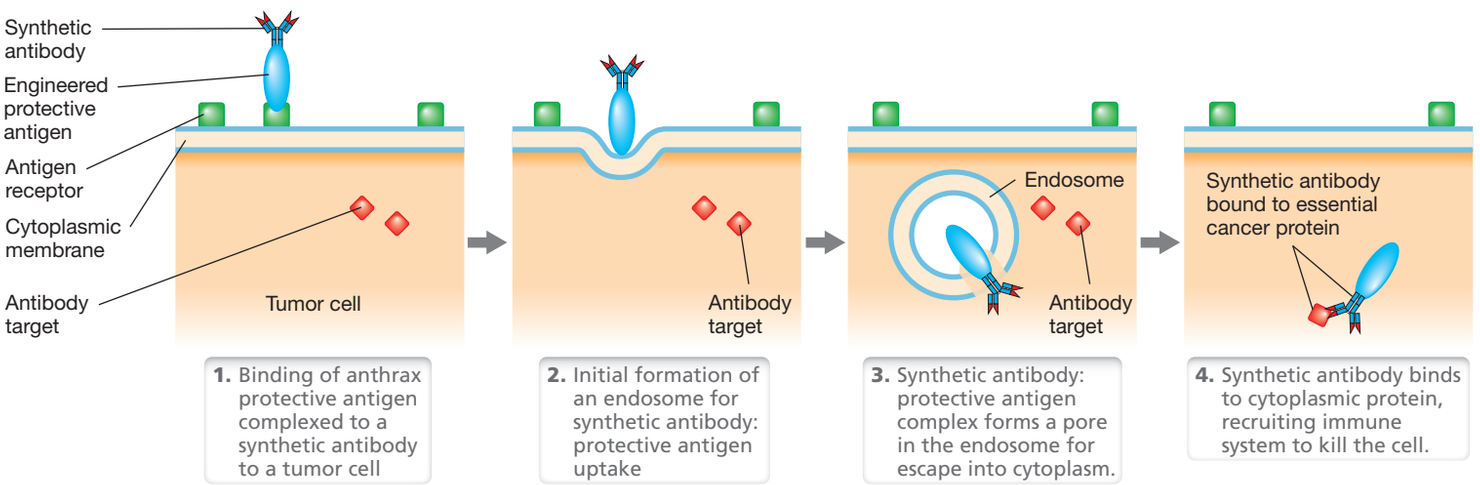


Figure 12.29 Engineered anthrax toxin. The protective antigen component of the anthrax toxin is engineered to carry a synthetic antibody. This engineered protective antigen specifically binds to a cell receptor on target cancer cells. After binding to the receptor, the engineered complex is taken up into the cell through an endosome. Following release into the cytoplasm, the synthetic antibody binds to an essential cellular protein, triggering the cancer cell's death through the host's immune response. (Anthrax toxin is discussed in more detail in Sections 30.9 and 32.8.)

Stimulating the immune system to fight cancer may well turn out to be a mechanism in a whole new line of anticancer therapies. The novel system devised here—combining antitumor antibodies with a delivery system crafted from a highly toxic bacterium—shows both the power and the promise of genetic engineering to accelerate the war on cancer. The immunological principles behind this and other important new anticancer therapies will be developed in Chapter 28.

We now transition from medical applications of genetic engineering to consider commercial applications in the final two sections of Part II of the chapter.

Check Your Understanding

- What are the important differences among a recombinant live attenuated vaccine, a vector vaccine, and a subunit vaccine?
- How can beneficial members of the microbiome be used as therapeutic agents?
- What feature of some pathogenic bacteria makes them attractive for use as engineered cancer treatments?

12.9 Mining Genomes and Engineering Pathways

Complex environments, such as fertile soil, contain vast numbers of uncultured microbes and their genes that are ripe for harvesting by genetic engineering. In addition, microbial metabolic pathways can be altered, embellished, or otherwise modified to change their characteristics and improve their efficiency. Here we explore how genetic engineering can both mine environmental genomes and alter metabolic pathways.

Environmental Gene Mining

Just as the total gene content of an organism is its *genome*, the collective genomes of an environment are its *metagenome* (◀ Section 10.7 and ▶ Section 19.8). *Gene mining* is the process of identifying and isolating potentially useful genes from the environment without the need to culture the organisms that contained them. In gene mining, DNA (or RNA) isolated directly from environmental samples is cloned into suitable vectors to construct a *metagenomic library* (**Figure 12.30**). If RNA is isolated, it must first be converted to cDNA by reverse transcriptase (Figure 12.3).

Screening of environmental metagenomic libraries has identified novel genes encoding enzymes that can degrade various pollutants and enzymes that make novel antibiotics. Retrieval of gene clusters encoding entire metabolic pathways—such as for antibiotic synthesis—requires vectors such as **bacterial artificial chromosomes (BACs)** (we discussed the general principles of artificial chromosomes in Section 12.2). BACs are similar to plasmids except that they can carry large inserts of DNA. BACs are especially useful for screening samples from rich environments, such as soil, where vast numbers of unknown genomes are present and correspondingly large numbers of genes are available to screen (Figure 12.30).

Several lipases, chitinases, esterases, and other degradative enzymes with novel substrate ranges and other properties have been isolated by this approach, and such enzymes have many industrial applications. Enzymes with improved resistance to industrial production conditions, such as high temperature, high or low pH, and oxidizing conditions, are especially valuable and desirable. Metagenomics can also target products with a particular combination of properties, such as a heat-stable lipase. Lipases hydrolyze fats, but their industrial production and use often require that they remain active at high temperatures. To isolate a thermostable lipase, a metagenomic library was prepared from a hot spring sample and the DNA was transformed into cells of *Escherichia coli*. Recombinant colonies expressing lipase activity were then selected and analyses indicated that certain of them remained active at 90°C. The gene encoding the heat-stable lipase was then introduced into an expression vector for commercial production of the enzyme.

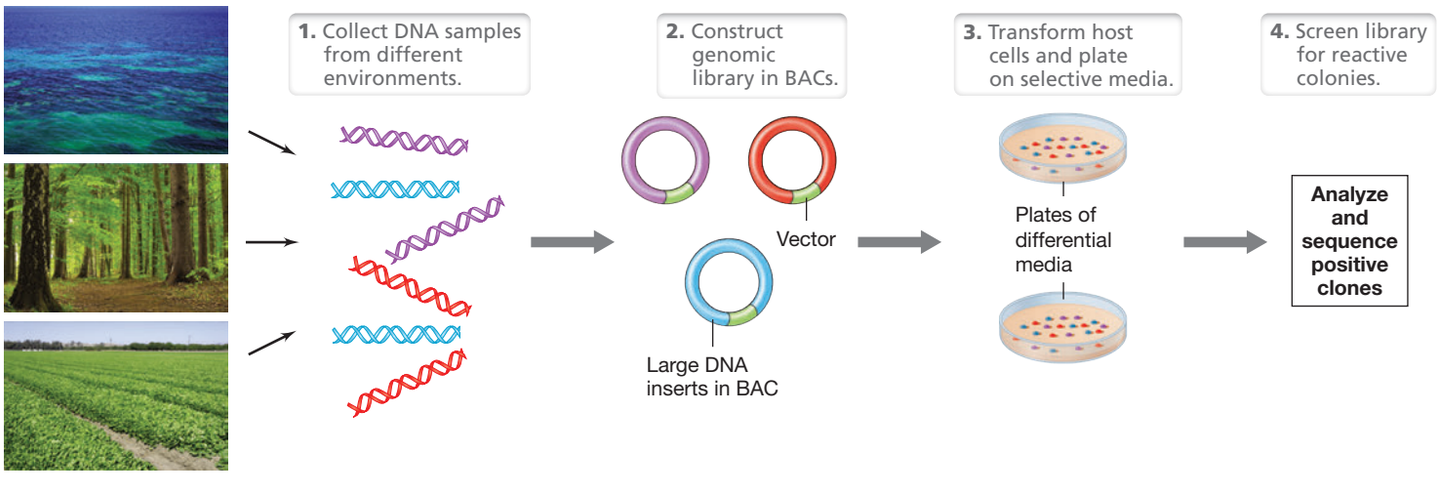


Figure 12.30 Metagenomic search for useful genes in the environment. DNA samples are obtained from different environments, such as seawater, forest soil, and agricultural soil. A metagenomic library is constructed using bacterial artificial chromosomes (BACs) and screened for genes of interest. Possibly useful clones are analyzed further.

Figure 12.31 Application of CinderBio hyperstable enzymes for the cleaning of creamery equipment. These heat-stable enzymes clean industrial food-processing equipment as well as or better than traditional cleaning methods and do not generate large amounts of toxic wastewater.

By metagenomic mining of extreme environments, several useful heat- and acid-stable enzymes have been isolated for cleaning food-processing equipment in the food industry (**Figure 12.31**). To prevent foodborne infections, food-processing equipment must be rigorously cleaned, and cleaning protocols typically employ rigorous acid and base treatments, detergents, and sanitizers, all of which consume large amounts of chemicals and generate large volumes of wastewater that must be treated. By contrast, cleaning equipment with enzymes that function optimally near the boiling point of water in dilute acids (Figure 12.31) requires fewer chemicals and less water and more effectively removes microbial biofilms than standard cleaning practices.

Pathway Engineering: The Indigo Synthesis Example

Pathway engineering is the process of assembling a new or improved biochemical pathway using genes from one or more organisms. Engineered microbes are used to make alcohols, solvents, food additives, dyes, antibiotics, and many other products. They may also be used to degrade agricultural waste, pollutants, herbicides, and other toxic or undesirable materials. Here we discuss improving or modifying *existing pathways*, and later we explore the use of synthetic biology to create *entirely new* pathways (Section 12.11).

An interesting example of pathway engineering is the production of indigo by *E. coli* (**Figure 12.32**). Indigo is an important dye used for treating wool and cotton; blue jeans, for example, are made of cotton dyed with indigo. Although indigo can be synthesized chemically, the heavy demand for indigo by the textile industry has spawned new approaches for its synthesis, including a biotechnological approach using pathway engineering.

Because the structure of indigo is very similar to that of the aromatic hydrocarbon naphthalene, enzymes that oxygenate naphthalene also oxidize indole to its dihydroxy derivative; the latter oxidizes spontaneously in air to yield indigo, a bright blue pigment. Enzymes for oxygenating naphthalene are encoded by several plasmids found in *Pseudomonas* and other soil bacteria. When genes from such plasmids were cloned into *E. coli*, the cells turned blue because they had incorporated the genes encoding the enzyme naphthalene oxygenase.

The indigo pathway consists of four steps, two enzymatic and two spontaneous (Figure 12.32). *E. coli* naturally synthesizes the enzyme tryptophanase that carries out the first of these steps, the conversion of tryptophan to indole. In the engineered *E. coli*, a second step converts indole to the product that converts to indigo spontaneously (Figure 12.32). For indigo production, tryptophan must be supplied to the recombinant *E. coli* cells, and for commercial application, this was accomplished by affixing cells to a solid support in a bioreactor and then continuously trickling a tryptophan solution obtained from waste protein sources over the cells. If the tryptophan solution is recirculated over the cells several times, indigo levels steadily increase until the dye can be harvested.

Although bioproduction of indigo is clearly possible, at present it is difficult to compete with the chemical production of more than 20 kilotons of indigo per year. Indeed, the two greatest challenges of pathway engineering are controlling the pathway and producing the desired compound at the yields necessary to be cost effective.

Check Your Understanding

- Explain why metagenomic cloning gives large numbers of novel genes.
- What types of environments are often sampled to prospect for industrial enzymes and why?
- How was *Escherichia coli* modified to produce indigo?

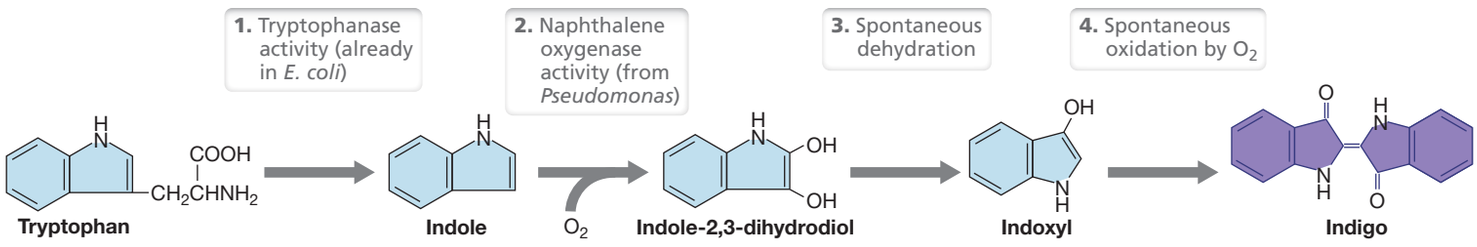


Figure 12.32 Engineered pathway for production of the dye indigo. *Escherichia coli* naturally expresses tryptophanase, which converts tryptophan into indole. Naphthalene oxygenase (originally from *Pseudomonas*) converts indole to dihydroxy-indole, which spontaneously dehydrates to indoxyl. Upon exposure to air, indoxyl dimerizes spontaneously to form indigo.

12.10 Engineering Biofuels

With the global supply of fossil fuels limited and environmental concerns growing about how climate change will affect our planet, renewable energy sources such as biologically produced fuels—*biofuels*—are in demand. The major biofuels in use today are ethanol, biodiesel, hydrogen, and methane. Select microorganisms can produce biofuels; however, the yield from wild-type organisms is often hindered by side reactions, toxic by-products, and missing enzymes for critical steps. For example, hydrogen (H_2) is produced by nitrogen-fixing bacteria through the activity of a nitrogenase enzyme complex (◀ Figure 3.28). However, most of this hydrogen is subsequently consumed by hydrogenase enzymes in wild-type nitrogen fixers. Thus, to enhance the production of biofuels, microorganisms have been genetically modified to optimize production. Here we discuss how genetic engineering has allowed for the use of alternative biofuel feedstocks, how enzyme replacement can yield new biofuels, and how phototrophic microorganisms can be harnessed as biofuel factories.

Bacterial Conversion of Switchgrass to Ethanol

Over 16 billion gallons of ethanol are produced per year in the United States from the fermentation of corn sugar by yeast (**Figure 12.33*a***). However, because corn requires considerable cost and energy inputs to grow and harvest and is a major food source for both humans and domesticated animals, alternative nonedible and low-resource-input plant materials are more desirable biofuel feedstocks. Much attention has been focused on fast-growing grasses such as *switchgrass* (*Panicum virgatum*) (Figure 12.33*b*) as a source of cellulose for the production of ethanol. However, switchgrass cellulose is integrated with other plant polymers such as hemicellulose and lignin and requires high-temperature, chemical, and enzymatic pretreatment to break the polymers down to fermentable sugars.

By tapping into both microbial diversity and genetic engineering, a bacterium has been discovered and genetically modified not only to break down switchgrass cellulose to fermentable sugars but also to ferment this sugar to ethanol. *Caldicellulosiruptor*, a gram-positive anaerobic and thermophilic bacterium, naturally produces cellulase and hemicellulase enzymes that can convert cellulose and hemicellulose to glucose. Unique proteins called *tāpirins* that extrude from the outer layer of the bacterium's peptidoglycan allow the cell to directly bind to raw switchgrass during the conversion process (Figure 12.33*c*). While *Caldicellulosiruptor bescii* grows optimally at 80°C and can ferment sugars, it naturally yields mostly acetate, lactate, and hydrogen as fermentation products. To directly convert switchgrass to ethanol, genetic engineers altered the terminal steps of the *C. bescii* glycolytic pathway (◀ Section 3.6 and Figure 3.11) by replacing genes encoding lactate dehydrogenase and other acidic fermentation products with a bifunctional acetaldehyde/alcohol dehydrogenase from another thermophile, *Clostridium thermocellum*. This shifted 70% of the *C. bescii* fermentation products to ethanol.

Use of thermophilic microorganisms for biofuel production has several advantages such as reduced risk of contamination with mesophiles and improved substrate solubility. It also makes the collection of any volatile products easier. For example, separating small amounts of desired products from large amounts of growth media (such as collecting ethanol during yeast fermentation of corn sugar) requires significant energy inputs to cool the bioreactors for growth of the organism and then later to heat and distill off the ethanol (Figure 12.33*a*). By contrast, since *C. bescii* grows optimally at 80°C—just above the boiling point of ethanol—commercial production of alcohol from cellulose by this genetically engineered bacterium requires little or no cooling and saves energy by the continuous emission of the desired product, ethanol.

Engineered Alkenes and Alkanes

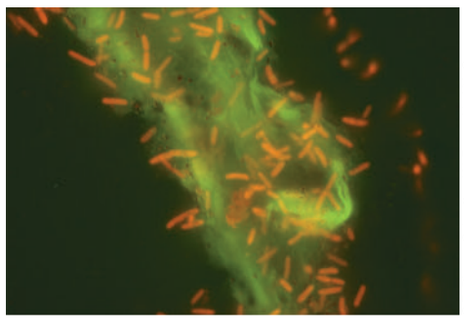
Petroleum contains a mixture of hydrocarbons of varying chain length. Propane (C_3H_8), produced from natural gas processing and petroleum refining, is a widely used heating and cooking fuel and a key fuel for agricultural applications. Using genetic engineering, scientists have modified strains of *Escherichia coli* to convert glucose into propane and some other petroleum hydrocarbons.

Hydrocarbon production in *E. coli* begins with the synthesis of a fatty aldehyde. This was done by heterologously expressing in *E. coli* the *Photorhabdus luminescens luxCED* genes, which encode enzymes that reduce fatty acids to their corresponding aldehydes (**Figure 12.34**). The activity of these fatty acid reductase, synthetase, and transferase enzymes yields a fatty aldehyde that can be converted to hydrocarbons

Chris Standlee and DOE/NREL

(a)

(b)



S.E. Blumer-Schuette, J.V. Zurawski, J.M. Conway, and R.M. Kelly

(c)

Figure 12.33 **Biofuels.** *(a)* A bioethanol plant in Nebraska (USA). In the plant, glucose from cornstarch is fermented by yeast to ethanol plus CO_2. The large tank in the foreground is the ethanol storage tank, and the pipes in the background are for distilling the alcohol from the fermentation broth. *(b)* Switchgrass, a source of cellulose as a feedstock for ethanol production. *(c)* Fluorescence photomicrograph of acridine orange–stained cells of the thermophilic and cellulolytic bacterium *Caldicellulosiruptor kronotskyensis* growing on switchgrass (a single cell measures about 0.6 μm × 3 μm). The green color is from the switchgrass plants.

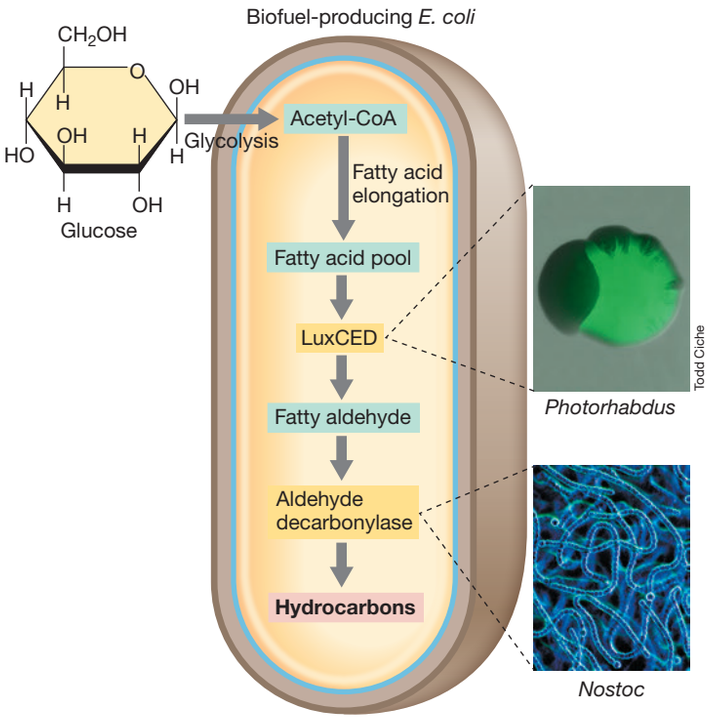


Figure 12.34 Hydrocarbon-producing *Escherichia coli*. *E. coli* naturally produces fatty acids from acetyl-CoA. By engineering a strain to express the LuxC (fatty acid reductase), LuxE (fatty acid synthetase), and LuxD (fatty acid transferase) proteins from the fluorescent bacterium *Photorhabdus luminescens* (inset: fluorescent colony), a fatty aldehyde intermediate is produced. This fatty aldehyde can then be converted to a hydrocarbon if the same strain has also been engineered to express the aldehyde decarbonylase enzyme from the filamentous cyanobacterium *Nostoc punctiforme* (inset photo).

by the enzyme aldehyde decarbonylase. However, *E. coli* lacks this enzyme as well. To overcome this limitation, genetic engineers cloned the aldehyde decarbonylase gene from the cyanobacterium *Nostoc punctiforme* into *E. coli*, allowing the engineered *E. coli* to convert linear fatty acids added to its growth medium into linear hydrocarbons (Figure 12.34).

Because branched-chain hydrocarbons yield higher octane numbers (which is good for gasoline engine performance), scientists expanded this engineered pathway by heterologously expressing enzymes that participate in the first step of the fatty acid elongation cycle (◀ Figure 3.35) from *Bacillus subtilis*. This allowed the engineered *E. coli* to use more branched-chain fatty acids as starting substrates and by doing so generate higher-octane fuels.

Microalgae and Biodiesel

Microalgae are unicellular phototrophic eukaryotes (▶ Section 18.16) that produce an abundance of bioactive compounds including lipids, fatty acids, and carotenoids. These products are made using only sunlight, CO_2, a few minerals, and water. Microalgae of interest to biotechnology include the green algal genera *Chlorella* and *Chlamydomonas*. These organisms produce significant amounts of storage lipids known as *triacylglycerides* (TAG), substances that can be chemically or enzymatically treated to yield *biodiesel*, a fuel for use in the diesel engines present in many trucks and heavy transport vehicles.

Improving TAG synthesis in the microalgal biosynthetic pathway required some genetic engineering breakthroughs, and **Figure 12.35*a*** illustrates the successful design of vectors that allow proteins to be targeted to the nucleus or chloroplast of cells of *Chlamydomonas*; other vectors have been developed that allow the mitochondrion or the endoplasmic reticulum to be targeted. Organelle targeting is facilitated through the fusion of *signal sequences* (◀ Section 6.12) to the protein of interest. A vector for the simultaneous expression of two foreign genes in separate cellular locations has also been generated (Figure 12.35*b*). These targeting vectors are critical for manipulating compartmentalized TAG biosynthetic activities such as control of gene expression by transcription factors (encoded by the nuclear genome), ATP-producing enzymes (encoded by the mitochondrial genome), and enzymes for initial fatty acid synthesis reactions

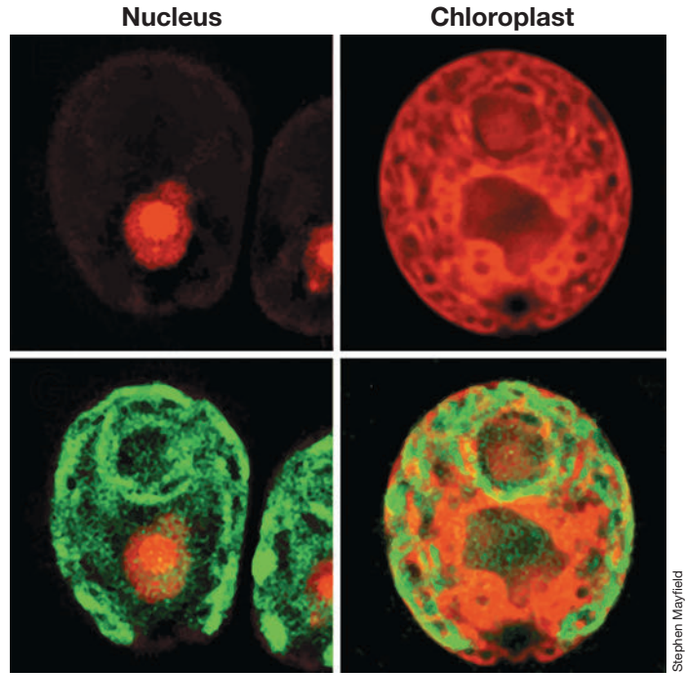


Figure 12.35 Genetic tools for engineering microalgae. *(a)* Fluorescent micrographs showing the expression of reporter genes in targeted regions of cells of *Chlamydomonas*. Top panel: A reporter gene encoding a red fluorescent protein that targets the nucleus (left) and chloroplasts (right). Bottom panel: both images overlaid with a green fluorescent stain for the chloroplast. *(b)* Dual expression of two reporter genes to different cellular locations using a single vector. A gene encoding a blue fluorescent protein is localized to the nucleus, while a gene encoding a red fluorescent protein is targeted to the endoplasmic reticulum in cells of *Chlamydomonas*. Adapted from Rasala, B.A., S-S. Chao, M. Pier, D.J. Barrera, and S.P. Mayfield. 2014 *PLoS ONE 9* (4): e94028.

(encoded by the chloroplast genome). Translation of proteins by ribosomes on the endoplasmic reticulum is also important for protein secretion.

Using microalgal triacylglycerides as a feedstock for biodiesel increases the possibilities for biofuel production, and the fact that the process is driven by the energy of sunlight makes it an environmentally attractive process. However, the major obstacle facing all biofuel production schemes—especially those that require sunlight—is the expense of the equipment and engineering necessary to scale production up to levels necessary to compete with the petroleum industry. Currently, about 90 million barrels of oil a day arrive on the highly volatile world energy market, and until oil supplies diminish to the point where a significant price hike is encountered, any biofuel will have a difficult time competing.

The last part of this chapter considers the "final frontier" for genetic engineering: the ability to synthesize cell parts or even entire cells from scratch in the revolutionary new field of synthetic biology.

Check Your Understanding

- How has *Caldicellulosiruptor* been modified to produce ethanol directly from switchgrass?
- What are the advantages of using thermophiles to produce biofuels?
- What has been the limiting factor in engineering microalgae to produce greater amounts of lipids?

III • Synthetic Biology and Genome Editing

Scientists can stitch together bits and pieces of DNA from various sources to create artificial genes, operons, or entire genomes and can edit these new genetic constructs at will to change the properties of existing organisms or even to fabricate an entirely synthetic organism.

The term *synthetic biology* refers to the use of genetic engineering to create novel biological systems out of available biological parts, often taken from several different organisms. These biological parts (promoters, enhancers, operators, riboswitches, regulatory proteins, enzyme domains, signal receivers, etc.) have been termed *biobricks*. Synthetic biology links biobricks together in various combinations to form modules capable of generating complex behaviors (Figure 12.1). While we will discuss some amazing examples of synthetic biology (including the formation of synthetic cells) in Sections 12.11 and 12.12, college students are also trying their hand at synthetic biology. An undergraduate competition called the *International Genetically Engineered Machine* (iGEM; https://igem.org/) occurs annually worldwide. Teams in this competition have used synthetic biology to engineer products ranging from probiotic bacteria that secrete the anti-inflammatory cytokine interleukin-10 to help treat irritable bowel disease (cytokines and interleukins are discussed in Chapters 26 and 27), to microfluidic systems that "print" (assemble) genetic circuits and transform them into cells of *Escherichia coli*.

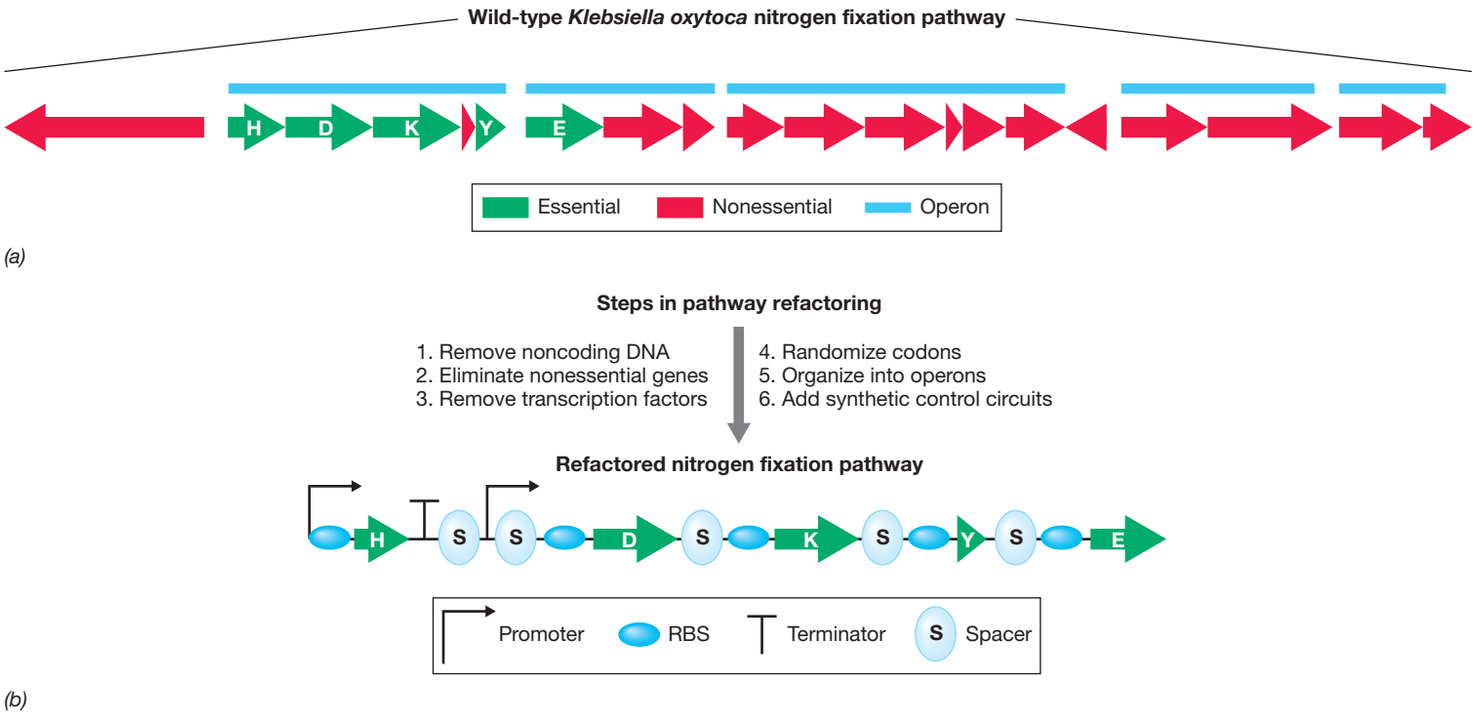


Figure 12.36 Synthetic biology and pathway refactoring. *(a)* Chromosomal operons encoding the nitrogen fixation pathway in *Klebsiella oxytoca*. Direction of arrows indicates direction of transcription and color-coding is as indicated. H, *nifH*; D, *nifD*; K, *nifK*; Y, *nifY*; E, *nifE*. *(b)* Refactored "minimalist" nitrogen fixation pathway. RBS, ribosome-binding site. Data modified from Smanksi, M.J., Zhou, H., Claesen, J., Shen, B., Fischbach, M.A., and Voigt, C.A. 2016. *Nat. Rev. Microbiol. 14,* 135.

Another powerful new and rapidly developing technology allows the precise "editing" of genomes in living cells, a technique that has revolutionized biotechnology. In Section 12.13 we explore how the microbial immune system has been leveraged to edit any genome and the remarkable applications of this genome editing technology.

12.11 Synthetic Metabolic Pathways, Biosensors, and Genetic Circuits

A major focus of synthetic biology thus far has been the construction or modification of metabolic pathways. High-value products are often expensive because purifying them from their original source, typically plants, is costly. The pathways responsible for these products include proteins and RNAs that regulate the synthesis, export, and protection from harmful effects of the product. Thus the DNA encoding pathways for high-value products can range from 1000 to 100,000 base pairs of DNA, which makes it impossible to clone the necessary genes into a model system.

However, by using various enzyme and regulatory biobricks, synthetic biologists can reconstruct or *refactor* natural pathways into artificial ones that allow for easy expression in model systems. This refactoring ultimately leads to the efficient conversion of cheap and abundant substrates into high-value products. **Figure 12.36** illustrates refactoring of the 20-gene pathway encoding nitrogen fixation (◀ Section 3.12) in *Klebsiella oxytoca*; the result is a streamlined and optimized five-gene pathway. While genetically modified organisms (GMOs) are used for refactoring and the production of high-value products, no foreign DNA is present in the products.

Engineering a Major Food Product

Vanillin, one of the most popular flavoring agents in the world, is a secondary metabolite extracted from the seedpods (vanilla beans) of orchids of the genus *Vanilla*. Natural vanillin is expensive because of the slow growth of orchids, their relatively low output, and the high production costs of cultivating and harvesting the beans. By analyzing the natural metabolic pathway for the production of vanillin, genetic engineers have synthesized strains of *Escherichia coli* and yeast that can synthesize the flavoring agent from glucose. Vanillin synthesis in *E. coli* requires five heterologously expressed enzymes, and once the necessary biobricks were incorporated into the bacterium, it produced vanillin identical in structure and taste to naturally produced vanillin. (However, some argue that naturally produced vanilla contains additional compounds extracted from the vanilla beans that contribute to its unique taste.)

Mastering Microbiology
Art Activity: Figure 12.33 Artemisinin synthesis through synthetic biology

"Synbio vanillin," as the *E. coli* product has been called, is commercially available as an inexpensive source of vanillin and has been used primarily for flavoring ice cream and baked goods. As the second most expensive spice in the world (behind saffron), natural vanilla is an especially costly ingredient for bulk use such as in ice cream. Synbio vanillin is a good example of how synthetic biology can be used to convert inexpensive feedstocks (corn sugar) into high-value products.

Synthetic Pharmaceuticals: Artemisinin and Malaria

Pharmaceuticals are often derived from natural products; aspirin, for example, was originally obtained from willow bark. While aspirin is now chemically synthesized, not all pharmaceuticals can be synthesized economically. One example is the antimalarial drug *artemisinin*. Malaria, which is caused by protozoans of the genus *Plasmodium*, is transmitted by mosquitoes and infects nearly 500 million people each year, primarily in tropical and subtropical countries (▶ Section 34.5). While various traditional antimalarials are in use, the parasite has evolved resistance to many of these and thus new antimalarial drugs are constantly needed. Artemisinin is an alternative antimalarial that is produced in limited amounts by the cultivated sweet wormwood plant *Artemisia annua*. To ensure availability of the drug, the *Semi-Synthetic Artemisinin Project* was initiated with the goal of engineering a microorganism for the synthesis of artemisinic acid, used for production of artemisinin (**Figure 12.37**).

A. annua naturally produces artemisinic acid by converting acetyl-CoA to farnesyl diphosphate (FPP; a 15-carbon intermediate) using the mevalonate biosynthetic pathway. FPP is then oxidized to artemisinic acid and dihydroartemisinic acid through an intermediate called *amorphadiene*. Initially, the plan was to have *E. coli* produce artemisinic acid through fermentation. Synthetic biologists made numerous attempts to engineer *E. coli* with the correct biobricks to produce artemisinic acid through permutations of the natural pathway, inhibiting natural competing enzymes, mutating genes for codon optimization (Section 12.3), and modifying fermentation conditions, but no strategy emerged that could yield the amorphadiene intermediate at sufficient levels. However, by transferring the necessary metabolic pathway biobricks to a modified strain of the baker's yeast *Saccharomyces cerevisiae* along with metabolic adjust-

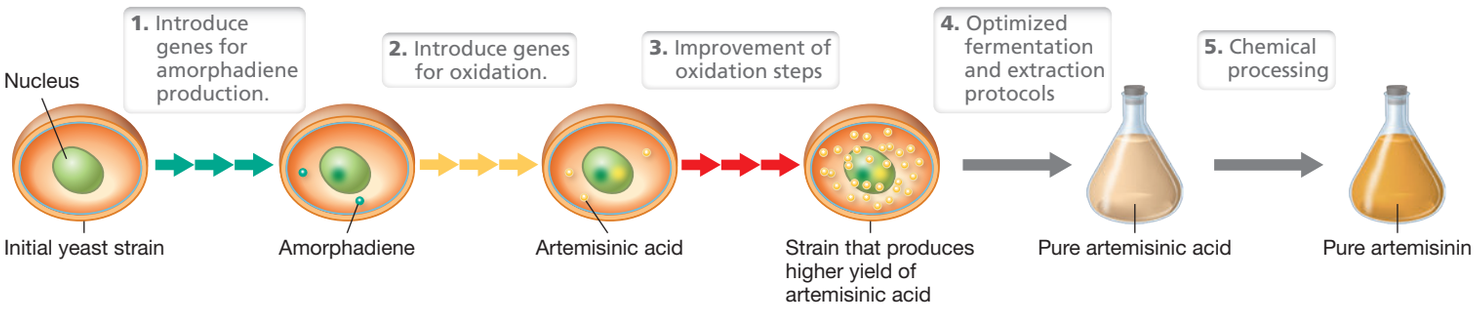


Figure 12.37 Artemisinin synthesis through synthetic biology. A summary of the engineering steps used to modify a *Saccharomyces cerevisiae* yeast strain to produce artemisinic acid. Colored arrows represent expression of genes from the plant *Artemisia annua* and other genetic modifications.

ments to divert carbon flux toward the final product, synthetic biologists designed a yeast strain that produces large amounts of artemisinic acid that can then be chemically converted to artemisinin (Figure 12.37).

Synthetic biology has also been successful at synthesizing powerful painkilling drugs. For example, genetic engineers modified yeast to produce the chemical *thebaine*, a precursor to morphine and hydrocodone, from glucose. This was accomplished by the heterologous expression in yeast of 21 different genes that originated from sources as diverse as plants, a rat, and the gram-negative bacterium *Pseudomonas*. The synthesized thebaine can then be chemically converted to a suite of pain-relieving drugs and marketed by pharmaceutical companies.

Biosensors and Genetic Circuits: Photographic *Escherichia coli*

An early excursion into the world of synthetic biology was the use of genetically modified *E. coli* to produce photographs using light sensing and a *genetic circuit*, another name for a gene regulatory network. The engineered bacteria are grown as a lawn on agar plates, and when an image is projected onto the lawn, unilluminated bacteria make a dark pigment while illuminated bacteria do not. The result is a primitive photograph of the projected image (**Figure 12.38**).

Construction of the photographic *E. coli* required synthetic biologists to create three key biobricks to form a sensor linked to a genetic circuit: (1) a light sensor or detector and signaling module; (2) a pathway to convert heme (already present in *E. coli*) into the photoreceptor pigment phycocyanobilin (an accessory light-harvesting pigment of cyanobacteria, ▶ Section 14.4); and (3) an enzyme encoded by a gene whose transcription can be switched on and off to make the dark pigment (Figure 12.38*a*). The photoreceptor is a fusion protein (Section 12.5) in which the sensing half is the light-detecting part of the phytochrome protein from the cyanobacterium *Synechocystis*. This required phycocyanobilin is not naturally made by *E. coli*; hence the need to install the biobrick that contained the pathway to make phycocyanobilin.

The other half of the fusion protein is the signal transmission domain of the EnvZ sensor protein from *E. coli*. EnvZ is part of a two-component regulatory system, its partner being OmpR (◀ Section 7.5). Normally, EnvZ activates the DNA-binding protein OmpR, and the latter in turn activates target genes by binding to the promoter. The hybrid protein was designed to activate OmpR in the dark but not in the light. This is because phosphorylation of OmpR is required for activation, and red light converts the sensor to a state in which phosphorylation is inhibited. Consequently, the target gene is *off* in the light and *on* in the dark. When a mask is placed over the Petri plate containing a lawn of the engineered *E. coli* cells (Figure 12.38*b*), cells in the dark make a pigment that cells in the light do not, and in this way a "photograph" of the masked image develops (Figure 12.38*c*).

The pigment made by the *E. coli* cells results from the activity of the lactose-degrading enzyme β-galactosidase, naturally present in *E. coli*. The target gene, *lacZ*, encodes this enzyme. In the dark, *lacZ* is expressed and β-galactosidase is made. The enzyme cleaves the lactose analog S-gal (3,4-cyclohexenoesculetin-β-D-galactopyranoside; similar to X-gal in Section 12.2) present in the growth medium to release galactose and a black dye. In the light, the *lacZ* gene is not expressed, no β-galactosidase is made, and so no dye is released. Contrast in the photograph is controlled by how much light cells see, which is governed by the nature of the mask that is used (Figure 12.38*c*).

Although bacterial photographs can hardly compare with digital photographs, the knowledge gained from assembling the biobricks required for bacterial photographs—work that is now many years old—helped form a foundation for the deployment of synthetic approaches in more complex biological systems, to which we turn now.

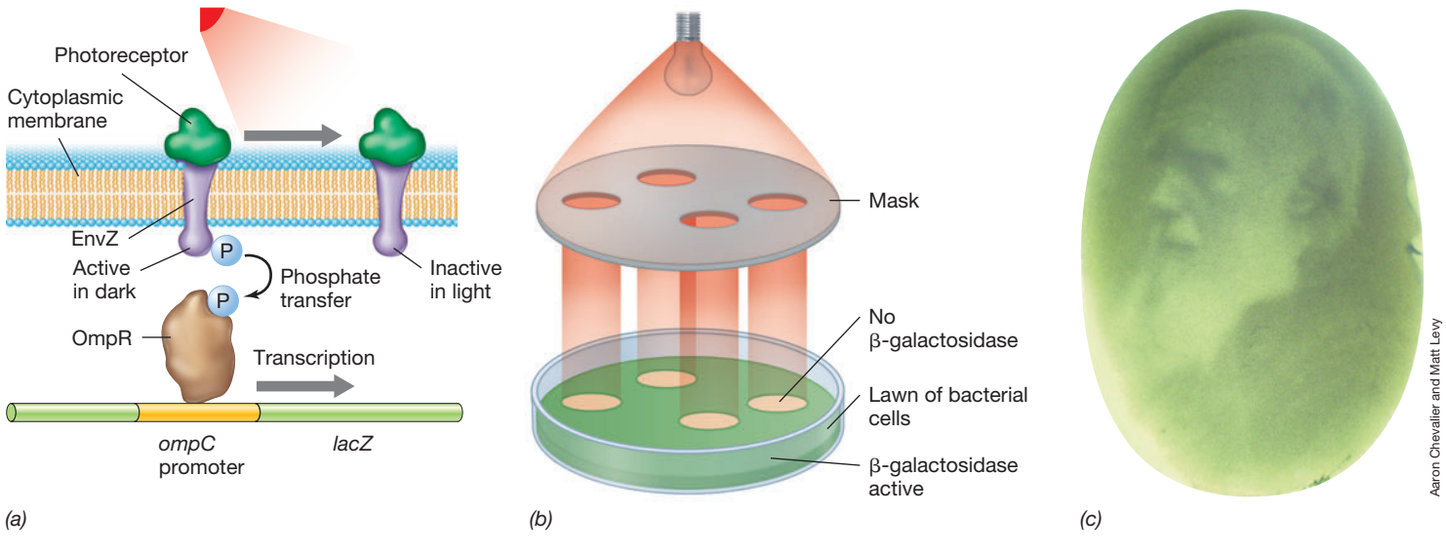
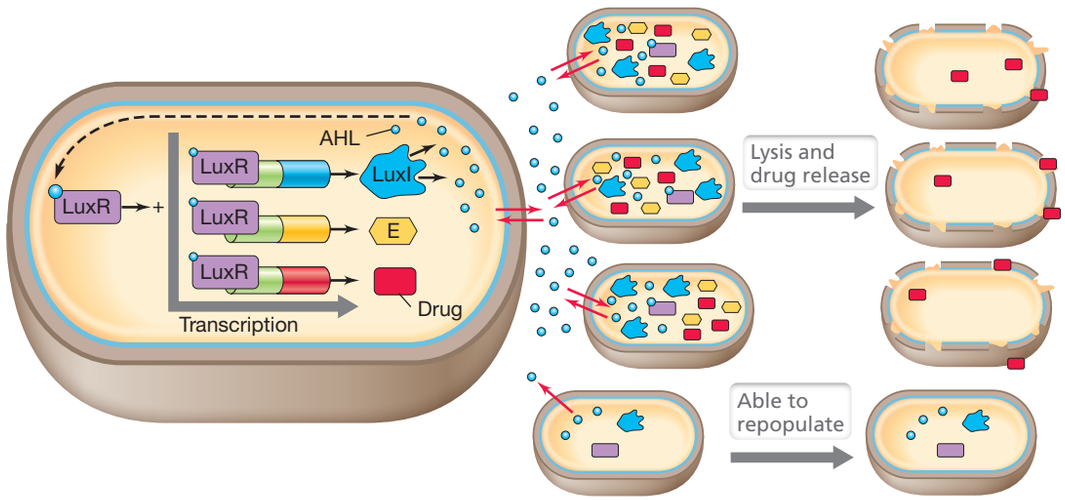


Figure 12.38 Bacterial photography. *(a)* Light-detecting *Escherichia coli* cells were genetically engineered using components from cyanobacteria and *E. coli* itself. Red light inhibits phosphate (P) transfer to the DNA-binding protein OmpR; phosphorylated OmpR is required to activate *lacZ* transcription (*lacZ* encodes β-galactosidase). *(b)* Procedure for making a bacterial photograph. The opaque portions of the mask correspond to zones where β-galactosidase is active and thus to the dark regions of the final image. *(c)* A bacterial photograph of a portrait of the eminent biologist Charles Darwin.

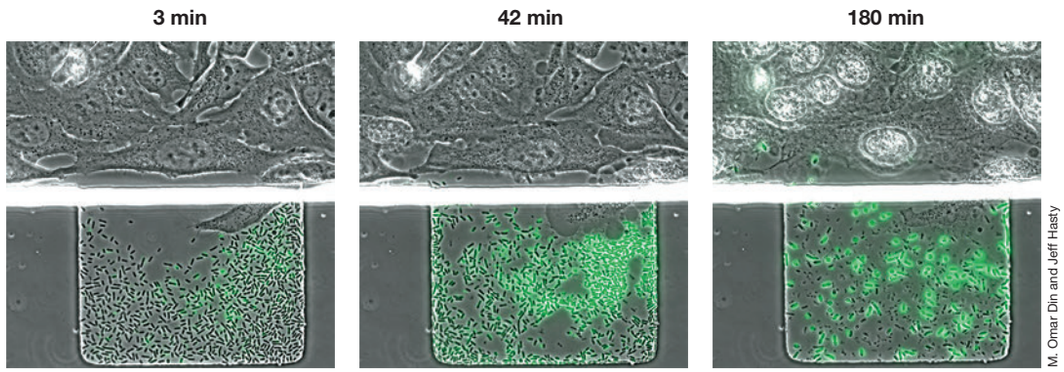
Biosensors and Genetic Circuits: Synchronized and Cyclical Drug Delivery

In Section 12.8 we discussed how features of the human microbiome (Chapter 24) can be used to deliver therapeutic agents to treat diseases including cancer. Synthetic biology is revolutionizing these treatment approaches through the design of synchronized genetic circuits that not only control the level of therapeutic delivery, but also the population level of the engineered microbe. We demonstrate this using the human pathogen *Salmonella enterica* (*typhimurium*), a bacterium that targets the gastrointestinal tract (▶ Section 33.10).

We have already seen how certain genetically modified gastrointestinal bacteria can grow and release anticancer drugs in the hypoxic environment of tumor cells (Section 12.8 and Figure 12.26*a*), and a similar anticancer strategy using *S. enterica* (*typhimurium*) has been developed. Advanced genetics can also be performed on this bacterium, allowing for the strain to be manipulated not only to deliver drugs to tumors but to do so using an "internal clock." To design a *S. enterica* (*typhimurium*) strain that releases an anticancer drug in a controlled and cyclical manner, genetic engineers took advantage of the natural ability of some microbes to communicate with others of their own kind through quorum sensing (◀ Section 7.7). Because a transcriptional activator (LuxR) is able to use the quorum-sensing molecule *N*-acyl homoserine lactone (AHL; ◀ Figure 7.18) as its inducer to activate transcription (◀ Section 7.3), a genetic circuit could be developed to link promoter regions of DNA that bind LuxR to genes that encode not only the production of AHL, but also the production of an anticancer drug and a cell lysis enzyme (**Figure 12.39*a***). Because AHL can freely diffuse into and out of cells, this genetic circuit results in AHL levels accumulating as the cell density increases. As AHL levels increase, the inducer molecule binds to LuxR and this



(a)



(b)

Figure 12.39 Genetic circuits and synchronized therapeutic agents. *(a)* A tumor-targeting bacterial strain of *Salmonella* was modified to produce and sense the quorum-sensing molecule *N*-acyl homoserine lactone (AHL) in response to population increase. Because AHL diffuses freely in and out of cells (red arrows), the cellular concentration increases as the surrounding cell density increases. Increased AHL levels lead to production of a lysis protein (E), an anticancer drug, and more AHL due to their corresponding genes being linked to a promoter that binds to the activator LuxR-AHL complex. This genetic circuit results in a positive feedback loop leading to cell lysis and drug release in the subpopulation of cells that encounter a specific threshold concentration of AHL. This cell lysis occurs in synchrony. Cells that did not encounter the required level of AHL survive to repopulate and initiate another cycle. *(b)* Time series of a coculture of *Salmonella enterica* (*typhimurium*) containing the part *a* genetic circuit, which is also linked to GFP for visualization (bottom chamber of panel), and HeLa cancer cells (top chamber of panel). The panels show an increase in bacterial cell concentration (42 minutes) and lysis and cell death (180 minutes). Bacterial cell lysis and drug release coincide with HeLa cell death (white cells) at 180 minutes. Intact bacterial cells at 180 minutes are able to grow and reinitiate the time course cycle. HeLa cells are an immortal cell line used in medical research.

activator–inducer complex binds to the promoters of genes encoding four components: LuxI (AHL synthesis), the anticancer drug, a fluorescent protein (GFP) to monitor the dynamics of the circuit, and the lysis protein E from bacteriophage ϕX174 (◀ Figure 11.6).

As the population of the engineered strain reaches a critical threshold, almost all cells in the population produce the components of the genetic circuit (Figure 12.39*a*). This leads to not only production of the anticancer drug, but also its release due to the production of the lysis E protein. However, a few outliers will exist in the population because of *phenotypic heterogeneity* (◀ Section 8.12), and these survivors will repopulate the tumor region (Figure 12.39*a*). The end result of this elegant genetic circuit is periodic bacterial lysis and drug delivery in response to a quorum-sensing clock.

Figure 12.39*b* illustrates the synchronized drug delivery circuit in action using a laboratory coculture system that allows cervical cancer HeLa cells to grow in a chamber above the engineered *S. enterica* (*typhimurium*) strain. The two cell types are separated and not allowed to mix, but molecules and drugs are able to move freely between the two growth chambers. As the engineered *S. enterica* (*typhimurium*) cell density increases, so does the level of GFP used to monitor the cells. This level of GFP decreases as the engineered microbes lyse from activation of the genetic circuit. Cell lysis leads to release of the anticancer drug and ultimately to HeLa cell death (Figure 12.39*b*).

By using biobricks encoding products and regulatory regions from various microorganisms, synthetic biology has produced a synchronized and cyclical therapeutic agent. However, while modified, this engineered strain of *S. enterica* (*typhimurium*) still possesses most of its wild-type genome. We turn now to approaches to engineering cells with synthetic genomes.

Check Your Understanding

- What are biobricks?
- What organism has been genetically modified to produce precursors to the drugs artemisinin and morphine?
- How was *Escherichia coli* modified to produce a photograph?

12.12 Synthetic Cells

The synthesis of an entire cell from scratch can be considered the pinnacle of synthetic biology. This has not happened yet, but a related feat was announced in 2010: A group of scientists from the J. Craig Venter Institute (JCVI, California, USA) had produced a "synthetic" bacterium. However, the organism produced was not the result of assembling various biobricks to form a living organism—true de novo cell synthesis—but instead was the product of the artificial construction of a small bacterial genome from a known genome sequence and the insertion of this synthetic genome into a different bacterial species to yield viable cells (**Figure 12.40**).

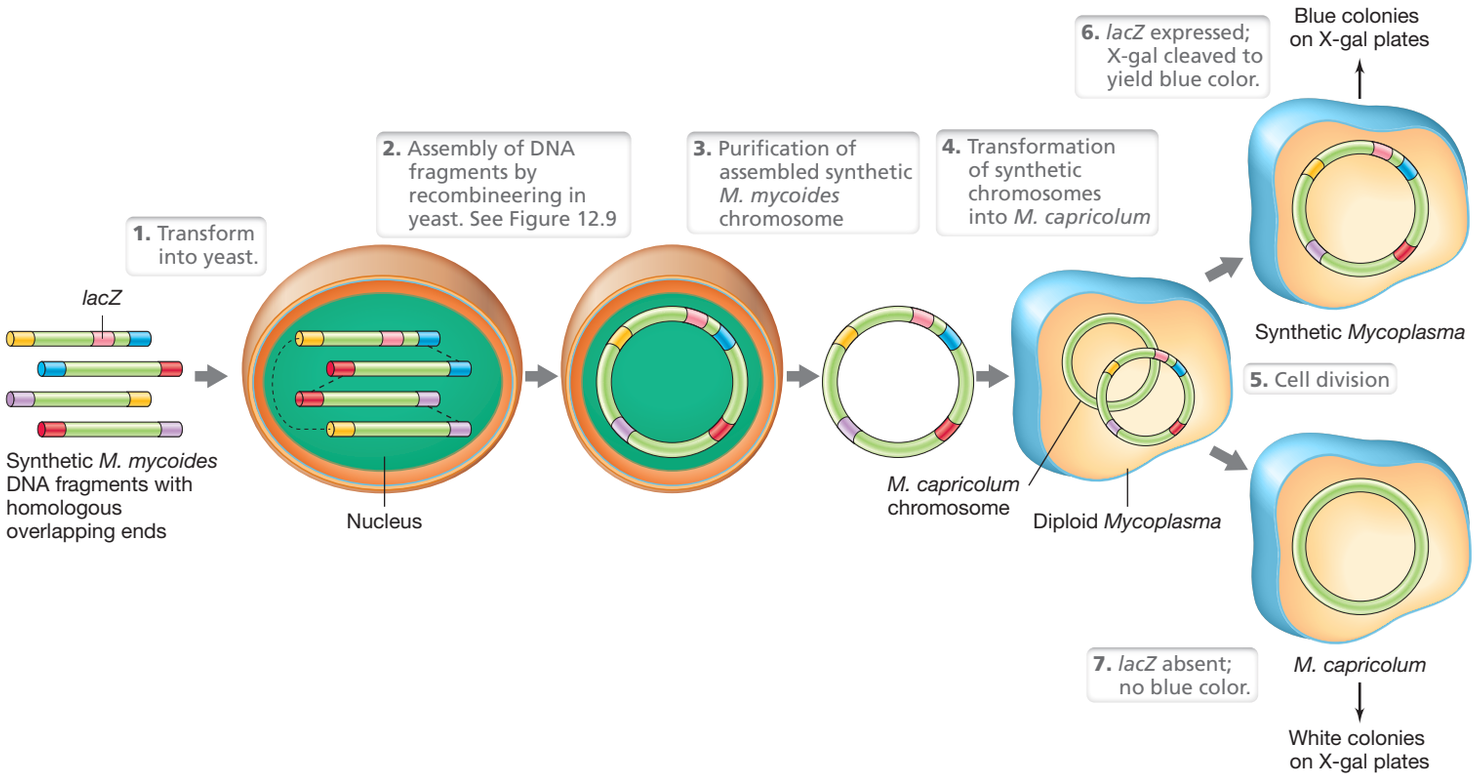
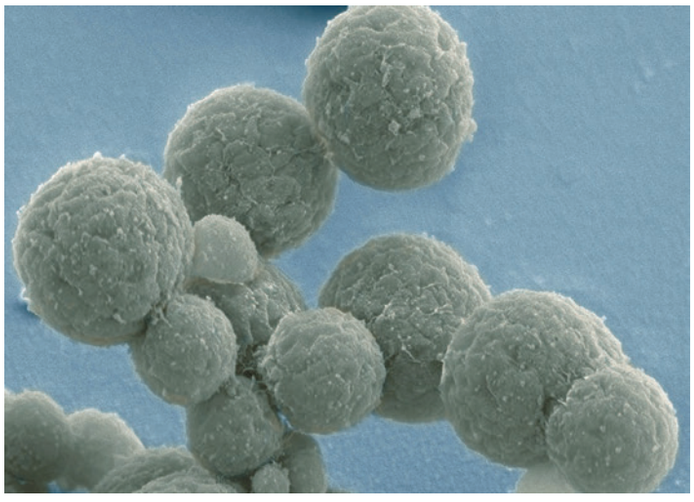


Figure 12.40 Formation of a synthetic *Mycoplasma* cell. DNA fragments corresponding to the desired *Mycoplasma mycoides* chromosome are synthesized with overlapping ends (represented by the same colors). These DNA fragments are linked together using the homologous recombination machinery of *Saccharomyces cerevisiae*, with sites of homologous recombination indicated by lines. Once assembled, the synthetic chromosome is transformed into a cell of *Mycoplasma capricolum*. This results in a cell with two separate chromosomes. After cell division, one cell possesses the synthetic chromosome (considered the synthetic *M. mycoides*) and the other remains a wild-type *M. capricolum*. The cell containing the synthetic chromosome was identified because it contained the reporter gene *lacZ* and thus produced blue colonies (see Figure 12.10).

Construction of a synthetic cell was accomplished by synthesizing a 1.08-million-base-pair (Mbp) genome based on the known genome sequence of the bacterium *Mycoplasma mycoides*. This small circular chromosome was pieced together from linear fragments of DNA containing homologous ends and the homologous recombination complex of yeast cells. The fully assembled 901-gene synthetic chromosome was then purified and transformed into a cell of *Mycoplasma capricolum* (Figure 12.40). The synthetic chromosome contained a reporter gene, *lacZ*, absent from the *M. capricolum* genome, which caused colonies containing the synthetic chromosome to turn blue (see Figure 12.10); this was needed to distinguish *M. capricolum* cells containing the *M. mycoides* genome from those containing the *M. capricolum* genome after cell division (Figure 12.40). When cells in the blue colonies were examined, they showed all of the properties of the original *M. mycoides* cell. This new cell was named JCVI-syn1.0. Although JCVI-syn1.0 was not constructed solely from biobricks (the *M. capricolum* host cell contributed ribosomes, various enzymes, and other important cytoplasmic components), the experiment did prove that an entire genome could be transplanted from one species to another.

Since the original creation of JCVI-syn1.0, synthetic biologists have expanded their work to determining the minimal genome necessary for a free-living cell. Using comparative genomics and prior knowledge about specific gene sequences, microbiologists at JCVI designed and synthesized several minimal genomes that they hypothesized would sustain life. To their dismay, none of these resulted in a viable cell. So instead, they generated modules of DNA corresponding to a *Mycoplasma* genome and sewed different combinations together to form synthetic genomes. Once viable cells were obtained from transplanting these genomes as described above, nonessential genes from the smallest genome were identified by transposon mutagenesis. After removing these unnecessary genes, a synthetic minimal cell coined JCVI-syn3.0 was created (**Figure 12.41**). This autonomous life form possesses a 531-kilobase genome encoding just 473 genes; JCVI-syn3.0 thus contains a genome smaller than that of any other free-living cell.



Thomas Deerinck and Mark Ellisman

Figure 12.41 Synthetic biology and the design of a minimal cell. Scanning electron micrograph of cells of the autonomous strain JCVI-syn3.0 possessing a synthetic genome containing 473 genes.

While this work showcases the amazing advancements in synthetic biology and the potential for creating designer cells with novel functions, a surprising mystery surrounds this minimal cell: the roles for almost a third of JCVI-syn3.0's genes remain unknown, highlighting how much we still need to learn about the genetic foundation of a living cell.

Check Your Understanding

- How were linear synthetic fragments of DNA assembled to form a *Mycoplasma* genome?
- How were *Mycoplasma* transformants with the synthetic chromosome selected for?

12.13 Genome Editing and CRISPRs

In Chapter 9 we discussed *c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeat (CRISPR) systems and their role in protecting *Bacteria* and *Archaea* from foreign DNA and maintaining genome integrity. Microbiologists studying a CRISPR system called *CRISPR/Cas9* (Cas9 refers to CRISPR *a*ssociated protein 9) in the bacterium *Streptococcus pyogenes* discovered that the system could also recognize and cleave a specific DNA sequence within other cells and that foreign DNA could be inserted into the cut site. This discovery has revolutionized biology.

The CRISPR/Cas9 system provides the most powerful and precise tool yet for altering eukaryotic genomes in living cells. Indeed, *genome editing*, as it has come to be known, has been used successfully to edit the genomes of plants, animal embryos, and human cell lines. Here we explore how this system works, its benefits to synthetic biology, and how it can be used as a *gene drive* to propagate specific genes throughout a population of organisms.

Sequence Targeting by the Cas9 Protein

As was illustrated in Figure 9.37, CRISPR systems possess Cas proteins that function as endonucleases when guided to a piece of nucleic acid by the complementary binding of *CR*ISPR RNAs (crRNAs). By designing a synthetic RNA molecule that both recruits the *Streptococcus* Cas9 protein and binds to the desired target DNA sequence, genetic engineers have harnessed the power of the CRISPR/Cas system to cut specific DNA sequences in the genome of virtually any cell. At the cut site, the DNA can either be ligated (yielding a gene deletion) or used for inserting new DNA (**Figure 12.42*a*, *b***).

The synthetic RNA molecule used for gene editing is called a *synthetic guide RNA* (sgRNA), and Figure 12.42*c* shows a model of the Cas9 protein from *S. pyogenes* binding both the sgRNA and target DNA. For complete Cas9 endonuclease activity, a short *p*rotospacer *a*djacent *m*otif (PAM; ◀ Figure 9.37) must also occur on the target DNA. Without this PAM sequence, the Cas9 protein will bind to the region where the sgRNA binds but will not cut. When the Cas9 protein does cut DNA, its two endonuclease domains (shown in purple in Figure 12.42*c*) cooperate to cut both DNA strands, and this generates double-stranded breaks (for simplicity, Figure 12.42*a* and *b* have single strands—shown as tubes—representing double strands). Depending on the target cell, various methods of delivering the CRISPR system can be used. Genes corresponding to the

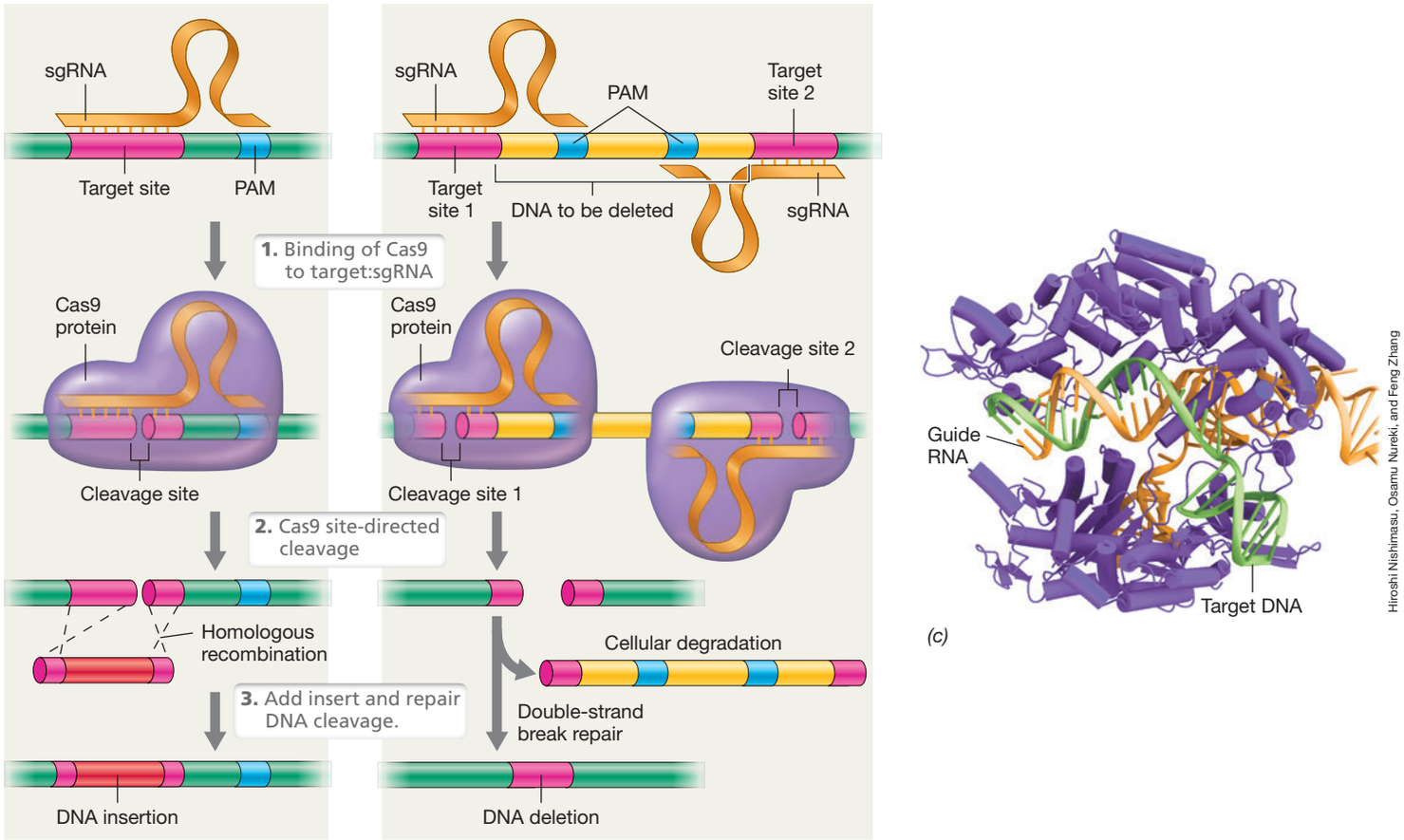


Figure 12.42 CRISPR/Cas9 genome editing. sgRNA represents the synthetic guide RNA, while PAM represents a protospacer adjacent motif. Note that each genome target site must possess a PAM sequence for DNA cleavage to occur. *(a)* Insertion of foreign DNA into a targeted site of the genome. An sgRNA is synthesized to bind to a single target site on the genome through complementarity. This binding of the sgRNA to the DNA stimulates the Cas9 protein to cleave the genome at the target site. Foreign DNA with ends homologous to the cleavage site can be incorporated into the cut site through homologous recombination. This results in a genomic insertion. *(b)* Deletion of a genomic region. Two separate target sites flanking the DNA to be deleted are selected. After the design, addition, and binding of sgRNAs corresponding to these regions, Cas9 protein–dependent DNA cleavage occurs. This results in a double-stranded break in the target chromosome and a free piece of DNA. The double-strand break is then ligated by the cell's DNA double-strand break repair pathway, while the free piece of genomic DNA is degraded. This results in a genomic deletion. *(c)* Crystal structure of the *Streptococcus pyogenes* Cas9 protein. The target DNA is shown in green and the sgRNA in orange.

designed sgRNA and Cas9 protein are often cloned into a plasmid under regulatory control of a strong promoter. Alternatively, the sgRNA and mRNA corresponding to the Cas9 protein can be generated in vitro. In either case, the materials are injected directly into target cells to trigger the gene editing process.

The presence of a PAM sequence close to the desired cleavage site is often the only limitation to cutting a specific DNA sequence. However, the PAM sequence is just three nucleotides in length and thus occurs with frequency in most genomes. CRISPR systems from other bacteria also recognize different PAMs, so alternative Cas proteins may be employed if needed. To delete a region of DNA, two target cleavage sites flanking the DNA sequence to be deleted must be identified and corresponding sgRNAs designed (Figure 12.42*b*). By contrast, only one cleavage site and corresponding sgRNA are needed to insert DNA (Figure 12.42*a*). To edit a region of a chromosome, sgRNAs are designed to bind to target DNA. This binding stimulates the Cas9 protein to cleave the target site if a PAM sequence is nearby (Figure 12.42*a*, *b*).

While a Cas9 protein and sgRNA can be used to cut DNA at specific sites (Figure 12.42), how is new DNA *inserted* at the cleavage site and how does the DNA get ligated back together? These tasks are accomplished by harnessing the cell's own DNA repair machinery. If a piece of DNA containing sequences with homology to the cut site is added to the system, homologous recombination will be used to incorporate the DNA (◀ Section 9.5), yielding a genomic insertion (Figure 12.42*a*). If the goal is only to delete the chromosomal region between two cut sites, the nonhomologous double-strand DNA break repair pathway will be employed to ligate the DNA following the deletion event (Figure 12.42*b*). The mechanism used by this DNA repair pathway makes it possible for genetic engineers to create an insertion or a deletion of as few as one nucleotide in a gene sequence through the cleavage of a single target site. No additional piece of DNA is needed because one nucleotide is either added or removed during the re-ligation of the two strands of DNA.

CRISPR Editing in Practice

Applications of CRISPR genome editing have seen a meteoric rise since its discovery in 2013. By designing a Cas9-encoding gene to possess codons optimized for the organism of interest (Section 12.3) and engineering sgRNA to target the gene of interest, almost any organism's DNA can be edited. In fact, CRISPR gene editing has exploded in crop plants including rice, sorghum, wheat, corn, and soybeans. The CRISPR/Cas9 system has also been employed in tomato plants by targeting a gene region in which a mutation leads to leaves that are needle-like or wiry (**Figure 12.43**). In this study, DNA encoding the Cas9 protein and sgRNA was introduced into the plant cells using *Agrobacterium* and the Ti plasmid system (Figure 12.20). Because the resulting mutations are stable and heritable, CRISPR genome editing has also been used to engineer numerous beneficial vegetable and fruit modifications including drought-resistant lettuce, grapes with increased levels of vitamin C, and disease-resistant oranges.

While a Cas9 system has been used to excise the genome of the retrovirus HIV (the causative agent of AIDS, ◀ Section 11.11) from the genome of infected human cells in vitro, Cas proteins from other bacteria can be used to target HIV or other specific viral RNAs. These RNA-targeting Cas proteins have been harnessed to not only create highly sensitive and portable diagnostic tools for detecting viruses such as Zika (see Chapter 10 Explore the Microbial World, "DNA Sequencing in the Palm of Your Hand"), but also to target RNA viruses inside of cells. For example, the Csy4 CRISPR-associated protein from the bacterium *Pseudomonas* is an endoribonuclease that processes the long CRISPR transcript (◀ Figure 9.37). The Csy4 protein has been modified to recognize and destroy free HIV RNA in infected cells (**Figure 12.44**). Similarly, the CRISPR/Cas9 system from the bacterium *Francisella novicida* has been reprogrammed to target the single-stranded RNA genome of hepatitis C virus (a liver pathogen and a cause of liver cancer) in a human cell line.

Not only has CRISPR genome editing been used to delete, interrupt, and insert DNA sequences into a single location, it can also be used to target multiple genetic loci. An impressive example of this is the removal of 62 copies of the porcine endogenous retrovirus from swine cells. This retrovirus generates an immune response in humans and is one of the factors preventing the use of swine organs for transplants in humans (swine are anatomically very similar to humans). Although other swine proteins also induce an immune response in humans, genetic engineers predict that in the foreseeable future they will be able to edit away all of these factors to produce immune-friendly pig embryos for human organ production.

Currently, CRISPR genome editing appears to have very few limitations. In fact, fertility clinic human embryos that were nonviable and could not result in a live birth have even been modified. This landmark accomplishment and reports that scientists in China may have successfully created CRISPR-modified babies resistant to HIV, smallpox, and cholera have raised serious ethical questions regarding the use of CRISPR editing in humans. But the technique may be the key to eradicating a host of devastating genetic diseases before a baby carrying the genes for one or more of these diseases is born. However, more studies are needed to verify gene target specificity and the response of the human immune system to Cas proteins.

Joyce Van Eck and Zachary Lippman

Yijun Qi and Biao Ding

Figure 12.43 CRISPR editing of tomato genome. Interruption of the tomato SlAGO7 gene encoding an argonaute homolog results in plants that have wiry or spindly leaves (left) compared to a normal tomato plant (right).

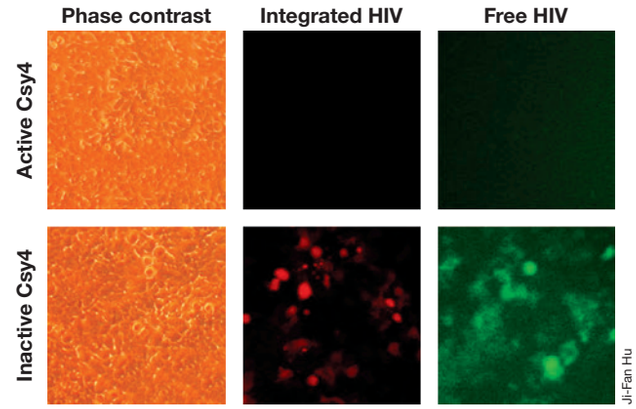


Figure 12.44 CRISPR-mediated inhibition of HIV infection. The results of using the *Pseudomonas* Csy4 endoribonuclease CRISPR protein to target RNA-based HIV in infected human embryonic kidney cells. Red indicates HIV provirus integration into infected cells, while free HIV virions are indicated in green. Top panel: expression of an HIV–Csy4 targeting vector. Bottom panel: expression of a mutated vector containing a nonfunctional Csy4 protein. Adapted from Guo, R., H. Wang, J. Cui, G. Wang, W. Li, and J-F Hu. 2015. *PLoS ONE 10*(10): e0141335.

Gene Drives and Engineered Mosquitoes

Not only can Cas proteins be used to edit almost any gene, the system can also be used as a *gene drive* to spread specific mutations throughout entire populations over generations. Gene drives, which only occur in sexually reproducing organisms, result in an allele (different forms of a gene) being inherited more than the normal frequency of 50%. Gene drives do occur in nature, but they are often facilitated by selfish DNA such as transposons (◀ Section 9.11). Thus, the spread and chromosomal location of the mutation is difficult to control. By contrast, CRISPR and Cas9 gene editing allows for the inheritance of the allele to be specifically controlled. The spread of a specific allele is based on diploid organisms possessing two copies of most genes, the ability of the Cas9 protein to be targeted to a specific site by a synthetic guide RNA (sgRNA), the endonuclease activity of Cas9 resulting in double-stranded DNA breaks, and the homology-directed DNA repair pathway.

Figure 12.45*b* illustrates the spread of an allele through a mosquito population by normal Mendelian inheritance. By contrast, Figure 12.45*c* shows how linking genes for Cas9 and the sgRNA to

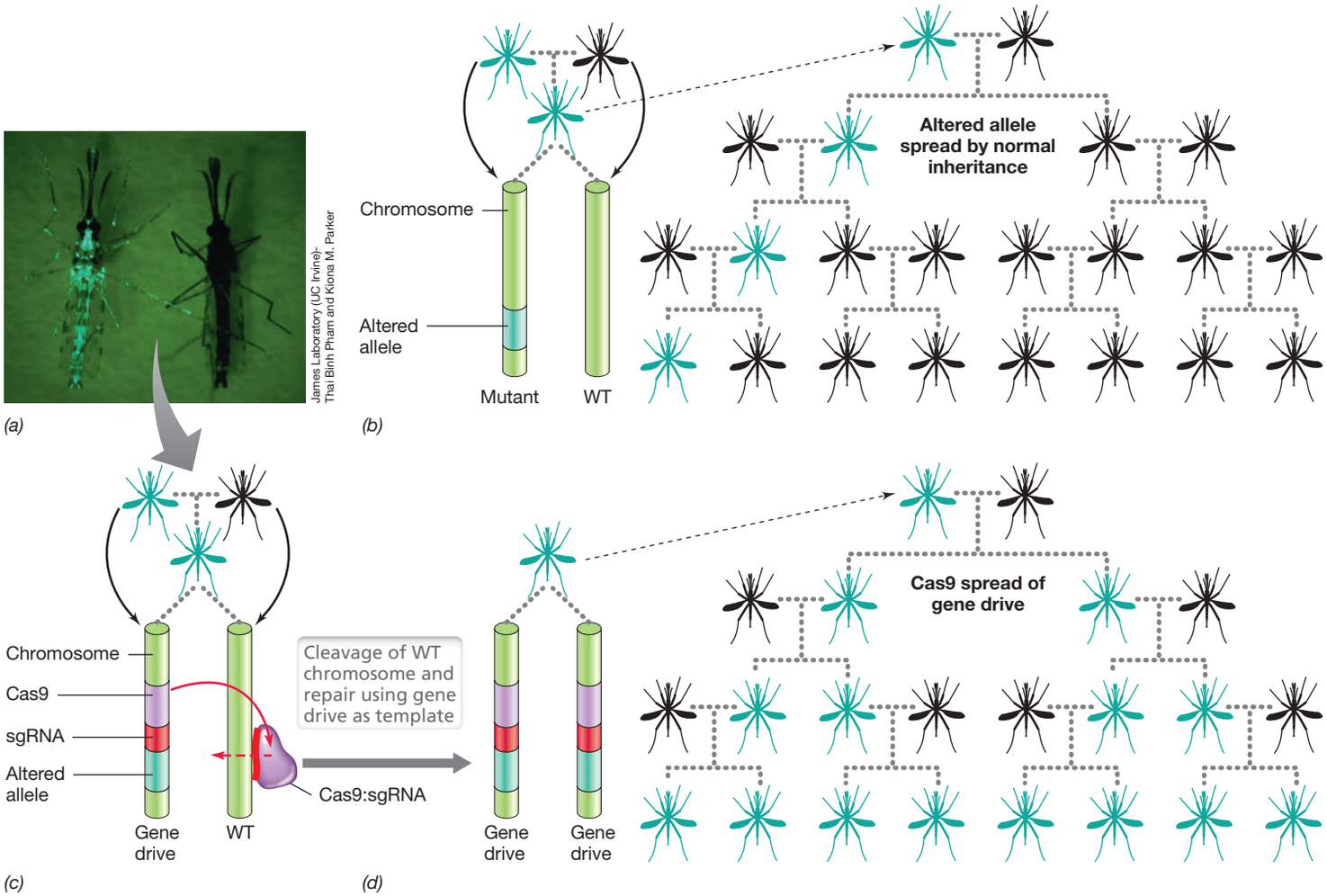


Figure 12.45 Spread of gene drives by Cas9 technology. *(a)* The mosquito *Anopheles stephensi* containing a green fluorescent protein (GFP) allele linked to a Cas9 gene drive (left) compared to wild type (right). *(b)* Normal inheritance of an altered allele (in this example, the gene encoding GFP) in mosquitoes through Mendelian genetics. Mating of a modified insect with a wild-type insect results in a heterozygote and only a 50% chance of the allele being transferred from parent to offspring. *(c)* By linking the allele to genes for Cas9 and synthetic guide RNA (sgRNA), the genetic modification in one copy of the chromosome spreads to a wild-type chromosome through the activity of Cas9 gene editing. The sgRNA guides the Cas9 endonuclease (solid red arrow) to cut the wild-type chromosome at a specific location, and the double-strand break (dashed red arrow) results in DNA repair of the cut chromosome using the altered allele as a template for DNA replication. *(d)* The altered allele linked to the Cas9 system becomes a gene drive as its inheritance occurs over 50% of the time, which eventually leads to the genetic modification occurring in the entire population.

a specific allele results in movement of the allele from one chromosomal copy to the other. Once the cell expresses the Cas9 and sgRNA genes, the gene drive results in a double-stranded break in the wild-type chromosomal copy. This break in the DNA is lethal unless repaired by the homology-directed repair system, which works by copying DNA from the intact chromosome into the broken region. Thus, the Cas9-based gene drive ultimately results in the spread of the allele throughout the entire population as heterozygotes become homozygous for both the mutant allele and the Cas9 gene drive (Figure 12.45*d*).

The applications for gene drives are countless, but one that is showing promise for public health is the modification of mosquitoes. While Figure 12.45*a* shows a mosquito carrying a Cas9 gene drive linked to an allele for the green fluorescent protein (GFP) for easy visualization, gene drives are being developed to prevent mosquitoes from carrying diseases such as malaria. The system can also be used to prevent the reproduction of disease-carrying mosquitoes by targeting an allele that results in female development. By linking this allele to a Cas9 gene drive, scientists have produced a population of mosquitoes unable to lay eggs after eight generations. While more scientific work is needed to determine the safety of releasing gene drives into nature, synthetic biologists now have the potential to eliminate malaria as a human disease and save millions of lives.

However, because the applications of gene drives are not just limited to pests like mosquitoes and have the potential to affect whole species, scientists have turned their attention to exploring ways to control the movement and reverse the activity of gene drives. Questions such as "Should scientists be altering genomes in ways that could have negative ecological consequences?" and "Can gene drives in one species be transmitted to other species?" are serious ones that need to be addressed. One way of protecting against these possibilities—effective biocontainment—is considered next to conclude this chapter.

Check Your Understanding

- What characteristics of the Cas9 protein make it an efficient DNA editing tool?
- What is the role of the sgRNA in genome editing? How is recombinant DNA inserted into a genome using CRISPR editing?
- Give an example of how a gene drive can slow or halt transmission of an infectious disease.

12.14 Biocontainment of Genetically Modified Organisms

Throughout this chapter we have focused on genetically engineering microbes as factories for the synthesis of high-value products, including specific enzyme systems for gene editing. While these applications of genetic engineering are clearly beneficial, environmental concerns remain that genetically modified organisms (GMOs) may spread their modified genes to wild-type populations with possible adverse consequences. Such concerns have slowed the acceptance of GMOs, and have been used to ban the sale of GMOs in certain countries. How can synthetic biology help solve these problems? Containment is one obvious answer and we consider this now.

Early Containment Schemes

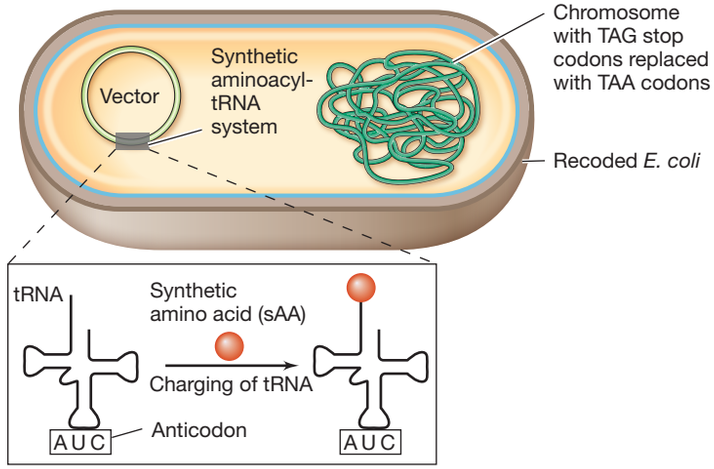
Through the years, various schemes have been proposed for containing GMOs. For example, both the use of auxotrophic strains and the induction of genes encoding self-toxins have been attempted with GMOs, but for both strategies, successful and reliable implementation has been a problem. Recall that an auxotroph is a mutant derivative of a microbial species that has a nutritional requirement; without the nutrient, the auxotroph cannot grow (◀ Section 9.1). This dependency is the theory behind the proposal to use auxotrophs to contain GMOs. However, in nature, auxotrophic strains can often survive by cross-feeding off the metabolites of other organisms; in addition, the possibility always remains that an auxotrophic GMO could revert to the wild type by back mutation and lose its nutritional dependencies.

In Section 8.12 we saw how certain bacteria produce a growth-inhibiting toxin that slows cell growth to help ensure survival of the population under stressful conditions. This has also been explored as a mechanism for biocontainment. That is, if a GMO were to escape confinement within a bioreactor (where all growth conditions are ideal for production of the desired product), the toxin would kick in and trigger the dormant state, from which the escaped GMO could not recover. However, in nature, where a cell has to compete not only with cells of its own kind but also with other microbial species, survival strongly selects for toxin gene mutations; if such a mutation occurred quickly, the toxin system could be disarmed and the GMO might survive. Similarly to modifying bacteria for toxin production, a modified bacterial strain can be rewired to produce a lysis protein to control population levels, as we discussed in Section 12.11. However, a subset of cells will always be out of sync during expression of the lysis protein and persist to repopulate (Figure 12.39*a*).

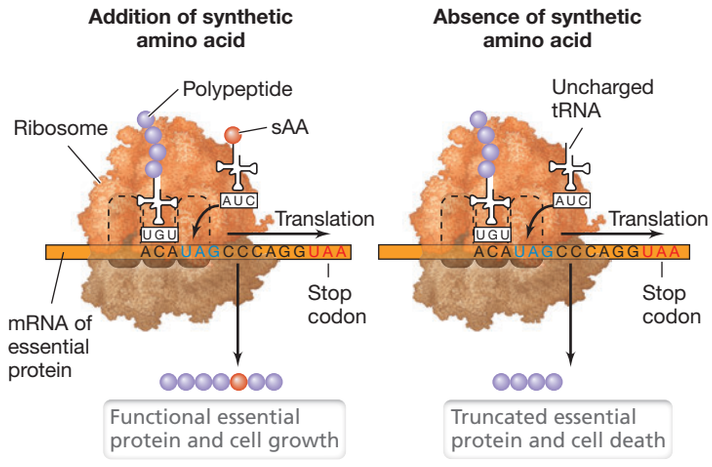
Neither the auxotroph nor the toxin and lysis approaches adequately solve the problem of GMO containment. Moreover, none of these mechanisms address the possibility of engineered DNA being released through industrial waste streams and finding its way into other microorganisms by horizontal gene transfer (Chapter 9). Thus, to more thoroughly and safely address the containment problem, genetic engineers have tapped into synthetic biology itself to devise novel methods of controlling GMOs in the environment, and we consider one now.

Rewiring the Genetic Code

A novel approach to prevent genetically modified bacteria from surviving outside of their bioreactor or other containment is to recode the genome of the GMO so the bacterium *can only grow if supplied with a synthetic amino acid* (**Figure 12.46**). This recoding results not only in biocontainment, but also in the ability to make novel proteins not found anywhere in nature. This requires rewiring the organism's genetic code and translational machinery (Chapter 6) to



(a)



(b)

Figure 12.46 Recoding and control of genetically modified *Escherichia coli*. *(a)* The chromosome of an *E. coli* cell is genetically modified (recoded) to replace all TAG stop codons with TAA stop codons. The recoded *E. coli* stably maintains a vector expressing a tRNA with an AUC anticodon and an aminoacyl-tRNA synthetase (◀ Figure 6.32) that charges the tRNA with a synthetic amino acid (sAA). *(b)* Control of cell growth by inserting a UAG codon into mRNAs of essential proteins. If the synthetic amino acid is added to the growth medium, the recoded *E. coli* will translate functional essential proteins. If the synthetic amino acid is not present, an uncharged tRNA will bind to UAG codons engineered into essential proteins. This results in truncated essential proteins and ultimately cell death.

synthesize proteins containing the synthetic amino acid; a significant feat, but synthetic biology has already accomplished it.

In the first step of recoding a cell, scientists replaced all the TAG (UAG on the RNA) stop codons associated with open reading frames on the *Escherichia coli* chromosome with the TAA (UAA on the RNA) stop codon. They also deleted the gene for release factor 1 that terminates translation when the ribosome encounters a UAG on the mRNA. Because this manipulation placed an alternative stop codon at the end of genes that had TAG codons, proteins of the correct length are still produced during translation and the recoded cell grows normally. But this genetic manipulation also freed up the UAG stop codon to be reassigned another translational function.

To engineer dependence on a synthetic amino acid, genes for an aminoacyl-tRNA synthetase (◀ Section 6.8) that recognizes the synthetic amino acid (sAA) and a corresponding tRNA with an AUC anticodon were expressed from a vector (Figure 12.46*a*). This resulted in tRNAs with the AUC anticodon carrying the sAA. A set of essential genes was then modified to contain the TAG codon in positions where incorporation of the sAA would not affect protein activity. Thus for the recoded bacterium to translate mRNAs possessing the UAG codon, the cell must be fed the artificial amino acid (Figure 12.46*a*). If the growth environment does not have the sAA (as would be the case if the GMO escaped to nature), uncharged tRNAs will enter into the ribosome when a UAG codon is encountered; if this occurs during the translation of an essential protein, translation will stall and a truncated (and nonfunctional) protein will result (Figure 12.46*b*). This will lead to death of the cell and puts ultimate control of the recoded GMO into human hands. Because the TAG codon was placed in three essential genes, it is extremely unlikely that sufficient mutations could arise to remove the GMO's dependence on the presence of the sAA.

While this strategy for biocontainment is dependent on the incorporation of the synthetic amino acid in regions of the protein that do not affect activity, synthetic biologists are using the same strategy to make proteins with novel activities and characteristics. By using amino acids with unique binding properties, there is no limit to the type of "alien" proteins that can be produced by synthetic biology. Some practical examples include modified drugs that are assimilated more easily or are less toxic, and improvements in the catalysis or specificity of enzymes for use in biotechnology. In addition to rewriting the genetic code, synthetic biology can also harness the CRISPR genome editing system to modify organisms to prevent their unwanted spread.

Continued advances in synthetic biology and the control of GMOs will not only allow for more widespread use of these organisms for synthesizing desired products and therapeutic agents in carefully controlled production settings but may also trigger the more extensive use of GMOs in general to solve urgent problems that remain in medicine, agriculture, and the environment.

Check Your Understanding

- Why is the use of auxotrophy not a good method for controlling the growth of a genetically modified organism?
- How can a tRNA be engineered to encode for a synthetic amino acid?
- Why is it unlikely that GMOs recoded to depend on a synthetic amino acid will mutate to no longer depend on the exogenously supplied synthetic amino acid?

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CHAPTER REVIEW

I • Tools of the Genetic Engineer

12.1 The polymerase chain reaction is a procedure for amplifying DNA in vitro and employs heat-stable DNA polymerases. This amplified DNA is often used for cloning purposes and can be visualized by gel electrophoresis. Complementary nucleic acid sequences may be detected by hybridization.

Q How is DNA amplified in a polymerase chain reaction (PCR) procedure? Why do DNA polymerases from thermophilic microbes significantly improve the PCR procedure?

12.2 The isolation of a specific gene or region of a chromosome by molecular cloning is done using a cloning vector. Plasmids are useful cloning vectors because they are easy to isolate and purify and are often able to multiply to high copy numbers in bacterial cells. The choice of a cloning host depends on the final application. In many cases the host can be a bacterium, but in others, it is essential that the host be a eukaryote.

Q How does the insertional inactivation of β-galactosidase (LacZ) allow the presence of foreign DNA in a plasmid vector such as pUC19 to be detected?

12.3 Many cloned genes are not expressed efficiently in a foreign host. Expression vectors have been developed that both increase transcription of the cloned gene and control the level of transcription. To achieve very high levels of expression of eukaryotic genes in bacteria, the expressed gene must be free of introns. This can be accomplished by synthesizing cDNA from the mature mRNA encoding the protein of interest or by making an entirely synthetic gene. Protein fusions are often used to stabilize or solubilize the cloned protein.

Q What is the significance of reverse transcriptase in the cloning of animal genes for expression in bacteria?

12.4 Synthetic DNA molecules of desired sequence can be made in vitro and used to construct a mutated gene directly or to change specific base pairs within a gene by site-directed mutagenesis. Also, genes can be disrupted by inserting DNA fragments, called cassettes, into them, generating knockout mutants.

Q What does site-directed mutagenesis allow you to do that normal mutagenesis does not?

12.5 Reporter genes are genes whose products are easy to assay or detect. They are used to simplify and increase the speed of genetic analysis. In gene fusions, segments from two different genes, one of which is usually a reporter gene, are spliced together.

Q What is the key property of a reporter gene?

II • Making Products from Genetically Engineered Microbes: Biotechnology

12.6 The first human protein made commercially using engineered bacteria was human insulin. Recombinant bovine somatotropin is sometimes used in the United States to increase milk yield in dairy cows.

Q What classes of mammalian proteins are produced by biotechnology? How are the genes for such proteins obtained?

12.7 Genetic engineering can make plants resistant to disease and improve product quality. The Ti plasmid of the bacterium *Agrobacterium tumefaciens* can transfer DNA into plant cells. Genetically engineered commercial plants are called genetically modified organisms (GMOs).

Q What is the Ti plasmid and how has it been of use in genetic engineering?

12.8 Many recombinant vaccines have been produced or are under development. These include live recombinant, vector, and subunit vaccines. Properties associated with pathogens can be used to develop cancer-treating therapeutic agents.

Q What is a subunit vaccine and why are subunit vaccines considered a safer way of conferring immunity to viral pathogens than attenuated virus vaccines?

12.9 Genes for useful products may be cloned directly from DNA or RNA in environmental samples without first isolating the organisms that carry them. In pathway engineering, genes that encode the enzymes for a metabolic pathway are assembled. These genes may come from one or more organisms, but the engineering must achieve regulation of the coordinated sequence of expression required in the pathway.

Q How has metagenomics improved the discovery of novel useful products in biotechnology?

12.10 While select microorganisms can produce biofuels in small amounts, others can be modified to produce various biofuels through pathway engineering. This modification often requires genes from multiple microorganisms. New tools for genetically manipulating microalgae have also been developed to facilitate biofuel production.

Q How can biodiesel be produced from microalgae-biosynthesis products?

III • Synthetic Biology and Genome Editing

12.11 Instead of modifying or improving a single existing pathway, synthetic biology focuses on engineering novel biological systems by linking known biological components together in various combinations. These modifications can result in the production of high-value products and synchronized biofactories.

Q How does synthetic biology differ from engineering of *Escherichia coli* to produce indigo?

12.12 Through the use of recombineering techniques, linear fragments of DNA can be synthesized and assembled into a genome. These synthesized genomes can then be transplanted into bacterial cells, displacing the parent genome and thus creating a synthetic cell.

Q How has the creation of a synthetic cell allowed microbiologists to probe the minimal genome required for autonomous life?

12.13 Not only can CRISPR systems be used as a prokaryotic immune system, they can also be modified to edit the genomes of eukaryotes. CRISPR requires a guide RNA to locate the genes to be cut and Cas proteins to cut the DNA. The resulting double-stranded breaks are suitable for the insertion of foreign DNA.

Q How has CRISPR editing technology been applied to targeting virus-infected eukaryotic cells?

12.14 With the advancements in genetic engineering, methods for controlling genetically modified organisms are imperative. One promising method is the use of synthetic biology to recode organisms for dependence on the presence of synthetic amino acids.

Q What are some mechanisms for controlling a genetically modified organism other than making it dependent on synthetic amino acids?

APPLICATION QUESTIONS

1. Suppose you have just determined the DNA base sequence for an especially strong promoter in *Escherichia coli* and you are interested in incorporating this sequence into an expression vector. What steps would you take and which tools would you use to be sure that this promoter actually worked as expected in its new location?

2. You have just discovered a protein in mice that may be an effective cure for cancer, but it is present only in tiny amounts. Describe the steps you would use to produce this protein in therapeutic amounts. Which host would you want to clone the gene into and why? Which host would you use to express the protein in and why?

3. Describe how you could use a soil sample and metagenomics to isolate a new antifungal protein that protects wheat from fungal infection. What steps could you take in the laboratory to modify this antifungal protein to be more effective against a broad range of fungal plant pathogens? Suppose you want to create genetically modified wheat that expresses your improved antifungal protein; what general steps would you take to introduce the foreign gene using CRISPR technology?
4. Describe how you could recode *Escherichia coli* to produce novel proteins containing more than the standard 22 amino acids.

CHAPTER GLOSSARY

Attenuate to decrease or eliminate the virulence of a pathogen or virus

Bacterial artificial chromosome (BAC) a circular artificial chromosome with a bacterial origin of replication

Biotechnology the use of organisms, typically genetically altered, in industrial, medical, or agricultural applications

Cassette mutagenesis creating mutations by the insertion of a DNA cassette

Complementary DNA (cDNA) DNA made from an RNA template during the reverse transcription PCR (RT-PCR) procedure

DNA cassette an artificially designed segment of DNA that usually carries a gene for resistance to an antibiotic or some other convenient marker and is flanked by convenient restriction sites

Expression vector a cloning vector that contains the necessary regulatory sequences to allow transcription and translation of cloned genes

Gel electrophoresis a technique for separation of nucleic acid molecules by passing an electric current through a gel made of agarose or polyacrylamide

Gene disruption (also called gene knockout) the inactivation of a gene by insertion of a DNA fragment that interrupts the coding sequence

Gene fusion a structure created by joining together segments of two separate genes, in particular when the regulatory region of one gene is joined to the coding region of a reporter gene

Genetically modified organism (GMO) an organism whose genome has been altered using genetic engineering; the abbreviation GM is also used in terms such as GM crops and GM foods

Genetic engineering the use of in vitro techniques in the isolation, alteration, and expression of DNA or RNA and in the development of genetically modified organisms

Green fluorescent protein (GFP) a protein that fluoresces green and is widely used in genetic analysis

Heterologous expression the transcription and translation of a gene or genes from one organism in a different organism

Hybridization the joining of two single-stranded nucleic acid molecules by complementary base pairing to form a double-stranded hybrid DNA or DNA–RNA molecule

Molecular cloning the isolation and incorporation of a fragment of DNA into a vector where it can be replicated

Northern blot a hybridization procedure where RNA is the target and DNA or RNA is the probe

Nucleic acid probe a strand of nucleic acid that can be labeled and used to hybridize to a complementary molecule from a mixture of other nucleic acids

Operon fusion a gene fusion in which a coding sequence that retains its own translational signals is fused to the transcriptional signals of another gene

Pathway engineering the assembly of a new or improved biochemical pathway using genes from one or more organisms

Polymerase chain reaction (PCR) the artificial amplification of a DNA sequence by repeated cycles of strand separation and replication

Polyvalent vaccine a vaccine that immunizes against more than one disease

Protein fusion a gene fusion in which two coding sequences are fused so that they share the same transcriptional and translational start sites

Recombinant DNA a DNA molecule containing DNA originating from two or more sources

Reporter gene a gene used in genetic analysis because the product it encodes is easy to detect

Restriction enzyme an enzyme that recognizes a specific DNA sequence and then cuts the DNA; also known as a restriction endonuclease

Shuttle vector a cloning vector that can replicate in two different organisms; used for moving DNA between unrelated organisms

Site-directed mutagenesis the construction in vitro of a gene with a specific mutation

Southern blot a hybridization procedure where DNA is the target and RNA or DNA is the probe

Subunit vaccine a vaccine that contains only a specific protein or two from a pathogen

Synthetic biology arranging biobricks to form new genetic elements for insertion into microbes or higher organisms

T-DNA the segment of the *Agrobacterium tumefaciens* Ti plasmid that is transferred into plant cells

Ti plasmid a conjugative plasmid in the bacterium *Agrobacterium tumefaciens* that can transfer genes into plants

Transgenic organism a plant or an animal with foreign DNA inserted into its genome

Vector (as in cloning vector) a DNA molecule that is replicated by a host cell and used to carry cloned genes or other DNA segments for genetic engineering

Vector vaccine a vaccine made by inserting genes from a pathogenic virus into a relatively harmless carrier virus

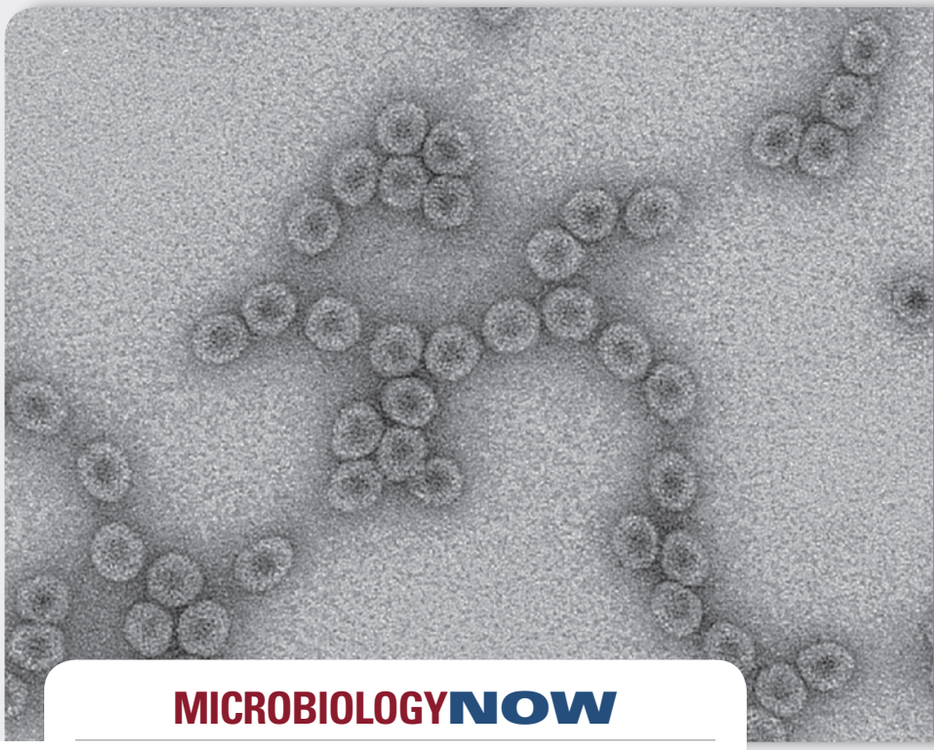
Yeast artificial chromosome (YAC) an artificial chromosome with a yeast origin of replication and a centromere sequence

13 Microbial Evolution and Genome Dynamics

I Early Earth and the Origin and Diversification of Life 429

II Mechanisms of Microbial Evolution 439

III Microbial Phylogeny and Systematics 448



MICROBIOLOGY**NOW**

Exploring Viral Genesis

Unlike cells, viruses cannot perform metabolic reactions or conserve energy, yet viruses thrive in a world filled with cells. Viruses can evolve with frightening speed, and a tremendous diversity of viruses exist. Indeed, it is likely that every cellular organism can be a host for several different viruses.

While we have learned much about viruses, we know little about their evolution. Most viral genomes have few genes—indeed, the smallest have as few as three—and as a result, it is difficult to reconstruct the evolutionary history of all viruses as has been done for cells. Hence, we can only speculate about viral origins. All viruses might have diverged from one ancestral virus that appeared soon after the origin of cellular life, some 3.8 to 4.3 billion years ago. However, another possibility is that viruses have multiple origins, appearing spontaneously again and again over the history of life.

To address these hypotheses, we must understand mechanisms that promote viral genesis. One important step in viral genesis is the origin of the viral capsid. All capsids are formed by self-assembly of one or a few proteins into a hollow molecular structure. Similar structures already exist in cells. For example, consider flagella and carboxysomes; both are hollow structures formed by self-assembling proteins. Another example is the enzyme lumazine synthase produced by the bacterium *Aquifex aeolicus*. Copies of this protein spontaneously assemble into capsid-like protein shells, and, with a few simple modifications in the laboratory, can be altered to package their own mRNA, resulting in virus-like nucleocapsids containing an RNA genome (see photo, capsids are 31 nm in diameter).

This discovery suggests that it could be surprisingly easy for viruses to originate spontaneously by mutation of cellular proteins that captured bits of nucleic acid. Thus, it is indeed possible that the viral diversity we observe today has many different origins.

Source: Terasaka, N., et al. 2018. Laboratory evolution of virus-like nucleocapsids from nonviral protein cages. *Proc. Natl. Acad. Sci. 115*: 5432.

I • Early Earth and the Origin and Diversification of Life

Microbes first appeared on Earth about 4 billion years ago and proceeded to colonize every habitable environment and oxygenate the planet. Cells of *Bacteria* and *Archaea* combined in some way to give rise to eukaryotic cells, and the evolutionary path that all cells have traveled is recorded in the nucleotide sequences of their DNA.

Evolution is a process that unifies all branches of biology because all living things undergo change over time and all have descended from the same universal ancestor. In this chapter, we tackle the topic of microbial evolution and describe methods for unraveling evolutionary relationships between microorganisms. We will also see how microbial evolution informs microbial systematics, a system we will use to describe relationships among microorganisms as we explore the diversity of the microbial world in the following five chapters.

In these first sections, we consider the possible conditions under which life arose, the earliest evidence for cellular life, and its divergence into three evolutionary lineages: ***Bacteria***, ***Archaea***, and ***Eukarya***. Although much about these events and processes remains speculative, geological and molecular evidence has combined to build a plausible scenario for the earliest events in the **evolution** of life and for the fundamental impacts that microbes have had on the history of our Earth.

13.1 Formation and Early History of Earth

The Earth of 4 billion years ago (bya) would be foreign and inhospitable to human eyes, but this sterile wasteland of blasted rock and boiling seas was the incubator from which all life sprang. Since few fossils exist to tell the story of the early Earth, most of what we know is inferred from chemical and isotopic analyses of ancient rocks and minerals. However, we will see in this chapter that we can infer ancient evolutionary events from living organisms by comparing their molecular structures and sequences to determine their common history of descent. The story of life begins not long after the dawn of our solar system with the formation of Earth itself.

Origin of Earth

Earth formed about 4.5 bya (**Figure 13.1**), based on analyses of slowly decaying radioactive isotopes. Our planet and the other planets of our solar system arose from materials making up a disk-shaped nebular cloud of dust and gases released by the supernova of a massive old star. As a new star—our sun—formed within this cloud, it began to compact, undergo nuclear fusion, and release large amounts of energy in the form of heat and light. Materials left in the nebular cloud began to clump and fuse due to collisions and gravitational attraction, forming tiny aggregates that gradually grew larger to form clumps that eventually coalesced into planets. Energy released in this process heated the emerging Earth as it formed, as did energy released by radioactive decay within the condensing materials, forming a planet Earth of fiery hot magma. As Earth cooled over time, a metallic core, rocky mantle, and a lower-density, thin surface crust formed.

The inhospitable conditions of early Earth, characterized by a molten surface under intense bombardment by asteroids and other objects from space, persisted for up to 500 million years. The intense heat and the absence of liquid water indicate that early Earth was completely devoid of life. Furthermore, the early Earth was anoxic, and the absence of O_2 means that Earth lacked an ozone layer, allowing intense ultraviolet (UV) light to sterilize its surface. Water on Earth originated from volcanic outgassing of the planet's interior and from innumerable collisions with icy comets and asteroids. Given Earth's heat at the time, this water would have been present only as water vapor. No rocks dating to the origin of Earth have yet been discovered, presumably because they have undergone geological metamorphosis. However, ancient crystals of the mineral zircon ($ZrSiO_4$) have been discovered that were formed on the early Earth, and these materials have given us a glimpse of conditions on Earth prior to the origin of life.

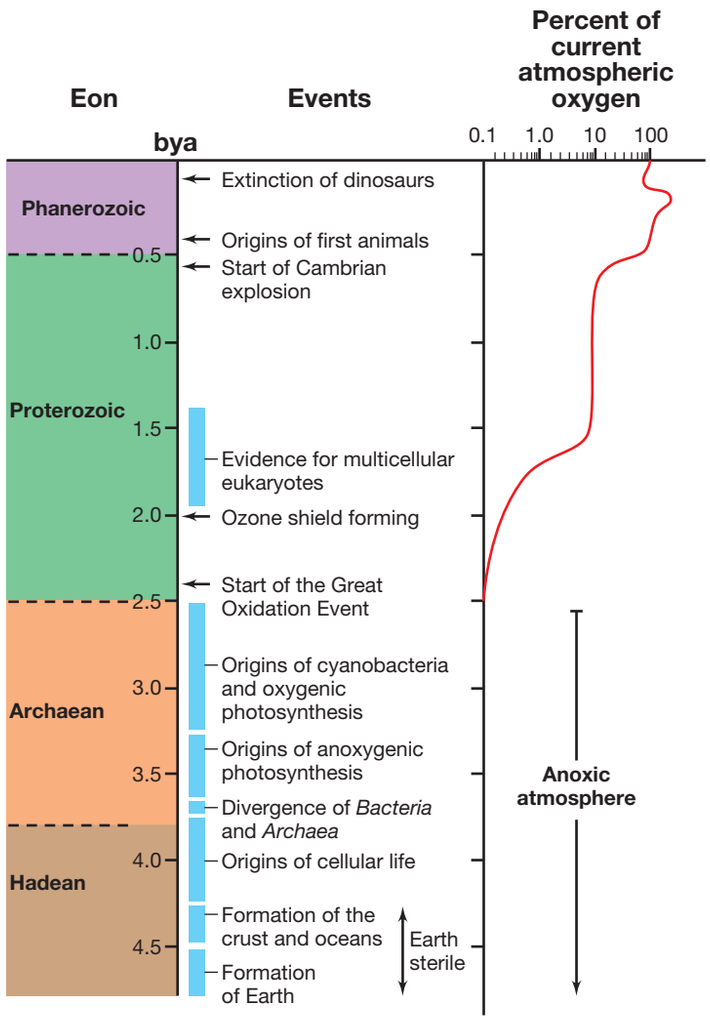


Figure 13.1 Major landmarks in biological evolution, Earth's changing atmospheric chemistry, and microbial metabolic diversification. The oldest date for the origin of life is fixed by the time of Earth's origin, and the minimum time for the origin of oxygenic photosynthesis is fixed by the Great Oxidation Event, about 2.4 billion years ago (bya). Note how the oxygenation of the atmosphere from cyanobacterial metabolism was a gradual process, occurring over a period of about 2 billion years. Compare this figure with the introduction to the antiquity of life on Earth shown in Figure 1.10.

Liquid water is a requirement for life, and its eventual presence on Earth made possible the origin of life. Analyses of ancient zircon crystals, including impurities trapped in these crystals and their oxygen isotope ratios (we discuss the use of isotopic analyses as indications of living processes in Section 19.10), provide evidence for a solid crust and liquid water on Earth as early as 4.3 bya (Figure 13.1). Additional evidence of water comes from ancient sedimentary rocks, since sedimentary rocks are only formed under water. Among the oldest surviving sedimentary rocks are those found in southwestern Greenland, dating to 3.86 bya. These ancient rocks indicate the presence of early oceans. These rocks also contain the fossilized remains of cells (**Figure 13.2**), as well as carbon isotope ratios that provide evidence for life. Furthermore, graphite inclusions having carbon isotope ratios of biogenic origin are found in zircon minerals that are between 3.7 and 4.1 bya. These lines of evidence show that liquid water appeared prior to 4 bya and soon thereafter the first life forms appeared.

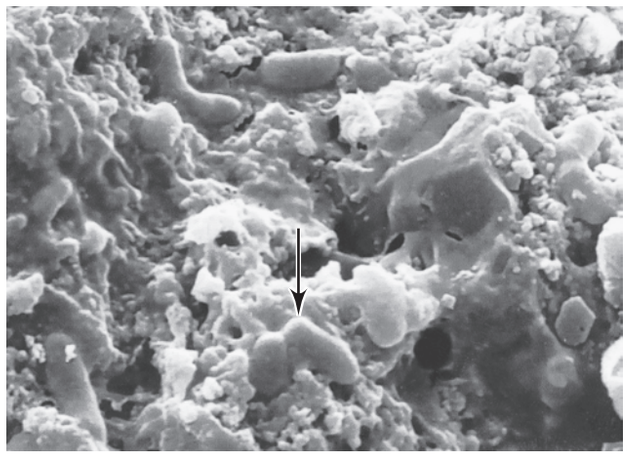


Figure 13.2 Ancient microbial life. Scanning electron micrograph of microfossil bacteria from 3.45-billion-year-old rocks of the Barberton Greenstone Belt, South Africa. Note the rod-shaped bacteria (arrow) attached to particles of mineral matter. The cells are about 0.7 μm in diameter.

Origin of Cellular Life

The origin of life on Earth remains the greatest of mysteries, obscured by the depths of time. Few rocks survive unaltered to testify about this period of Earth's history. Experimental evidence indicates that organic molecules such as RNA nucleotides, amino acids, and lipids can form spontaneously under conditions that were present on the early Earth, providing the preconditions needed for the first living systems. However, conditions on Earth's surface more than 4.1 bya, in particular the extremely hot temperatures and high levels of ultraviolet radiation, were likely hostile to the origin of life as we know it.

Mastering Microbiology
Art Activity: Figure 13.3 Submarine mounds and their possible link to life origins

One hypothesis holds that life may have originated at hydrothermal systems on the ocean floor (**Figure 13.3**). Deep on the ocean floor, protected from the lethal UV radiation at Earth's surface, conditions would have been less hostile and more stable than on land. A steady and abundant supply of energy in the form of reduced

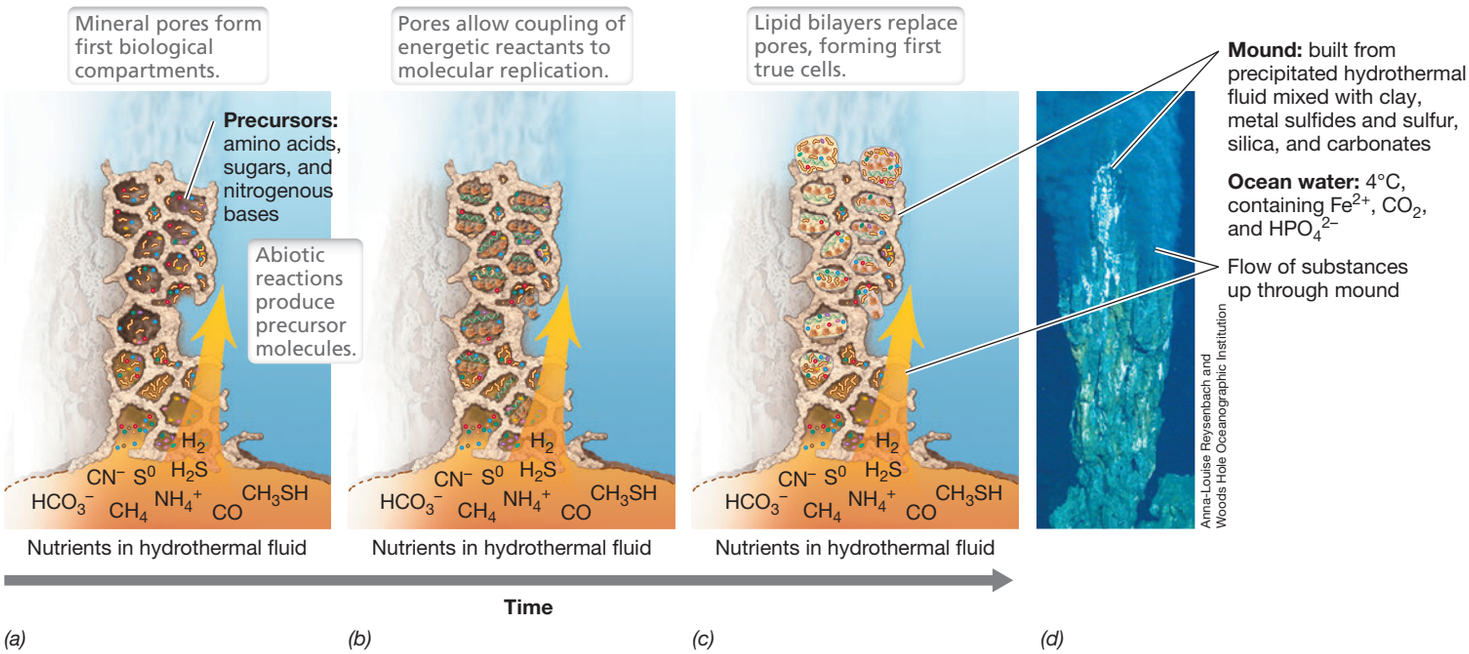


Figure 13.3 Submarine mounds and their possible link to the origin of life. Deep-sea hydrothermal vents are a likely site for the origin of life on Earth. They are stable environments conducive to life and they provide a source of chemical energy, precursors required for the formation of biological molecules, and mineral pores that can serve as compartments to house pre-cellular biochemical reactions. *(a)* Precursor molecules form abiotically and accumulate within mineral pores. *(b)* Chemical gradients that form within mineral pores provide a source of energy that drives the replication of pre-cellular biological molecules. *(c)* Membranes eventually take over the role of mineral compartments, leading to the formation of the first cells. *(d)* Photo of an actual hydrothermal mound. Hot, mineral-rich hydrothermal fluid mixes with cooler, more oxidized ocean water, forming precipitates of Fe and S compounds, clays, silicates, and carbonates. These mineral precipitates form pores that could have served as energy-rich compartments to facilitate the evolution of pre-cellular forms of life.

inorganic compounds—hydrogen (H_2), hydrogen sulfide (H_2S), and elemental sulfur (S^0), for example—would have been available at these hydrothermal systems. The unique geochemistry of these sites can support the abiotic production of molecules critical for the emergence of life, such as amino acids, lipids, sugars, and nucleotides. Furthermore, minerals that form in these systems can produce compartmentalized structures that may have been necessary for conserving energy prior to the emergence of biological membranes. Whether on the seafloor or elsewhere, some form of prebiotic chemistry must have facilitated the development of the first self-replicating systems, the precursors to cellular life.

Molecules of RNA were likely a central component of the first self-replicating systems, and it is possible that life began in an *RNA* (rather than DNA) *world* (**Figure 13.4**). RNA molecules can perform diverse biochemical functions. For example, RNA molecules are the backbone of certain molecules essential to all cells, such as ATP (used in energy transfer), NADH (used in electron transfer), and coenzyme A (used in many biosynthetic reactions) (Chapter 3). RNA can also bind small molecules (such as nucleotides and amino acids), can catalyze certain simple biochemical reactions, and can be the template for its own synthesis. In addition, RNA is able to catalyze protein synthesis through the activities of ribosomal RNAs, tRNAs, and mRNAs (◀ Section 6.10). Hence, it is not unreasonable to imagine that the earliest forms of life relied exclusively on RNAs and had little or no need for DNA or proteins.

Eventually, however, proteins synthesized by RNA molecules replaced the catalytic role of RNAs in primitive cells (proteins can catalyze many reactions that RNA cannot) and at some later point, DNA, a molecule that is inherently more stable than RNA and therefore a better repository of genetic (coding) information, assumed the role of the genome and became the template for RNA synthesis (Figure 13.4). The earliest cellular forms of life likely possessed elements of this three-part system of DNA, RNA, and protein, in addition to a membrane system capable of conserving energy and preventing cell leakage. The *last universal common ancestor* (*LUCA*) of all extant life likely existed at 3.8–3.7 bya, the point at which *Bacteria* and *Archaea* diverged and life began to diversify into the cellular forms we would recognize today.

The Last Universal Common Ancestor

Following the origin of cells, microbial life likely experienced a long period of metabolic diversification, exploiting the various energy resources available on Earth. For much of Earth's history the planet, including all of its oceans, was anoxic (Figure 13.1). Thus, the energy-generating metabolism of primitive cells would have been exclusively *anaerobic*. Furthermore, organic matter was largely absent and so early cells must have been chemolithotrophic, using substances such as H_2 and H_2S spewed out of volcanoes and thermal springs as electron donors. H_2 is also produced abiotically when pyrrhotite (FeS), an abundant volcanic mineral on the early earth, reacts with H_2S to yield pyrite (FeS_2) and H_2. Regardless of the source of H_2, this simple molecule is thought to have been the basis of a primitive energy metabolism based on the proton motive force (**Figure 13.5**). Elemental sulfur (S^0), also abundant on the early Earth, was likely one of the first acceptors of electrons from H_2 oxidation; the reduction of S^0 to yield H_2S is exergonic and can be accomplished by a single enzyme (Figure 13.5). Because organic carbon

Mastering Microbiology
Art Activity: Figure 13.4 Events hypothesized to precede the origin of cellular life

UNIT 3

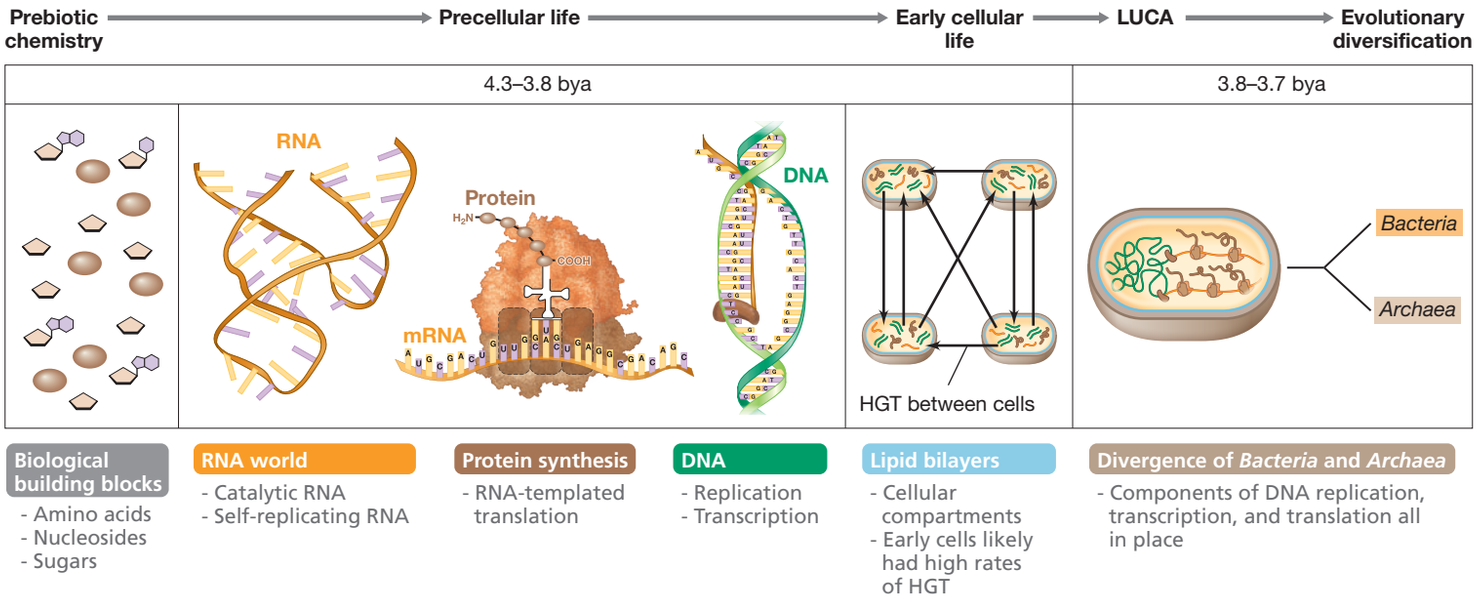


Figure 13.4 Events hypothesized to precede the origin of cellular life. The earliest self-replicating biological systems may have been based on catalytic RNA. At some point RNA enzymes evolved the capability to synthesize proteins, and proteins became the main catalytic molecules. Conversion from RNA- to DNA-based genomes required the evolution of DNA and of RNA polymerases. The lipid bilayer is the site of electron transport, and the evolution of this structure was likely important for energy conservation, in addition to containing and protecting biomolecules. The last universal common ancestor (LUCA), which preceded the divergence of *Bacteria* and *Archaea* (see Figure 13.9), was a cellular organism that had a lipid bilayer and used DNA, RNA, and protein. Horizontal gene transfer (HGT) may have allowed rapid transfer of beneficial genes among early forms of life.

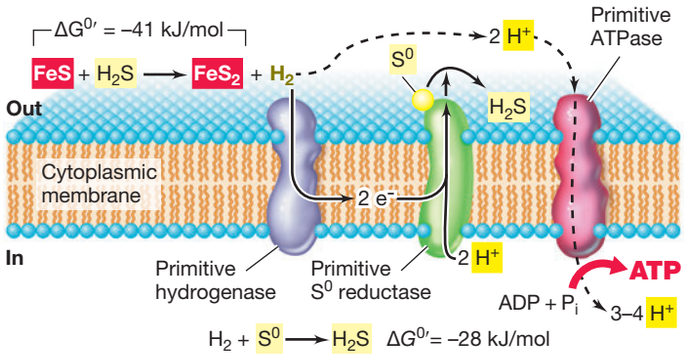


Figure 13.5 A possible energy-conserving scheme for primitive cells. The earliest cells could have generated energy by using H_2 as electron donor and S^0 as electron acceptor, both of which are abundant in hydrothermal systems and are also emitted from volcanoes. H_2 can also be formed from an abiotic reaction between FeS and H_2S, both of which were common on the early Earth. Such reactions could have allowed early cells to colonize diverse habitats.

was scarce on the early Earth, these primitive chemolithotrophs likely used CO_2 and N_2 as major sources of C and N for biosynthesis (see autotrophy and nitrogen fixation, Chapter 3).

By analyzing the gene content of genomes from living organisms across the tree of life, we can identify those genes that were most likely present in the earliest cells. A total of just 355 genes trace back to the last universal ancestor. These genes indicate that LUCA was an anaerobic organism that lived in a high-temperature environment rich in sulfur and iron and used H_2 as a source of energy and CO_2 as a source of carbon. These traits suggest that LUCA could have lived in a hydrothermal environment (Figure 13.3). Unsurprisingly, these cells had all the genetic machinery required for DNA replication, transcription, and translation, as well as ATP synthase. These early cells had the capacity to metabolize H_2 and CO_2, likely producing either acetate (acetogens, ▶ Section 14.14) or methane (methanogens, ▶ Section 14.15) as their waste products. These early forms of chemolithotrophic metabolism along with the reduction of S^0 (Figure 13.5), all driven by H_2 as electron donor, would likely have supported the production of large amounts of organic compounds from autotrophic CO_2 fixation. Over time, these organic materials would have accumulated and provided the conditions needed for the evolution of chemoorganotrophic bacteria with diverse metabolic strategies to conserve energy from the oxidation of organic compounds.

Eventually Earth became a highly oxic planet, with an atmosphere containing the high levels of O_2 that we breathe today (Figure 13.1). Microbes catalyzed this key geochemical transition in Earth's history, and we explore this topic now.

Check Your Understanding

- What characteristics would have made the surface of Earth inhospitable to the formation of life 4.5 billion years ago?
- How do we know when oceans were first present on Earth? Why is the presence of oceans significant to the origins and diversification of life?
- What lines of reasoning support the hypothesis that the first self-replicating systems were based on RNA molecules?

13.2 Photosynthesis and the Oxidation of Earth

The evolution of photosynthesis was a biological breakthrough that radically changed the chemistry of Earth. Phototrophic organisms use energy from the sun to oxidize molecules such as H_2S, S^0, Fe^{2+}, or H_2O and to synthesize complex organic molecules from carbon dioxide or simple organic molecules (▶ Sections 14.2 and 14.3). Over time, the products of photosynthesis accumulated in the biosphere, stimulating the further diversification of microbial life. Earth's first phototrophs were anoxygenic (cells that do not produce O_2, ▶ Sections 14.5 and 15.4–15.7), but from these evolved the *Cyanobacteria*, the earliest O_2-producing (oxygenic) phototrophs (Figure 13.1, ▶ Section 15.3).

Fossilized microbial formations called **stromatolites** can be found in rocks that are 3.5 bya, providing some of the earliest conclusive evidence of life on Earth (**Figure 13.6**). Stromatolites, or "layered rocks," are formed when microbes cause the deposition of carbonate or silicate minerals that promote fossilization (we discuss microbial mats in ▶ Section 20.5). Stromatolites were diverse and common on Earth between 2.8 and 1 bya but declined dramatically in abundance over

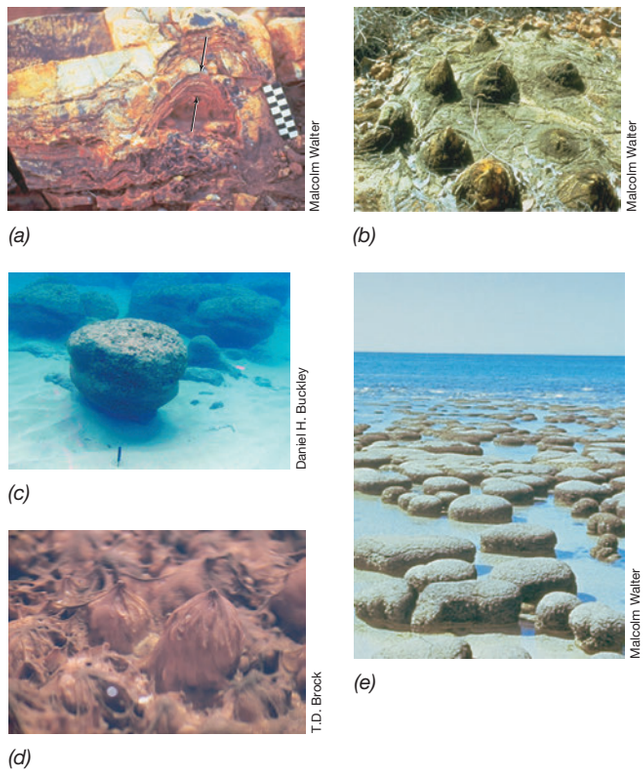
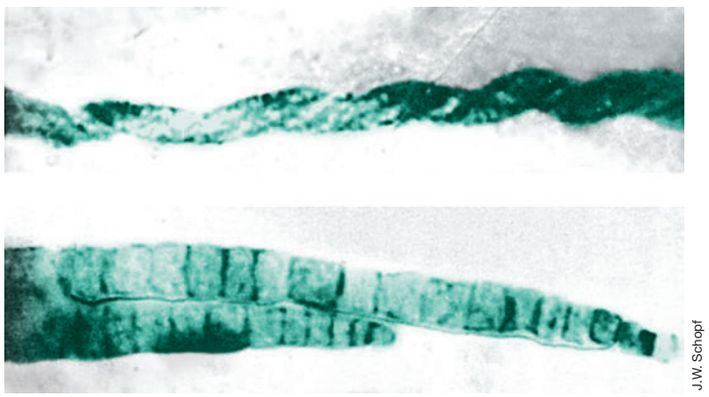


Figure 13.6 Ancient and modern stromatolites. *(a)* The oldest known stromatolite, found in a rock about 3.5 billion years old from the Warrawoona Group in Western Australia. Shown is a vertical section through the laminated structure preserved in the rock. Arrows point to the laminated layers. *(b)* Stromatolites of conical shape from 1.6-billion-year-old dolomite rock from northern Australia. *(c)* Modern stromatolites, Darby Island, Bahamas. The large stromatolite in the foreground is about 1 m in diameter. *(d)* Modern stromatolites composed of thermophilic cyanobacteria growing in a thermal pool in Yellowstone National Park, USA. Each structure is about 2 cm high. *(e)* Modern stromatolites from Shark Bay, Australia. Individual structures are 0.5–1 m in diameter.

the last billion years. Stromatolites are largely gone from Earth today, and yet modern examples of this ancient microbial ecosystem can still be found in certain shallow marine basins (Figure 13.6*c*, *e*) or in hot springs (Figure 13.6*d*). Phototrophic bacteria, such as cyanobacteria (▶ Section 15.3) and anoxygenic phototrophs, play a central role in the formation of modern stromatolites. Ancient stromatolites contain microfossils that appear remarkably similar to modern species of phototrophic bacteria (**Figure 13.7*a***). Hence, the earliest phototrophic organisms may have evolved in the *Bacteria* more than 3.5 bya, giving rise to the stromatolites we observe in the fossil record.

The first phototrophs were anoxygenic and likely used H_2S as electron donor for CO_2 fixation, generating elemental sulfur (S^0) as a waste product (▶ Section 14.5). How could the first phototrophs have evolved at a time when life existed mostly near hydrothermal systems? A clue came from the recent discovery of anoxygenic phototrophs living at hydrothermal vents in the complete darkness of the deep ocean. These phototrophs actually carry out photosynthesis using infrared radiation generated by the heat of hydrothermal vents. Likewise, the first photosynthetic organisms likely lived in the dark, at hydrothermal vents where H_2S and infrared radiation were abundant. Diversification of anoxygenic phototrophs led to species that were able to use a range of electron donors including Fe^{2+}, which was abundant throughout Earth's early oceans. The ability to use Fe^{2+} as an electron donor likely allowed early phototrophs to escape from hydrothermal systems and colonize shallow regions of Earth's early oceans where light was abundant but where overlying water still provided protection from UV radiation.



(a)

(b)

Figure 13.7 More recent fossil bacteria and eukaryotes. *(a)* One-billion-year-old microfossils from central Australia that resemble modern filamentous cyanobacteria. Cell diameters, 5–7 μm. *(b)* Microfossils of eukaryotic cells from the same rock formation. The cellular structure is similar to that of certain modern green algae, such as *Chlorella* species. Cell diameter, about 15 μm. Color was added to make cell form more apparent.

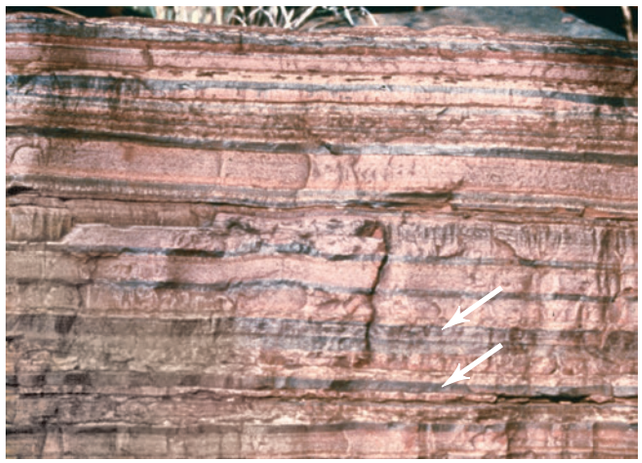
The ability to use solar radiation as an energy source allowed phototrophs to diversify extensively. By 2.5–3.3 bya, the cyanobacterial lineage evolved a photosystem capable of oxygenic photosynthesis (▶ Section 14.6) in which H_2O supplanted H_2S as the reductant for CO_2, thereby generating O_2 as a waste product. About a billion years later, eukaryotic oxygenic phototrophs appeared and can be seen in the microfossil record (Figure 13.7*b*). The origin of oxygenic photosynthesis and the rise of O_2 in Earth's atmosphere (Figure 13.1) caused the greatest change ever in the history of our biosphere and set the stage for the evolution of even newer and more complex forms of life that evolved to exploit the energy available from O_2 respiration. We look at the evidence for and consequences of Earth's oxidative transformation now.

The Rise of Oxygen: Banded Iron Formations

In the absence of O_2, most of Earth's iron would have been present in reduced forms (Fe^0 and Fe^{2+}), and large amounts of iron were dissolved in Earth's anoxic oceans. Molecular and chemical evidence indicates that oxygenic photosynthesis first appeared on Earth several hundred million years before significant levels of O_2 appeared in the atmosphere. However, the O_2 that cyanobacteria produced could not accumulate in the atmosphere because it reacted spontaneously with the reduced iron minerals in the oceans to make iron oxides. By 2.4 bya, O_2 levels had risen to one part per million, a tiny amount by present-day standards, but enough to initiate what has come to be called the *Great Oxidation Event* (Figure 13.1).

The metabolism of cyanobacteria yielded O_2 that oxidized reduced minerals containing Fe^{2+} to iron oxides containing Fe^{3+}. These iron oxide minerals became a prominent marker in the geological record. Iron oxides are poorly soluble in water and would have precipitated in the oceans, raining down onto the seafloor and forming sedimentary structures we see today as **banded iron formations** (**Figure 13.8**), laminated sedimentary rocks formed in deposits of iron- and silica-rich materials. In addition to cyanobacteria, anoxygenic phototrophs that oxidize Fe^{2+} likewise contributed to the formation of these banded iron formations. Much of the iron in rocks of Precambrian origin (>0.5 bya, see Figure 13.1) exists in banded iron formations, and today these minerals are mined as a major source of iron ore. During this span of Earth history, lasting more than 1.5 billion years, the presence of precipitating iron minerals could have caused the oceans to appear brown, or black, or even red rather than the blue color we know today. Only after the abundant Fe^{2+} on Earth was oxidized could O_2 begin to accumulate in the atmosphere, and not until 600–900 million years ago did atmospheric O_2 reach present-day levels (~21%, Figure 13.1).

As O_2 accumulated on Earth, the atmosphere gradually changed from anoxic to oxic. Species of *Bacteria* and *Archaea* unable to adapt to this change were increasingly restricted to anoxic habitats because of the toxicity of O_2 and because it chemically oxidized the reduced substances upon which their metabolisms depend. However, the oxic atmosphere also created conditions for the evolution of various new metabolic schemes, such as sulfide oxidation, nitrification, and the various other aerobic chemolithotrophic processes described in Chapter 14. Microbes that evolved the capacity to respire O_2 gained



John M. Hayes

Figure 13.8 Banded iron formations. An exposed cliff made of sedimentary rock about 10 m in height in Western Australia contains layers of iron oxides (arrows) interspersed with layers containing iron silicates and other silica materials. The iron oxides contain ferric iron (Fe^{3+}) produced from ferrous iron (Fe^{2+}) primarily by the oxygen released by cyanobacterial photosynthesis.

Mastering Microbiology
Art Activity: Figure 13.9 The universal phylogenetic tree as supported by comparative SSU rRNA gene sequence analysis

a tremendous energetic advantage because of the high reduction potential of the O_2/H_2O couple (◀ Section 3.2 and Figure 3.4), and with more energy at their disposal, aerobes could reproduce far more rapidly than anaerobes.

The Ozone Shield

An important consequence of O_2 for the evolution of life was the formation of *ozone* (O_3). The sun bathes Earth in intense amounts of ultraviolet (UV) radiation, which is lethal to cells and can cause severe DNA damage (◀ Section 9.4). When O_2 is subject to UV radiation from the sun, it is converted to ozone, which strongly absorbs UV radiation in wavelengths up to 300 nm. The conversion of O_2 to O_3 creates an *ozone shield*, a barrier that protects the surface of Earth from much of the UV radiation from the sun. Prior to the generation of the ozone shield, the punishing UV irradiation from the sun would have made Earth's surface fairly inhospitable for life, restricting life to environments that provided protection from UV radiation, such as in the oceans or in the subsurface. However, as Earth developed an ozone shield, organisms could range over the terrestrial surface of Earth, exploiting new habitats and evolving ever-greater diversity. Figure 13.1 summarizes some landmarks in biological evolution and Earth's atmospheric chemistry as Earth transitioned from an anoxic to a highly oxic planet.

Microscopic examination of ancient rocks has revealed timelines for the origin of life and major changes in Earth's geochemistry. But what about modern organisms—how can we unravel their evolutionary relationships? We consider next how this is possible.

Check Your Understanding

- Why is the origin of cyanobacteria considered a critical step in evolution?
- What caused the formation of banded iron formations?
- What lines of evidence indicate that microbial life was present on Earth 3.5 billion years ago?

13.3 Living Fossils: DNA Records the History of Life

The evolutionary origins of microorganisms remained a mystery until the discovery that certain molecular sequences are a record of evolutionary history (◀ Section 1.15). Mutations occur at random and accumulate over time, causing heritable changes in the sequence of DNA (Chapter 9) that drive the evolutionary process. Hence, organisms that share a recent ancestor have similar DNA sequences, and organisms that are more distantly related have DNA sequences that are more dissimilar. This means we can reconstruct the evolutionary history, or **phylogeny**, of a set of related DNA sequences by analyzing similarities in their nucleotide sequences (see Section 13.11).

The Universal Tree of Life

Carl Woese was the first to construct a **universal tree of life** that he inferred from nucleotide sequence similarity in the **ribosomal RNA (rRNA)** genes of diverse organisms (◀ Section 1.15). The universal tree of life (**Figure 13.9**) is a genealogy of life on Earth. It depicts the evolutionary history of all cells and clearly reveals the three-domain concept in which all cells can be classified as *Bacteria*, *Archaea*, or *Eukarya*. The root of the universal tree represents a point in time when the common ancestor of all life on Earth today, the last universal common ancestor, LUCA (Figures 13.4 and 13.9), was alive. The universal tree of life shows that the first living things were microorganisms, and that microbes were the dominant life form for most of the history of life on Earth.

Genomics (Chapter 10) supports the three-domain concept through phylogenetic analysis of most genes central to cellular function. For example, at least 60 genes (including rRNA genes) are shared by nearly all modern cells, and these genes were likely present in the universal ancestor. Most of these genes encode core functions in transcription, translation, and DNA replication. Across these conserved genes, those of *Eukarya* show greater sequence similarity to those of *Archaea* than to those of *Bacteria*. These and other data support the conclusion that the *Bacteria* and *Archaea* diverged prior to the origin of eukaryotic cells (Figure 13.9).

Horizontal Transfer and the Tree of Life

The manner in which the three domains were established remains a topic of debate. It is clear that the three domains represent the major evolutionary cell lineages that exist on Earth, and also clear that *Bacteria* and *Archaea* diverged prior to the divergence of *Eukarya* from *Archaea*. However, there are many examples of genes shared by any two of the three domains. One hypothesis is that early in the history of life, before the primary domains had diverged, *horizontal gene transfer* (Section 13.9 and Chapter 9) was extensive. During this time, any genes that provided a strong benefit likely were transferred rapidly among early forms of life (Figure 13.4). As the domains continued to diverge over time, barriers to unrestricted horizontal gene transfer evolved in order to maintain genomic stability. As a result, the previously promiscuous cell populations began to diverge into the primary lines of evolutionary descent we recognize today (Figures 13.4 and 13.9). However, the ability of genes to move horizontally between different microbial species

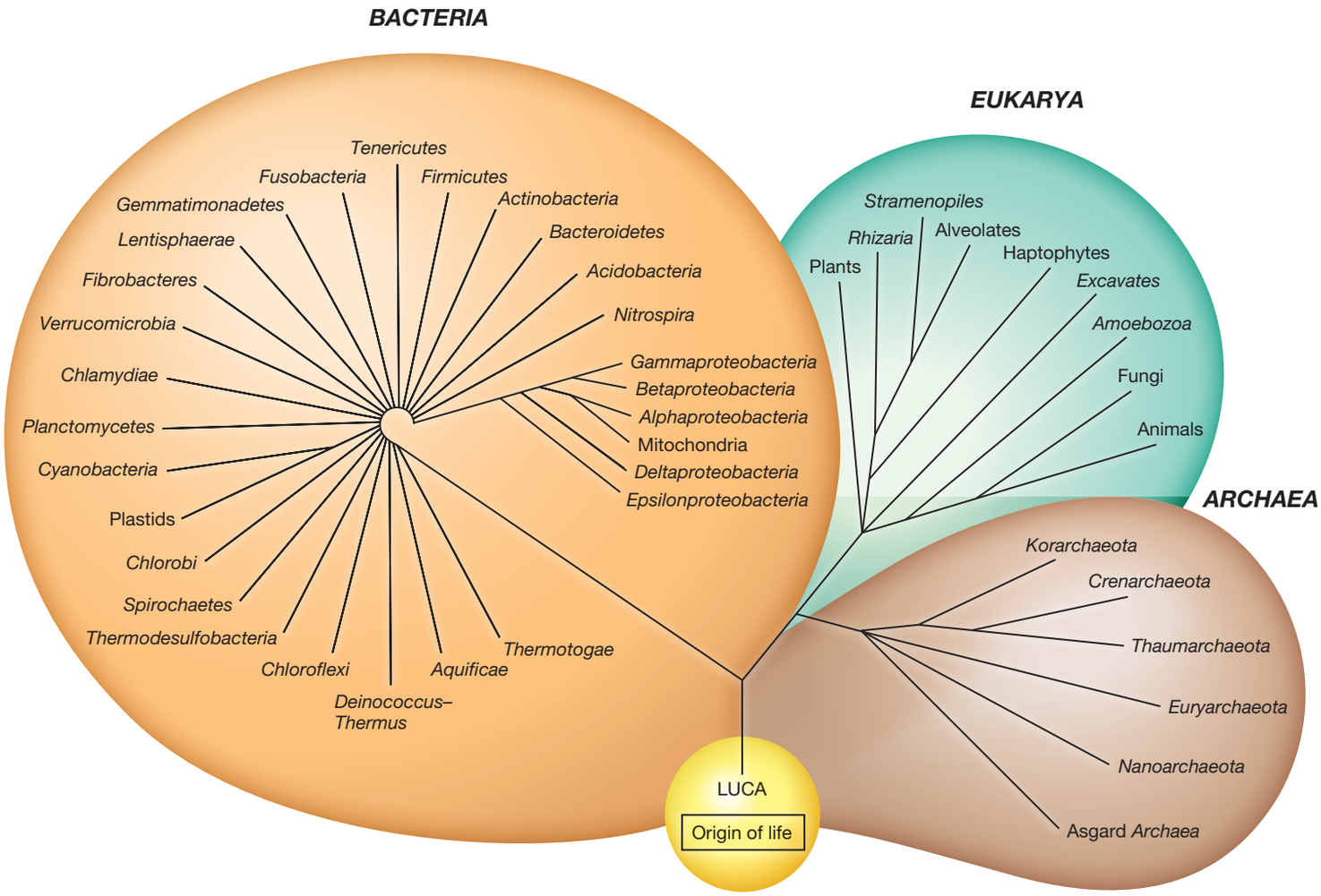


Figure 13.9 Schematic universal phylogenetic tree as supported by comparative analysis of small subunit (SSU) rRNA gene sequences. Only a few key organisms or lineages are shown in each domain. The branch lengths in this tree are arbitrary, and nodes have been collapsed to reflect phylogenetic uncertainty. At least 84 phyla of *Bacteria* have now been identified, although many of these have not yet been cultured. LUCA, last universal common ancestor (Figure 13.4). The procedures used to generate phylogenetic trees are discussed in Section 13.11.

complicates our understanding of microbial phylogeny, a concept we will return to later in the chapter.

It seems likely that the domains *Bacteria* and *Archaea* had already diverged by about 3.7 billion years ago (Figure 13.1). Following this, there was a further bifurcation, somewhere between 2.7 and 1.5 billion years ago, at which time the *Eukarya* diverged from *Archaea* to form a distinct domain. As each domain continued to evolve, certain traits became fixed within each group, giving rise to a multitude of genetic, physiological, and structural differences (Chapters 2, 9, 10, and 14–18). After nearly 4 billion years of microbial evolution, we see the grand result: three domains of cellular life that are each evolutionarily distinct and yet share certain features that reveal their common descent from a universal cellular ancestor.

Before we turn our attention to the evolutionary process per se, we focus briefly on the origin of the eukaryotic cell and eventually the domain *Eukarya*, a phylogenetically distinct group (Figure 13.9) whose cell structure contrasts with the prokaryotic cell structure of the *Bacteria* and *Archaea* (Chapter 2). The major properties and phylogentic diversity of microbial eukaryotes are discussed in Chapter 18.

Check Your Understanding

- What kinds of evidence support the three-domain concept of life?
- What is LUCA and what are some of its characteristics?
- Between *Bacteria* and *Archaea*, which domain shares more similarities with *Eukarya*?

13.4 Endosymbiotic Origin of Eukaryotes

The evolution of the eukaryotic cell marked a major milestone in cellular evolution that paved the way for the evolution of complex multicellular organisms. Here we consider the origin of the *Eukarya* and show how eukaryotes are genetic chimeras containing genes from both *Archaea* and *Bacteria*.

The Last Eukaryotic Common Ancestor and Its Genetic Makeup

For around two billion years, about half the time life has existed on Earth, the Earth was anoxic and all living things had prokaryotic cell structure. The origin of the eukaryotic cell and the evolution of multicellularity roughly coincide with the oxygenation of Earth's atmosphere, and this event may have facilitated eukaryote evolution. While the exact origins of the eukaryotic cell remain unclear, the oldest microfossils that have recognizable nuclei are about 2 billion years old. Multicellular and increasingly complex microfossils of algae are evident from 1.9 to 1.4 billion years ago (Figure 13.7*b*). By 0.6 billion years ago, with O_2 near present-day levels, large multicellular organisms, the Ediacaran fauna, were present in the sea. In a relatively short time geologically speaking, multicellular eukaryotes diversified into the ancestors of modern-day algae, plants, fungi, and animals during what has been coined the "Cambrian explosion" (Figure 13.1).

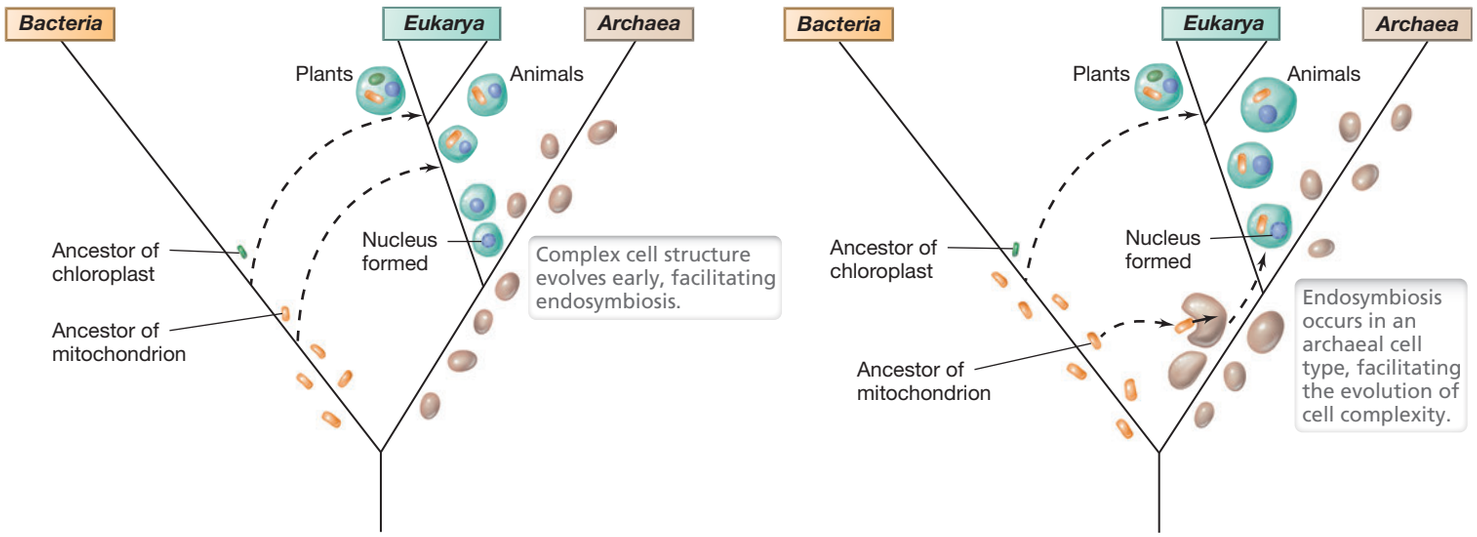
The exact pathway by which the eukaryotic cell emerged remains a major unresolved question in evolution; however, it is clear that the modern eukaryotic cell is a genetic chimera, containing genes from both *Bacteria* and *Archaea*. By analyzing the genomes of living organisms across the tree of life (Figure 13.9), we can identify those genes that were most likely present in the *last eukaryotic common ancestor* (LECA), the ultimate ancestor of all eukaryotic organisms.

A total of about 4000 genes trace back to LECA, and this organism had all the characteristic features of eukaryotic cells. The latter included a nucleus, mitochondria that respired O_2, organelles, a complex cytoskeleton, spliceosomal machinery and genes with introns (Chapter 6), and other eukaryotic characteristics (◀ Sections 2.13–2.15). More than 70% of these 4000 genes exist only in *Eukarya*, which clearly reveals the *Eukarya* to be a unique domain of life unambiguously differentiated from the *Bacteria* and *Archaea*. However, LECA shares its remaining genes with the *Bacteria* and *Archaea*. Of these genes, about two-thirds of them derive from *Bacteria*, whereas the remaining third are from *Archaea*. The genes of bacterial origin encode mostly metabolic functions, while the genes of archaeal origin encode the information-processing machinery of the cell. For example, eukaryotic cells share with *Archaea* molecular features of transcription and translation (Chapter 6), while features eukaryotes share with *Bacteria* include their ester-linked membrane lipids (◀ Section 2.1) and glycolytic pathway (◀ Section 3.6).

Endosymbiotic Theory

A well-supported explanation for the origin of organelles in the eukaryotic cell is the **endosymbiotic theory**, in which at least two scenarios have been proposed (**Figure 13.10**). This theory states that the mitochondrion of modern-day eukaryotes arose from the stable incorporation of a bacterium capable of aerobic respiration into the cytoplasm of an early eukaryotic cell. Endosymbiotic mitochondria would have been beneficial to these early eukaryotic cells because they increased the cell's respiratory capacity, and thus these early mitochondria-containing cells proceeded to become the ancestors of all living *Eukarya*. Virtually all eukaryotic cells have mitochondria (◀ Section 2.14), though these structures were subsequently lost in certain lineages of anaerobic microbial eukaryotes (Chapter 18).

A second endosymbiotic event, in an important lineage of the *Eukarya*, also had a major impact on the evolution of life. Chloroplasts arose, after the acquisition of mitochondria, from the



(a) **Serial endosymbiosis hypothesis** *(b)* **Symbiogenesis hypothesis**

Figure 13.10 Endosymbiotic models for the origin of the eukaryotic cell. *(a)* The serial endosymbiosis hypothesis proposes that the eukaryotic ancestor diverged from the archaeal line and possessed a nucleus and other features of eukaryotic cells prior to endosymbiosis with the bacterial ancestor of the mitochondrion. A later endosymbiosis with the cyanobacterial ancestor of the chloroplast gave rise to the eukaryotic ancestor of all plants and all other photosynthetic eukaryotes. *(b)* The symbiogenesis hypothesis proposes that the eukaryotic cell evolved from a symbiotic relationship between *Bacteria* and *Archaea*. The bacterial partner was eventually engulfed by its archaeal partner and evolved over time into the mitochondrion. The nucleus and other features of the eukaryotic cell evolved after establishment of endosymbiosis. A later endosymbiosis with the cyanobacterial ancestor of the chloroplast gave rise to the eukaryotic ancestor of all plants and all other photosynthetic eukaryotes. Note the position of the mitochondrion and plastids (chloroplasts are a type of plastid) on the universal phylogenetic tree (Figure 13.9).

stable incorporation of a cyanobacterium-like cell into the cytoplasm of a eukaryotic lineage, and this endosymbiotic event triggered the origin of photosynthesis within *Eukarya* (Figure 13.10). All phototrophic eukaryotes, including plants and algae, have descended from the lineage of cells that acquired endosymbiotic chloroplasts. Atmospheric oxygen is intimately associated with the endosymbiotic origins of organelles, being consumed by the ancestor of the mitochondrion and being produced by the ancestor of the chloroplast. These endosymbiotic events diversified the metabolism of early eukaryotic cells, with mitochondria providing aerobic respiration and chloroplasts providing the ability to exploit sunlight for energy. Moreover, the endosymbiotic origin of organelles set the stage for the diversification of *Eukarya* into the forms we know today.

Several lines of evidence support the endosymbiotic theory. The earliest evidence that suggested an endosymbiotic origin of organelles was that chloroplasts and mitochondria are about the same size as bacteria and they replicate independently within the cytoplasm of the eukaryotic cell. However, the endosymbiotic theory rests largely on evidence derived from molecular biology. First, mitochondria and chloroplasts contain their own genomes composed of bacterial genes. These genomes are circular like those of *Bacteria* and encode proteins of the respiratory chain (mitochondrion) and photosynthetic apparatus (chloroplast) as well as ribosomal RNAs and transfer RNAs. Second, both mitochondria and chloroplasts contain bacterial ribosomes. The **16S rRNA** gene sequences of mitochondria and chloroplasts are also characteristic of those from *Bacteria*, and sequence analyses place the ancestor of mitochondria in the **phylum** *Proteobacteria*, and the ancestor of chloroplasts in the phylum *Cyanobacteria* (Figure 13.9). (Phyla are the major lineages of organisms within the *Bacteria*, *Archaea*, and *Eukarya*; see Section 13.12 and Table 13.1.) Third, the same antibiotics that inhibit ribosome function in free-living *Bacteria* inhibit ribosome function in these organelles. Fourth, the eukaryotic nucleus contains genes derived from *Bacteria*. Genomic sequences of eukaryotic cells have revealed genes that encode functions specific to mitochondria and chloroplasts. Moreover, because these gene sequences more closely resemble those of *Bacteria* than those of *Archaea* or *Eukarya*, it is concluded that these genes were translocated to the nucleus from ancestors of mitochondria and chloroplasts during the initial stages of endosymbiosis.

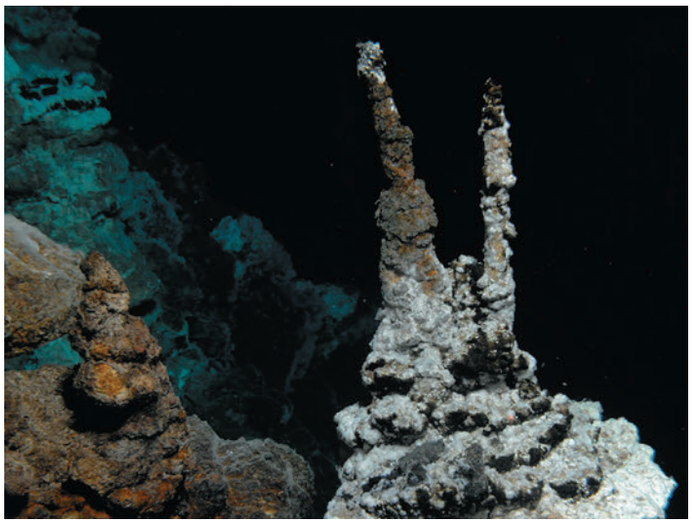
Formation of the Eukaryotic Cell

It is generally agreed that the acquisition of mitochondria was the critical event that led to the origin of the eukaryotic cell and establishment of the domain *Eukarya*. It is also thought that most of the bacterial genes found in eukaryotic genomes were transferred from the genome of the mitochondrial ancestor. However, the phylogenetic nature of the *cell* that acquired the mitochondrion remains a matter of debate; but even here, molecular evidence is paving the way to an answer.

The fact that the genes encoding DNA replication, transcription, and translation in *Eukarya* are derived from *Archaea* is generally used to support the hypothesis that the *Archaea* are ancestral to *Eukarya*. The recent discovery of *Lokiarchaeota*—a new phylum of *Archaea*—has provided fresh insights into the origin of *Eukarya*. *Lokiarchaeota* were discovered through metagenomic analyses (◀ Section 10.7) of microbial communities that inhabit deep marine sediments near a hydrothermal vent system known as Loki's Castle (**Figure 13.11**), located along the Mid-Atlantic Ridge between Greenland and Norway. The genomes of *Lokiarchaeota* contain a number of eukaryotic features that suggest the presence of a eukaryotic-like cytoskeleton (◀ Section 2.15) and the ability to synthesize intracellular membranes, traits that may have facilitated stable integration of an endosymbiont. Because of these strong genetic links to eukaryotes, it is thought that *Lokiarchaeota* may be close ancestors of the cell line that ultimately gave rise to the *Eukarya*.

The exact series of events that led to the eukaryotic cell is unknown, but two major pathways have been proposed (Figure 13.10). In the *serial endosymbiosis hypothesis*, eukaryotes arose from a complex nucleus-bearing cell line that diverged from the *Archaea* and later acquired mitochondria and chloroplasts by endosymbiosis (Figure 13.10*a*). Endosymbiosis is posited to have occurred when this cell line engulfed or was invaded by a bacterial cell that, rather than being destroyed, managed to survive and replicate as an endosymbiont within the cytoplasm of the host cell. According to this hypothesis, eukaryotic genes that resemble those of *Bacteria* were acquired in gene transfers from the endosymbiont to the nuclear genome.

A second hypothesis for the formation of the eukaryotic cell is called *symbiogenesis* and proposes that the eukaryotic cell arose from a symbiotic relationship between cells of *Bacteria* and *Archaea* that ultimately resulted in engulfment of the bacterial partner to form mitochondria (Figure 13.10*b*). One version of symbiogenesis proposes that the eukaryotic cell arose from an association between a H_2-producing species of *Bacteria* and a H_2-consuming species of *Archaea*. This "hydrogen hypothesis" proposes that the bacterial cell was a facultative aerobe and a chemoorganotroph that could grow by aerobic respiration or by syntrophic production of H_2 (▶ Section 14.22). The archaeal partner was an anaerobic chemolithotroph that required



Rolf B. Pedersen

Figure 13.11 Habitat of *Lokiarchaeota* and the origin of the *Eukarya*. *Lokiarchaeota* were discovered in a metagenomic survey of marine sediments near a hydrothermal vent system known as Loki's Castle (shown in photo). These *Archaea* share certain key similarities with *Eukarya* and might be the closest known relatives of the last eukaryotic common ancestor.

H_2 for growth. These symbiotic partners could have coevolved for an extended period prior to bacterial engulfment and formal development of the mitochondria.

The origin of the nucleus was critical to the evolution of the eukaryotic cell, yet it remains unclear whether the nucleus appeared before or after the endosymbiotic origin of mitochondria. One hypothesis for the origin of the nucleus is that its formation is associated with the evolution of RNA processing in *Eukarya*. In contrast to the genes of *Bacteria* and *Archaea*, eukaryotic genes frequently contain introns (◀ Section 6.6). For proper gene expression to occur in *Eukarya*, RNA splicing must remove introns prior to translation. Eukaryotic cells have a molecular complex called the *spliceosome* that performs RNA splicing and operates in the cell nucleus. Hence, the nuclear membrane may have evolved in *Eukarya* as a mechanism to separate spliceosomes (enzymes that can cut RNA) in the nucleus from ribosomes (major sources of RNA) in the cytoplasm.

Check Your Understanding

- What evidence supports the idea that the mitochondrion and chloroplast were once free-living members of the domain *Bacteria*?
- In what ways are modern eukaryotes a combination of attributes of *Bacteria* and *Archaea*?
- Describe the different hypotheses for the formation of the eukaryotic cell.

13.5 Viral Evolution

The tree of life does not contain viruses, and for good reason: Even though viruses show some of the properties of cells, viruses are not cells (Chapters 5 and 11). Among other cellular deficiencies, viruses lack ribosomes (and hence, ribosomal RNA), so it has been impossible to place viruses on the universal tree of life (Section 13.3 and Figure 13.9). Nevertheless, the question of when viruses first appeared on Earth and what their relationship is to cells remains an intriguing question and one to which several hypotheses have been proposed.

All known viruses require a host cell for their replication, and this leads to the natural conclusion that viruses evolved sometime *after* cells appeared on Earth. However, viruses have very few genes, they evolve rapidly, and their genes are only shared among closely related viruses. Hence there are no universally conserved viral genes. As a result, it is impossible to generate a tree of life that contains viruses, or even a universal phylogenetic tree of viruses (a phylogenetic tree of a group of viruses that share a common gene is possible, however, ◀ Figure 11.5*b*). Indeed, it is possible that viruses have multiple origins and that different types of viruses have evolved independently over the history of life.

Viruses as Selfish DNA

Mobile DNA elements such as plasmids and transposons are well known (see Section 13.9 and Chapter 9). These elements have been called "selfish DNA" because they replicate independently of the host chromosome, contain genes that enable their transfer between cells, have discrete host ranges, and make no contribution to an organism's fitness (and may even be harmful). Ultimately, viruses are not much different from these selfish DNA elements. For example, many viruses reside indefinitely in the host's cytoplasm or integrated into its genome and do not kill the host cell immediately or at all. The main differences between viruses and mobile DNA are that viruses typically have higher rates of replication, often resulting in the death of the host cell, and viral DNA is encased in a protein capsid that facilitates transmission between cells. There exists a diversity of viral capsid proteins, however, and so it is likely that different viral families have different evolutionary origins.

One clue as to viral origins comes from the discovery of *gene transfer agents*. Gene transfer agents are bacteriophage-like particles that transfer genes between cells (◀ Section 9.7). Gene transfer agents facilitate a type of horizontal gene transfer and are encoded by genes present on the chromosome. These genes produce a capsid within which is packaged a piece of chromosomal DNA. This capsid allows for gene transfer between cells, a trait that can be beneficial for cell populations. Gene transfer agents share ancestry with viruses, but the nature of this relationship remains disputed. One possibility is that viruses appeared first and gene transfer agents evolved from defective bacteriophages. But it is also possible that gene transfer agents appeared first and viruses evolved when some of these agents gained the ability to package the genes necessary for their own production and escaped from cellular control (**Figure 13.12*a***). It is clear that different gene transfer agents evolved independently in various lineages of bacteria and that these agents are very ancient. Hence, viruses may have evolved on multiple occasions as rogue mobile genetic elements that escaped from host control by exploiting preexisting genetic mechanisms for DNA replication and transmission (see MicrobiologyNow on page 428).

Viruses as a Vestige of Pre-Cellular Life

Structural features shared among viral proteins have been suggested as a method to probe viral evolution. While no genes or proteins are shared among all viruses, certain structural domains, those parts of proteins that have discrete conserved functions (◀ Section 6.7), have been found in a diverse array of viruses. It is possible that the analysis of these conserved structural domains will provide new insights into how viruses first appeared on Earth.

Analyses of conserved structural domains shared among viral proteins suggests an ancient origin of viruses. Such analyses provide evidence that the earliest viruses had RNA genomes and may have existed prior to the origin of cellular life (Figure 13.12*b*). Some form of self-replicating RNA must have existed in the "RNA world" prior to the origin of cells (Section 13.1). Viruses may have originated from within this RNA world and taken an alternate evolutionary path from that of cellular life. Some of this pre-cellular RNA may have adapted to the appearance of cells by becoming obligate parasites. Under this scenario, viruses are a holdover from the pre-cellular days of life and have been infecting cells ever since the origin of cellular life itself.

Check Your Understanding

- Why is it difficult to place viruses on the universal tree of life?
- Explain the hypothesis that viruses have evolved many different times from mobile DNA elements.
- Explain the hypothesis that viruses first evolved prior to the appearance of cellular forms of life.

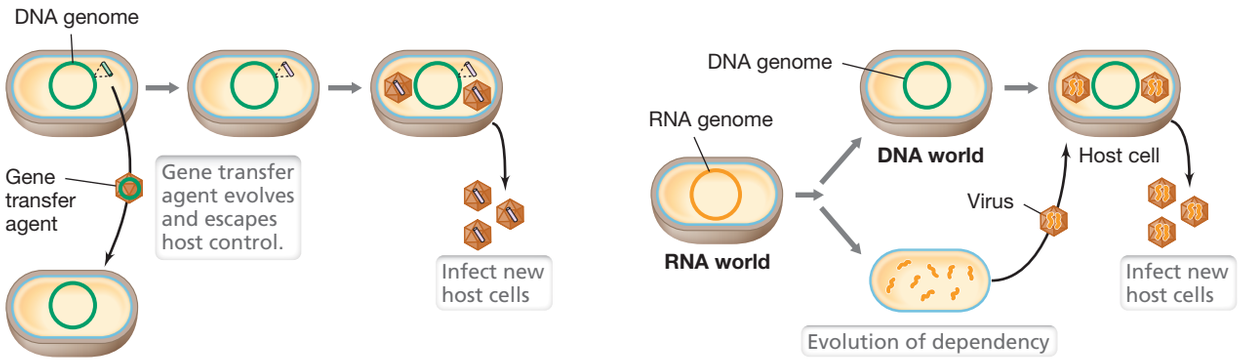


Figure 13.12 Hypothetical schemes for viral origins. *(a)* Gene transfer agents are mobile genetic elements that facilitate horizontal gene transfer by packaging host DNA and exporting it in a capsid. Random mutations in the DNA encoding gene transfer agents could have resulted in their escape from host control, leading to replication and transmission of infective agents that evolved into viruses. *(b)* Prior to the evolution of modern cells, during the RNA world (Figure 13.4), RNA-based life forms might have been highly interdependent. Following the origin of DNA, these RNA-based organisms could have evolved further dependency to the point that their replication could only take place within DNA-based cells, thereby resulting in the evolution of viruses.

II • Mechanisms of Microbial Evolution

Evolution modifies a microbe's genome over long time periods through mutations and genetic recombination. Organisms can also express new properties quickly when they receive genes from other cells. The ability of microbes to grow rapidly allows microbiologists to document evolutionary events in the laboratory.

While many of the basic principles of evolution are conserved across all domains of life, certain aspects of microbial evolution are uncommon in plants and animals. For example, *Bacteria* and *Archaea* are generally haploid and asexual, they have several mechanisms for horizontal gene transfer that result in the asymmetrical exchange of genetic material uncoupled from reproduction, and their genomes can be remarkably heterogeneous and highly dynamic. Despite these differences, we can study microbial evolution and explore the evolutionary history of life by directly comparing the genomes of living organisms. In this part of the chapter, we consider the processes that cause the diversification of microbial lineages and how these forces affect the evolution of microbial genomes.

13.6 The Evolutionary Process

In its simplest form, evolution is a change in **allele** frequencies in a population of organisms over time, resulting in descent with modification. Alleles are alternate versions of a given gene. New alleles and totally new genes arise as a result of mutation and recombination, and changes in allele frequencies can occur through a variety of processes. We see here how these simple mechanisms give rise to the origin and divergence of microbial species.

Origins of Genetic Diversity

As we have seen, **mutations** are random changes in DNA sequence that accumulate over time, and they are a fundamental source of the natural variation that drives the evolutionary process. Most mutations are neutral or deleterious, though some can be beneficial. Mutations take several forms including *substitutions*, *deletions*, *insertions*, and *duplications* (Chapter 9). Gene duplication events produce a redundant copy of a gene that can be modified by further mutation without losing the function encoded by the original gene (Section 13.8). Hence, duplications allow for the diversification of gene function.

Recombination is a process by which segments of DNA are broken and rejoined to create new combinations of genetic material (◀ Section 9.5). Recombination can cause reassortment of genetic material already present in a genome and is also required for the integration of DNA acquired through horizontal gene transfer. Recombination can be broadly classified as either *homologous* or *nonhomologous*. Homologous recombination requires short segments of highly similar DNA sequence flanking the region of DNA being transferred. By contrast, nonhomologous recombination is mediated by several mechanisms that share in common the fact that they do not require high levels of sequence similarity to initiate successful DNA integration.

Selection and Genetic Drift

New alleles and new genes result when mutation and recombination cause variation in gene sequences. Evolution occurs when different alleles change in frequency in a population over a span of many generations. Evolutionary biologists have described many different mechanisms that can govern this evolutionary process, but chief among them are the forces of *selection* and *genetic drift*.

Evolutionary selection is defined on the basis of **fitness**, the ability of an organism to produce progeny and contribute to the genetic makeup of future generations. Most mutations are *neutral* with respect to fitness and they have no effect on the cell, as a result of the degeneracy of the genetic code (◀ Section 6.9). These mutations generally accumulate in DNA over time. Some mutations are *deleterious*, and these decrease the fitness of an organism by disrupting gene function. Deleterious mutations are generally purged

from populations over time by natural selection. Some mutations can be *beneficial*, increasing the fitness of an organism, and these mutations are favored by natural selection, increasing in frequency in a population over time. An example of a beneficial mutation would be a mutation that induces antibiotic resistance in a pathogenic bacterium infecting a person undergoing antibiotic therapy. It is important to remember that all mutations occur by chance; the selective nature of the environment does not *cause* adaptive mutations but simply *selects* for the growth and reproduction of those organisms that have incurred mutations that provide a fitness advantage.

While Darwin proposed natural selection as the mechanism by which gene frequencies change over time, evolutionary change can occur through mechanisms other than selection. A chief example is **genetic drift** (Figure 13.13), a random process that can cause gene frequencies to change over time, resulting in evolution in the absence of natural selection. Genetic drift occurs because some members of a population will have more offspring than others simply as a result of chance. Over time, these chance events can result in evolutionary change in the absence of selection. Genetic drift is most powerful in small populations and in populations that experience frequent "bottleneck" events. The latter occur when a population experiences a severe reduction in population size followed by regrowth from the cells that remain. For example, genetic drift can be very important in the evolution of pathogens because each new infection is caused by a small number of cells colonizing a new host. Hence, pathogen populations can change rapidly as a result of random genetic drift (Figure 13.13).

Origins of Microbial Species

Microbial species can be very old and can encompass a wide variety of individual strains having diverse traits (we consider the microbial species concept in Section 13.12). The accumulation of mutations over time can be used to explore the evolutionary origins of species. Sequence changes due to mutation can be used as a **molecular clock** to estimate the time since a species has diverged from its closest relatives. Major assumptions of the molecular clock approach are

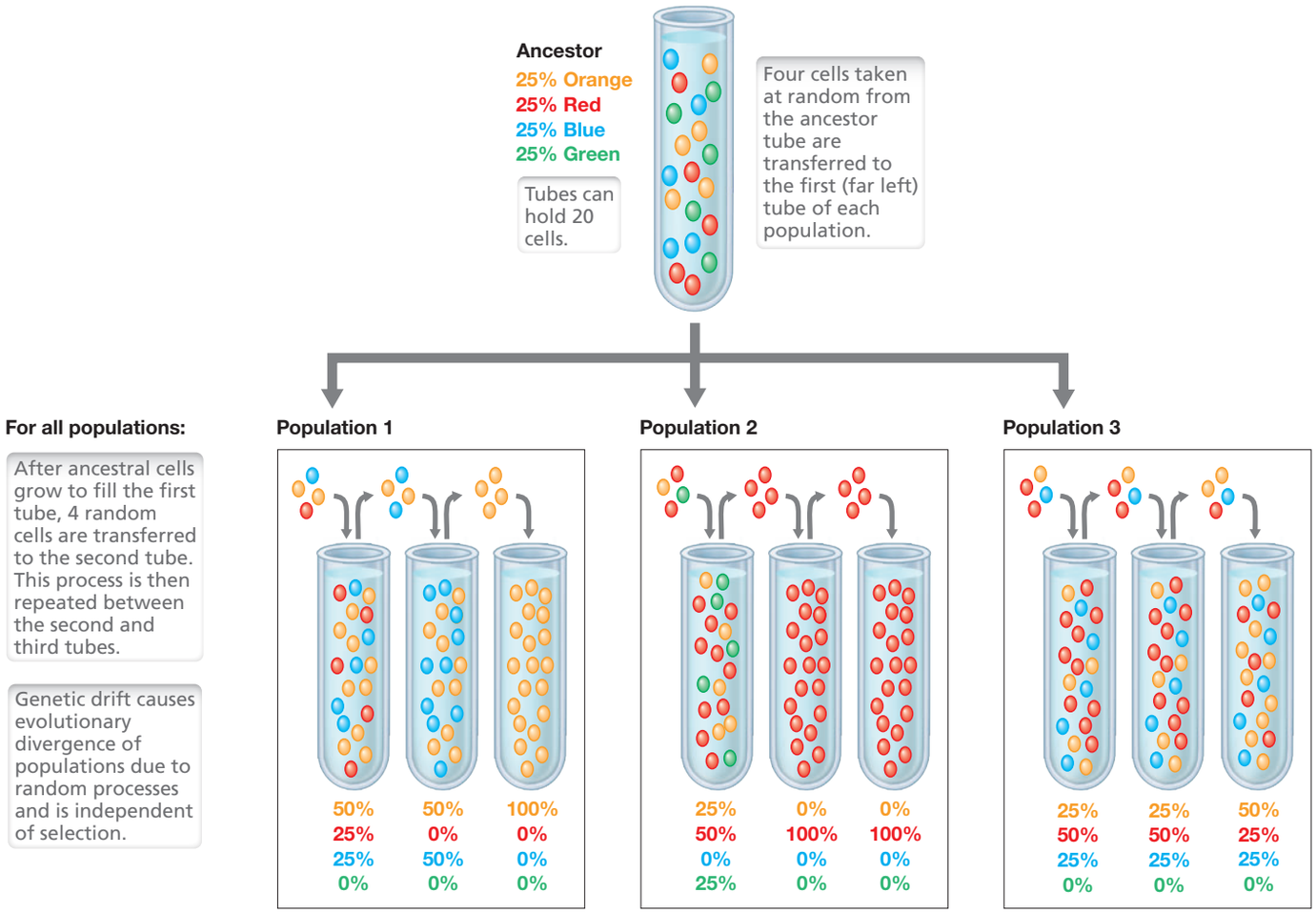


Figure 13.13 Genetic drift. Genetic drift is a random process that can cause gene frequencies in a population to change over time, causing evolution without natural selection. In this example, a population containing four different bacterial genotypes (indicated by different colors), each at equal frequency, is present in the ancestor tube. Four cells at random are then transferred to each of three new tubes and the cells allowed to grow to fill each tube. There is no difference in fitness between the cells and so they grow equally. Cells taken at random are then transferred in two successive rounds. Striking differences in genotype frequencies between the populations are observed after only three rounds of transfers.

that nucleotide changes accumulate in a sequence in proportion to time, that such changes are generally neutral and do not interfere with gene function, and that they are random. Molecular clock estimates are most reliable when they can be calibrated with evidence from the geological record. The most well documented of these have used obligate bacterial symbionts of insects (▶ Section 23.7) for which the insect host provides a fossil record to accurately date evolutionary events.

Molecular clock estimates make it possible to estimate the divergence of microbial species based on dissimilarity in their gene sequences. For example, molecular clock estimates indicate that the closely related species *Escherichia coli* and *Salmonella enterica* (*typhimurium*), which have 2.8% dissimilarity in their 16S rRNA gene sequences, last shared a common ancestor some 100–140 million years ago. Likewise, it is estimated that two well-characterized strains of *E. coli*, the harmless strain K-12 and the foodborne pathogenic strain O157:H7 (▶ Section 33.11), diverged about 4.5 million years ago. Many microbial species, like *E. coli*, can encompass an enormous number of different strains possessing a diversity of traits that collectively define the species. Molecular clock estimates reveal that microbial speciation takes a very long time. However, we will see in the next section that while speciation can take a long time, strains of a species can acquire new traits rapidly.

Check Your Understanding

- How do gene duplication and recombination result in genetic diversity?
- How are molecular clock estimates used to infer evolutionary origins of species?

13.7 Experimental Evolution

Experimental evolution is a field that employs laboratory experiments with microorganisms to investigate evolutionary processes. Such evolution experiments are enabled by the rapid growth of bacteria and the ability to preserve them indefinitely by freezing. The latter makes it possible to maintain a living "fossil record" of ancestral organisms that can be thawed later and grown to compare with evolved strains. Microorganisms typically form large populations and can reproduce quickly, producing a new generation in as few as 20 minutes for some species, and thus evolutionary events in microbial populations can often be observed in the laboratory on relatively short time scales. Here we consider two examples of rapid evolutionary change in bacteria, one involving the rapid *loss* of a characteristic trait in *Rhodobacter*, and one involving the *acquisition* of a new trait in *Escherichia coli*.

Loss of Function

Rhodobacter is a phototrophic purple bacterium that carries out anoxygenic photosynthesis (▶ Section 14.5) in illuminated anoxic environments in nature. When cultured anaerobically, the cells synthesize photopigments in both the presence and the absence of light because pigment synthesis is regulated by O_2 availability and not by light. In the light these pigments participate in photosynthetic reactions that lead to ATP synthesis, but in darkness, the pigments provide no benefit to the cell. Random mutations, by chance, occasionally generate *Rhodobacter* cells that produce reduced levels of photopigments. In nature, the ability to carry out photosynthesis provides a fitness benefit and wild-type cells outcompete such pigment-deficient mutants. However, when cultured in constant darkness, wild-type cells have no such advantage. In darkness, mutants that produce reduced levels of photopigments gain the advantage because they do not waste energy and resources synthesizing photopigments that provide them no benefit. As a result, photopigment mutants can grow at a faster rate and quickly take over *Rhodobacter* cultures grown in darkness because under these conditions, the mutants have greater fitness than wild-type cells (**Figure 13.14**). Mutations affecting photosynthesis occur at the same rate in the light as in the dark, but in the light the selection for the wild-type phototrophic phenotype is so strong that photopigment mutants are quickly lost from the population.

Gain of Function

The *E. coli* long-term evolution experiment (LTEE), which has been running since 1988, has tracked the evolution of 12 parallel lines of *E. coli* through more than 60,000 generations. The *E. coli* LTEE cultures have been grown aerobically on a minimal medium with glucose as a sole source of carbon and energy. *E. coli* is typically

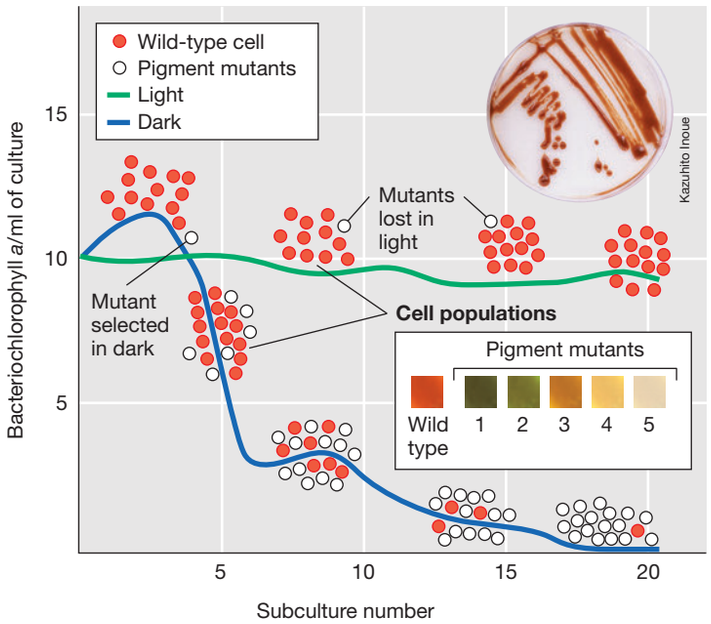


Figure 13.14 Survival of the fittest and natural selection in a population of phototrophic purple bacteria. Serial subculture of the purple bacterium *Rhodobacter capsulatus* in the light (green line) provides a benefit to wild-type phototrophic strains and they outcompete nonphototrophic mutants. In the dark (blue line), however, phototrophy provides no benefits and nonphototrophic mutants quickly outcompete wild-type cells still making bacteriochlorophyll and carotenoids. Photos: top, plate culture showing colonies of phototrophic cells of *R. capsulatus*; bottom, close-up photos showing the color of colonies of wild-type cells and five pigment mutants (1–5) obtained during serial dark subculture. Wild-type cells are reddish-brown from their assortment of carotenoid pigments. The color of mutant colonies reflects the absence (or reduced synthesis) of one or more carotenoids. Mutant strain 5 lacked bacteriochlorophyll and was no longer able to grow phototrophically. Mutant strains 1–4 could grow phototrophically but at reduced growth rates from the wild type. Data adapted from Madigan, M.T., et al. 1982. *J. Bacteriol. 150*: 1422.

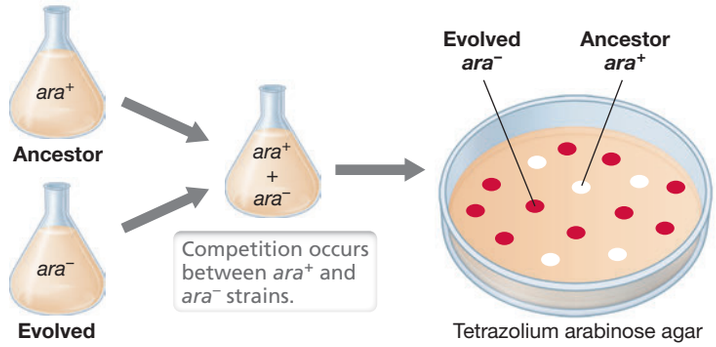
propagated in a rich medium that contains an excess of nutrients, and so the minimal glucose medium used in the LTEE represents a new adaptive environment in which *E. coli* can evolve over time.

In the LTEE, both the ancestor and the evolved lines were genetically engineered to contain a neutral marker that made their colonies either red or white. The marker made it possible to measure the fitness of evolved strains relative to the ancestor by putting them into competition with one another (**Figure 13.15*a***). Genome sequencing during the experiment revealed that mutations accumulated randomly over time in the evolved lines. However, the relative fitness of the evolved lines on minimal glucose medium increased dramatically over the first 500 generations as a result of selection acting on mutations beneficial in this new environment (Figure 13.15*b*). The fitness of the evolved lines continued to increase, albeit at a reduced rate, as a result of further selection over the course of the experiment. Most remarkably, after 31,500 generations, one of the evolved lines obtained the ability to use citrate as an energy source (Figure 13.15*c*). Citrate was present as a pH buffer in the growth medium and was not considered a potential carbon source for *E. coli*, because the inability to grow aerobically on citrate is a diagnostic trait used for identifying *E. coli*. However, the random accumulation of mutations in this one evolved line modified preexisting genes in such a way as to allow for the evolution of a new adaptive trait. The diverged strains can now exploit a new resource that was unavailable to the ancestral population. Since they can now catabolize both citrate and glucose, these cells grow to higher cell density than the ancestor (Figure 13.15*c*). The fact that only one of the 12 parallel lines evolved the ability to grow on citrate demonstrates the chance nature of evolution.

The transitions shown in these experiments remind us of how quickly evolutionary pressures can shift even major properties (such as metabolic strategies) of a microbial cell population. In the case of *Rhodobacter* (Figure 13.14) a mutation that is deleterious in the wild provides a selective advantage when the organism is grown in the laboratory in a continuously dark environment. Under this new condition, evolution causes *Rhodobacter* to lose unneeded metabolic machinery. In the case of *E. coli* (Figure 13.15), the accumulation of random mutations set the stage for genetic diversity in a population. That is, natural variation caused by spontaneous mutation generated a new trait—the ability to use citrate—and since the environment in which the cells were grown happened to contain citrate, this mutation provided a selective advantage to those cells. In the absence of citrate, these mutations would still occur at the same rate. However, in the absence of a selective benefit, cells able to use citrate would likely disappear from the population over time.

Check Your Understanding

- In the experiment of Figure 13.14, why did the cell population in the dark lose its pigments?
- Why did only one of twelve *Escherichia coli* populations in the LTEE eventually evolve the ability to grow on citrate? What does this tell us about the process of evolution?
- In the LTEE, if the rate of mutation in *E. coli* remains constant, then why does the fitness of *E. coli* improve rapidly upon initial addition to a new environment and then more slowly in later generations (as in Figure 13.15)?



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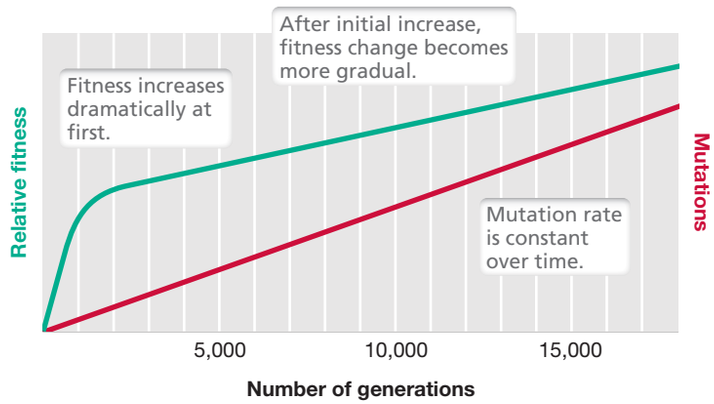

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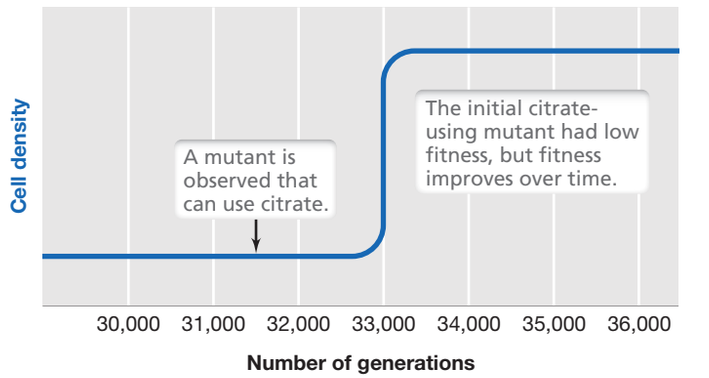


(c)

Figure 13.15 Long-term evolution of *Escherichia coli*. *(a)* In the *E. coli* long-term evolution experiment (LTEE), ancestral and derived lines differ in a mutation that affects their ability to use arabinose, allowing them to be differentiated by their colony color when grown on tetrazolium arabinose agar. *(b)* Competition experiments between evolved and ancestral strains show that relative fitness in minimal glucose media increases dramatically for evolved lines. *(c)* The ability to use citrate aerobically evolved in one of 12 LTEE lines. Cells growing on minimal and citrate allowed the mutant cell line to reach significantly higher cell densities. Relative fitness is a measure of the growth rate of the evolved strain to that of the ancestral strain. Note that the mutation rate in the *E. coli* cultures is constant and that mutations accumulate randomly over time.

13.8 Gene Families, Duplications, and Deletions

The duplication and deletion of genetic information within the genome plays a major role in microbial evolution. Genomes vary dramatically in size (Chapter 10), and duplication and deletion mutations are a major force that governs the size of microbial genomes. In addition, these mutations govern the gene content of the genome, allowing for removal of nonessential genes and the expansion of gene function. In order to examine the impact of deletions and duplications on the evolution of microbial genomes, we must first classify genes based on their shared ancestry so that we can compare the presence and absence of genes across genomes.

Duplications and Gene Families

A set of genes that have all descended from a single ancestral gene present in a shared ancestor are **homologs** (Figure 13.16). Homologous genes tend to have similar nucleotide sequences, and we can infer homology based on nucleotide similarity. Genes that are highly similar in sequence often have the same function, and homologous genes that share the same function are **orthologs**.

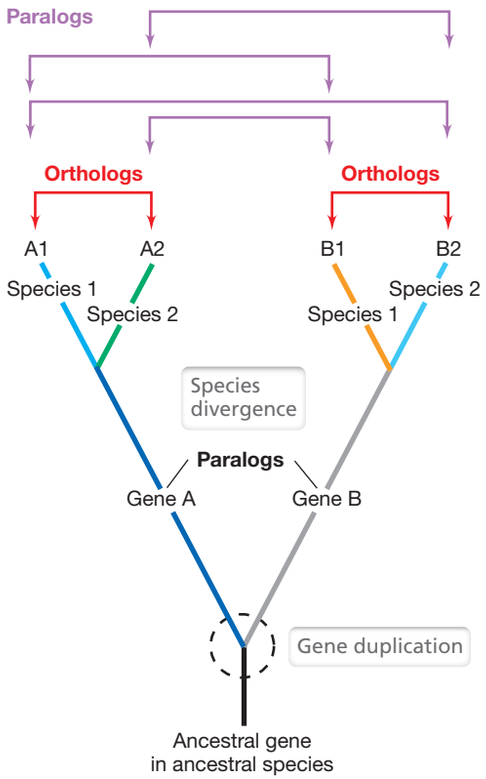


Figure 13.16 Homologs. This family tree depicts the evolution of a gene family composed of homologous genes (orthologs and paralogs), which means that all have evolved from a single ancestral gene present in an ancestral species. A gene duplication occurs in the ancestral species, allowing the redundant gene copy, gene B, to evolve a new function within the genome of the ancestral species (see Figure 13.17*a*). Over time, the ancestral species evolves into two new species whose gene sequences have diverged as a result of evolution. Genes A1 and A2 have the same functions and they have evolved from the same ancestral gene, and so they are orthologs, as are B1 and B2. Genes that have evolved from the same ancestral gene but have different functions are paralogs.

However, not all homologous genes have the same function. The accumulation of mutations over time can eventually cause genes to evolve new functions. Genes that are homologous but have different functions are **paralogs**. Over time a single ancestral gene sequence can diverge to have many different functions in many different organisms. A group of homologous genes is called a **gene family**, and gene families can contain diverse paralogs that all share a common evolutionary origin.

Gene duplication is thought to be a major driving force behind the evolution of gene families and the organisms that contain them (Figure 13.17*a*). A duplication mutation creates a redundant copy of a gene sequence in the genome. The original gene copy retains its function, making it possible for the second copy to accumulate mutations freely without a loss of gene function in the cell. In this way, evolution can "experiment" with one copy of the gene. Genomic analyses have revealed many examples of protein-encoding genes that were clearly derived from gene duplication. Figure 13.17*b* shows this for the enzyme RuBisCO, a key enzyme of autotrophic metabolism (◀ Section 3.12): An ancestral RuBisCO gene gave rise to paralogous enzymes with different but related catalytic activities.

Gene Deletions in Microbial Genomes

Gene deletions play an important role in microbial genome dynamics. It is a common misconception that evolution inevitably causes organisms to increase in complexity over time. In reality, evolution is both a give and a take proposition. Fitness changes are completely dependent on the environment, and fitness in some environments can actually be improved by deletion of genetic material.

It is common in prokaryotic cells for nonessential and nonfunctional materials to be deleted over evolutionary time. Deletions occur with far greater frequency than insertions or duplications in microbial genomes. This bias toward deletions is the force that maintains the small size of many microbial genomes and the reason why the genomes of *Bacteria* and *Archaea* are tightly packed with genes and contain relatively few noncoding sequences (◀ Section 10.3). Selection is the main force that counters the effect of deletions, preserving those genes that provide a fitness benefit to the cell.

Deletions are also thought to be a driving force for the tiny genomes observed in many obligate intracellular symbionts and intracellular pathogens (◀ Section 10.3 and ▶ Section 23.7). The genomes of these bacteria are highly streamlined because many metabolites are available in the cytoplasm of their host organisms. Hence deletion mutations that remove biosynthetic genes might have little effect on the fitness of an intracellular pathogen if those biosynthetic functions are provided by the host. Over time, deletion mutations eliminate redundant functions. Insect endosymbionts have carried this theme to an extreme and show the smallest of all cellular genomes (◀ Section 10.3, Table 10.1, and Figure 10.8). The same process is ultimately responsible for the minute genomes found in mitochondria and chloroplasts (◀ Section 10.4).

Deletion Mutations and the Evolution of Interdependence in Microbial Communities

A deletion mutation that prevents an organism from producing an essential nutrient such as a vitamin when growing in pure culture would be a lethal event. But in nature, microorganisms grow in

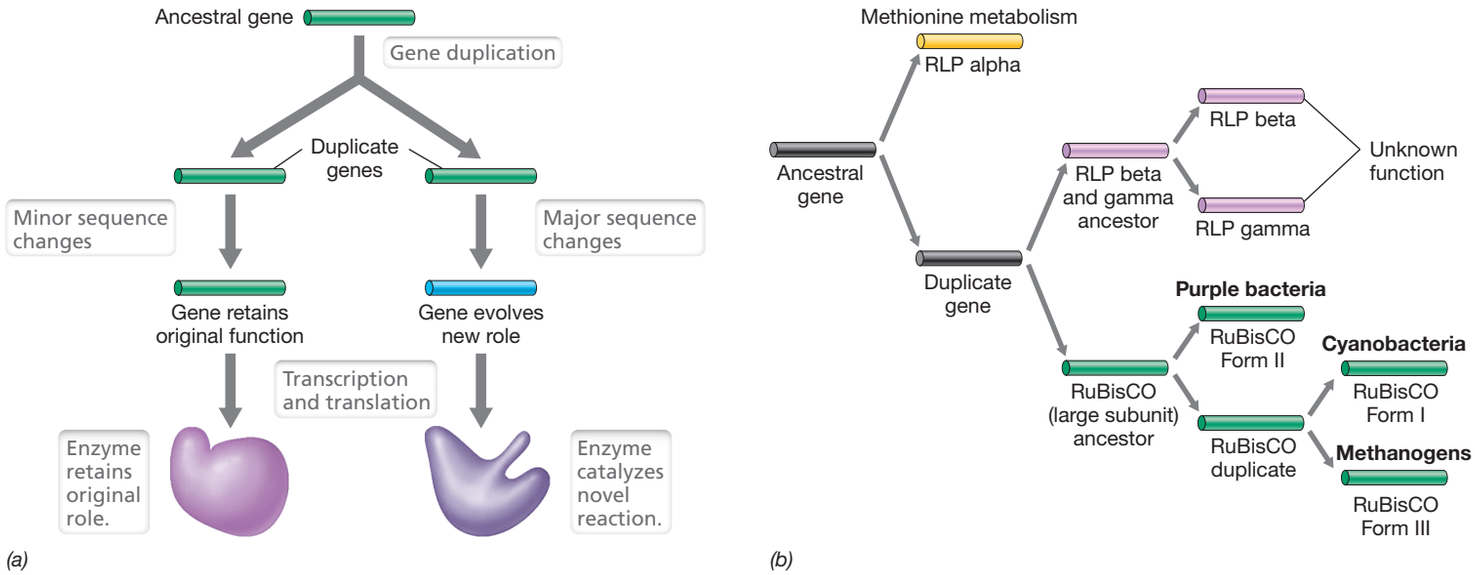


Figure 13.17 Evolution by gene duplication. *(a)* The principle of gene duplication. After duplication, the "spare" copy of a gene is free to evolve to encode a new function. *(b)* The large subunit of the enzyme RuBisCO, which fixes CO_2 during photosynthesis, is part of a gene family that contains many different genes. Different orthologous forms of RuBisCO (forms I, II, and III), which all retain the same function, are present in different microbial groups. However, RuBisCO is in turn derived from an ancestral gene (black bars) of unknown function that was duplicated to produce paralogous genes encoding an enzyme in methionine metabolism (yellow bar) and several genes whose function is still unknown (purple bars). RLP, RuBisCO-like protein.

microbial communities, and in such a setting, a deletion mutation that prevents an organism from producing an essential nutrient may not be lethal if the organism can obtain the nutrient from some other organism in the environment (**Figure 13.18**).

All genes impose some cost on the cell, and organisms living in a microbial community can delete a gene to avoid this cost if other community members provide the gene's function. Such deletions can increase an organism's fitness but promote the interdependency of members of the microbial community. Organisms having such "loss-of-function" mutations will remain competitive as long as they remain within the community but may be unable to grow if separated from the community in which they coevolved. This sharing of resources in natural communities explains the observation that many bacteria grown in pure culture require one or more growth factors. For example, regardless of where they are isolated from, all strains of the phototrophic purple bacterium *Rhodospirillum rubrum* require the vitamin

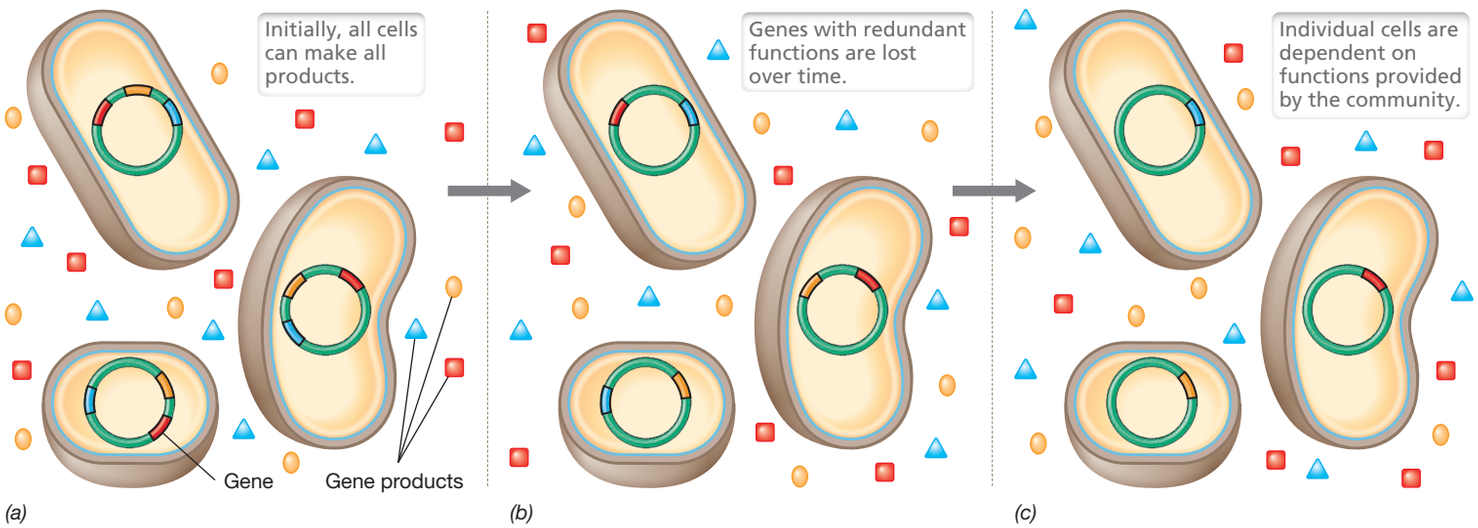


Figure 13.18 The evolution of interdependence in microbial communities. *(a)* Three species in a community each have three different genes that make extracellular products that benefit the whole community (a gene and its product are shown in the same color). *(b)* Over time, random mutations cause functions to be lost from the genomes. *(c)* As long as some members of the community continue to make each product, there will be no fitness cost when a single species loses a single gene. Over time, the three species become mutually dependent.

biotin when grown in pure culture, while all strains of *Rhodobacter capsulatus* (see Figure 13.14) require thiamine. In nature, these two phototrophs could coexist in the same microbial community if each phototroph (or other microbes in the community) secreted a small amount of the vitamin that the other phototroph required. Such gene deletions result in metabolic interdependencies within a microbial community (Figure 13.18), making deletions one of the factors that govern the microbial composition of a microbial community.

Many microbes can grow in pure culture without any growth factor additions. Such organisms preserve all essential functions and bear the costs of maintaining all gene functions; this puts them at a disadvantage relative to mutually dependent competitors when the competition takes place within a complex microbial community. However, cells that maintain their ability to grow independently earn a fitness bonus in that, unlike their mutually dependent competitors, they retain the option of dispersing to new habitats and growing there independently.

Check Your Understanding

- What is a homologous gene?
- Contrast gene paralogs with gene orthologs.
- In what way can a gene deletion cause an increase in the fitness of an organism?

13.9 Horizontal Gene Transfer

Microorganisms can exchange DNA between cells through the process of **horizontal gene transfer** (Figure 13.19), and this process has a tremendous impact on microbial evolution. We often think of evolution as a vertical process that takes place along the branches of a phylogenetic tree starting with an ancestor at the tree's root and ending with descendants at its topmost branches. However, horizontal gene transfer allows the transfer of DNA between distant branches of the tree. At least three mechanisms of horizontal gene transfer exist: *transformation*, *transduction*, and *conjugation* (Figure 13.19 and Chapter 9).

Horizontal gene transfer can be thought of as a type of recombination in which DNA from the donor is passed to the recipient and becomes part of the recipient's genome. Horizontal transfer is unlike sexual recombination because it is (1) unidirectional, with DNA passing from donor to recipient, (2) asymmetrical, in that only a small amount of DNA is usually transferred, and (3) not constrained by species boundaries because the donor and recipient can be distantly related.

Horizontal gene transfer can have a range of consequences depending on the mechanism of recombination used to incorporate the DNA into the genome. The effect of horizontal transfer on the genome is strictly additive when the newly acquired DNA is inserted by nonhomologous recombination. However, when the newly acquired DNA is incorporated by homologous recombination, the result can be *gene conversion*, in which the recipient's copy of a gene is replaced by the donor's copy.

Like most mutations, most genes acquired by horizontal gene transfer can be expected to be neutral or deleterious to the cell. Hence, horizontally acquired genes that do not convey a fitness benefit are deleted from the genome over time. In contrast, those genes that do confer a fitness benefit are likely to persist within the genome. Horizontal gene transfer allows a cell to "explore" a tremendous diversity of genetic information, and newly acquired genes can change over time as a result of further mutation and recombination. As a result, horizontal gene transfer is an effective means for microbes to acquire new functions and to evolve new traits.

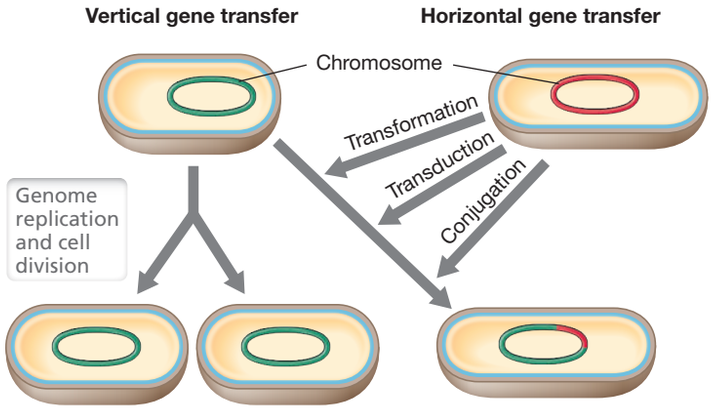


Figure 13.19 Vertical versus horizontal gene transfer. Vertical gene transfer occurs when cells divide. Horizontal gene transfer occurs when a donor cell contributes DNA to a recipient cell. In *Bacteria* and *Archaea*, horizontal transfer occurs through one of three mechanisms: transformation, transduction, and conjugation.

The Mobilome

Horizontal gene transfer is facilitated by the **mobilome**, the sum total of all mobile genetic elements in a genome. Mobile genetic elements are discrete segments of DNA that have genes encoding their own replication, recombination, and transmission. Mobile genetic elements include plasmids, prophages (integrated viral genomes), insertion sequences, transposons, and integrons (Chapter 9). Although the mobilome is not essential to the cell, it can nevertheless play an important role in genome evolution by shuffling genes within and between genomes.

Insertion sequences are simple mobile elements that consist of a *transposase* gene flanked by inverted repeats (◀ Section 9.11). Transposase is an enzyme that recognizes the inverted repeats and catalyzes nonhomologous recombination, allowing the insertion sequence to jump between different segments of DNA through a "cut and paste" mechanism. If multiple insertion sequences are present in a genome, these elements can generate chromosomal rearrangements such as deletions, inversions, or translocations (Figure 13.20). In addition, insertion sequences shared between a chromosome and a plasmid can facilitate the exchange of genes between these molecules.

Transposons are mobile genetic elements that are larger than insertion sequences but similar in function, having both terminal inverted repeats and a transposase gene (◀ Section 9.11). Transposons, however, contain multiple genes that can have a diversity of functions unrelated to transposition. Hence, transposons can move a wide variety of genes between different segments of DNA. Furthermore, some transposons contain genes that cause conjugative transfer, thereby promoting horizontal transfer between cells by conjugation. Because of this tendency, transposable elements are a strong driver of genome evolution.

Integrons also facilitate recombination and DNA exchange. Integrons contain an *integrase* gene flanked by a promoter and a

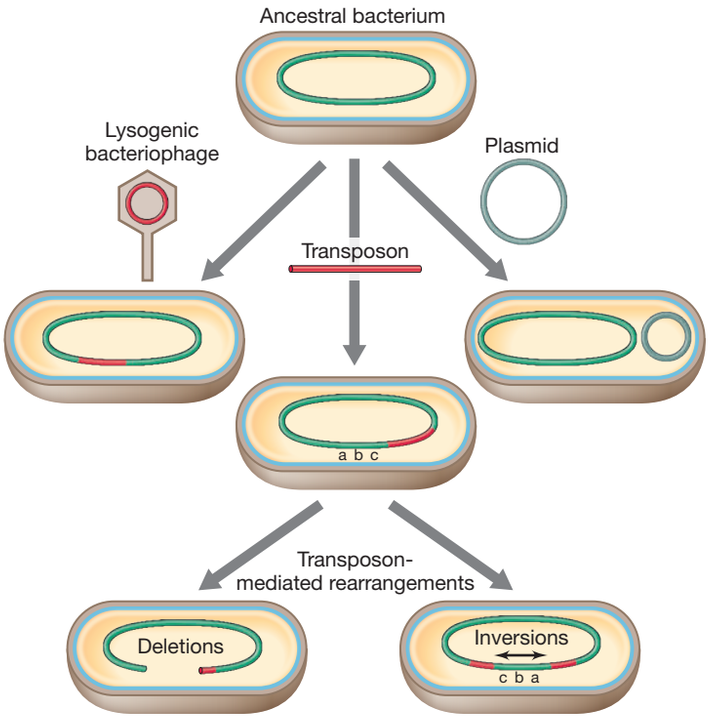


Figure 13.20 Mobile elements promote genome evolution. Mobile genetic elements facilitate the transfer of DNA from one organism to another, thus adding new genes to the recipient genome. The most common mobile genetic elements are plasmids, bacteriophages, and transposons. Mobile genetic elements can also facilitate chromosomal rearrangements. For example, transposase can cause deletions and inversions of host DNA next to sites of transposon insertion.

recombination site that contains direct repeats. Integrons typically contain a gene cassette consisting of one or more genes flanked by a series of corresponding recombination sites. The integron promoter drives the expression of genes within the cassette. The integrase catalyzes site-specific recombination of DNA at the integron recombination site. Hence, DNA sequences flanked by corresponding recombination sites can be inserted or excised from the integron gene cassette. Integrons are often found on other mobile elements such as plasmids or transposons. This mechanism can facilitate the rapid acquisition and rearrangement of new genes.

Detecting Horizontal Gene Flow

The rate of horizontal gene transfer per cell per generation is quite low, but the effect of horizontal gene transfer on genome composition is integrated over the entire evolutionary history of an organism dating back to the last universal common ancestor. As a consequence, the vast majority of genes in a microbial genome have been subject to horizontal gene transfer at some point in their evolutionary history. However, detecting horizontally transferred genes and unraveling their evolutionary history can be a challenge.

A variety of methods are used to identify horizontally transferred genes, but these methods typically take one of two different approaches: statistical analysis of sequence composition, or phylogenetic analysis. Microbial genomes often have distinguishing nucleotide sequence characteristics, much like a fingerprint, that can be used in their identification. These differences in sequence composition can be used to identify segments of DNA acquired from other organisms. One example is to compute the percentage of G + C (guanine–cytosine) base pairs in the genome (genomic GC content, ◀ Figure 10.8). In this case, horizontal gene transfer is signaled if the percentage of G + C base pairs in a segment of DNA differs significantly from that of the rest of the genome. In addition, genomes also contain DNA corresponding to each of the 64 possible codons at different frequencies, and these codon-use frequencies (codon bias, ◀ Section 10.2) can also be used to identify horizontally acquired genes. A variety of statistical tests can be applied to look for such differences in sequence composition across the genome.

Horizontally acquired genes can also be detected through phylogenetic analysis by making phylogenetic trees for different genes in the genome (Section 13.11). If a gene has a different pattern of ancestry from the rest of the genes in the genome, that is an indication that it was horizontally transferred. Horizontally transferred genes are most easily detected when they are transferred between highly dissimilar organisms and more difficult to detect when transferred between close relatives.

We move on now from the effects of horizontal transfers of individual genes or sets of genes to consider the evolution of the entire genome.

Check Your Understanding

- What is one way that you can identify genes acquired by horizontal gene transfer?
- List the three major mechanisms that mediate horizontal gene transfer between cells.
- How is an integron different from an insertion sequence? Which would be more likely to cause a major genome rearrangement?

13.10 The Evolution of Microbial Genomes

Comparative genomics makes use of whole genome sequencing technologies (Chapter 10) to analyze differences in gene content and DNA sequence composition across entire sets of genomes. Comparative analyses have revealed microbial genomes to be highly diverse and dynamic. A microbial species is composed of many individual strains that differ in gene composition. In the case of microbial genomes, those differences in gene composition can be substantial. **Figure 13.21** illustrates the diverse nature of microbial genomes within a single species of the genus *Salmonella*. All strains of *S. enterica*—an important human pathogen (▶ Section 33.10)—whose genomes have been sequenced share 2811 genes, but each strain of this species also has a unique set of *strain-specific* genes. The genomes of many microbial species, like *S. enterica*, are evolving constantly as diverse strains gain genes by horizontal gene transfer and lose genes as a result of deletions.

From genomic studies of several strains of a single species, it is now clear that the genome of a microbial species is really composed of two segments: the **core genome**, consisting of genes shared by all individual strains of a species, and the **pan genome**, consisting of the core genome *plus* genes that are not shared by all strains of a species (**Figure 13.22*a***). As we have seen, horizontal gene transfer of mobile genetic elements (Section 13.9) is widespread. The existence of the pan genome shows that microbes are constantly sampling genes from

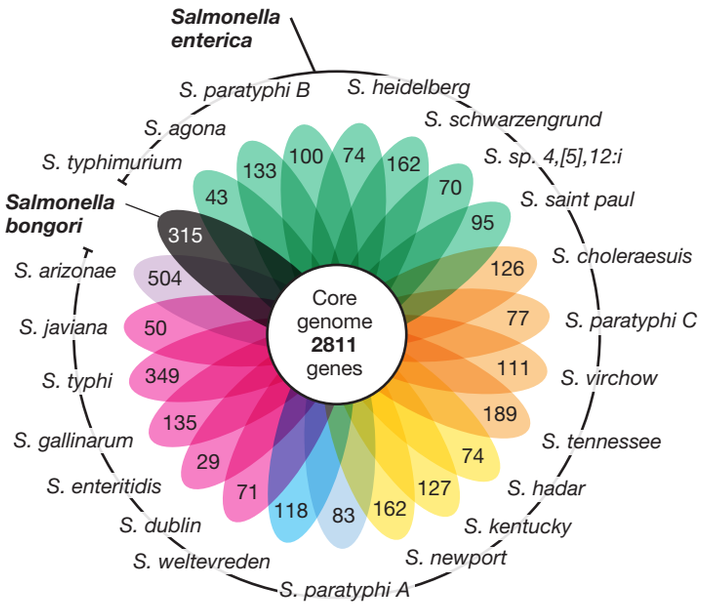


Figure 13.21 The *Salmonella enterica* pan genome includes a large number of strain-specific genes. The species *Salmonella enterica* contains many different strains that differ in various traits including their pathogenicity. While these strains all share the same 2811 core genes, each individual has some number of strain-specific genes. *Salmonella bongori* is a species distinct from *S. enterica*. Data from Jacobsen, A., R.S. Hendriksen, F.M. Aaresturp, D.W. Ussery, and C. Friis. 2011. The *Salmonella enterica* pan-genome. *Microb. Ecol. 62*: 487.

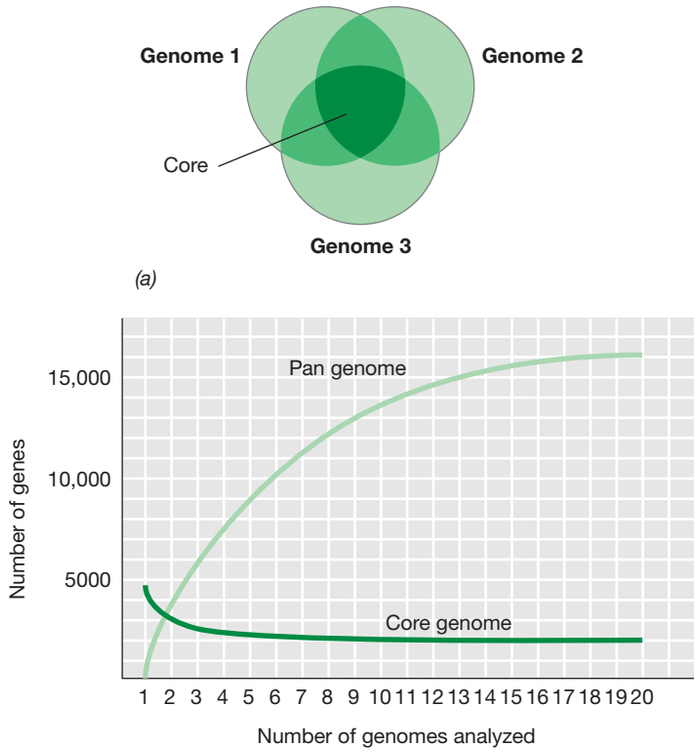


Figure 13.22 The core and pan genome concept. Microbial genomes are dynamic and heterogeneous. The first three genomes sequenced from different strains of *Escherichia coli* were found to have only 39% of their genes in common. The core genome is considered the set of genes that are shared by all members of a species (darkest green in part *a*), while the pan genome is the totality of all genes found in the different strains of a species (all the genes in genomes 1–3 in part *a*). The size of the core and pan genome can vary between species. In *E. coli* the core genome is composed of approximately 1976 genes *(b)*. The size of the pan genome in *E. coli* is not fixed, as each different strain has a unique complement of genes acquired from horizontal gene transfers. Data adapted from Touchon, M., et al. 2009. *PLoS Genetics 5*(1): e1000344.

other organisms in the environments in which they live. Consequently, there can be major differences in genome size, gene content, and functional traits (virulence, symbiosis, biodegradation, and the like) between individual strains within a single microbial species.

The Dynamic Nature of the *Escherichia coli* Genome

The dynamic nature of microbial genomes was revealed in dramatic fashion when the first genomes were sequenced from multiple strains of a single species. For example, genome sequencing of *Escherichia coli* strain K-12 and two pathogenic strains showed that only 39% of their genes were shared among all three genomes (Figure 13.22). The three genomes varied in size by more than a million base pairs and each contained a unique and diverse complement of genes acquired through horizontal gene transfer.

E. coli genomes have on average 4721 genes, with individual strains having as few as 4068 or as many as 5379 total genes. The core genome consists of only 1976 genes present in all strains, accounting for less than half the genes present in the average *E. coli* genome. The size of the core genome can be expected to decrease as the evolutionary distance of strains increases.

The number of unique genes observed in *E. coli*, and the size of its pan genome, continues to increase indefinitely as each new strain of *E. coli* has its genome sequenced. For example, a survey of genomes from 20 *E. coli* strains revealed a total of 17,838 unique genes (Figure 13.22*b*). Subtracting the contribution from the core genome, this indicates that as many as 15,862 genes are not shared by all strains. A great many of these genes have clearly been obtained from horizontal gene transfers rather than through vertical patterns of inheritance. Patterns of gene exchange appear to be governed by phylogenetic distance, with rates of gene exchange declining as the phylogenetic distance increases between genomes. In the core genome of *E. coli*, for example, it has been found that most horizontal gene transfer takes place between close relatives and occurs by homologous recombination of DNA segments of 50 to 500 base pairs in length.

Chromosomal Islands

Comparative genomic analyses have revealed that in addition to a gene or two, *entire genetic pathways* can be acquired as a result of horizontal gene transfer. These so-called **chromosomal islands** (also called *genomic islands*) contain clusters of genes for specialized functions (Figure 13.23). Several lines of evidence indicate that chromosomal islands derive from horizontal gene transfer. First, chromosomal islands are often flanked by inverted repeats, implying that the whole region was inserted into the chromosome by transposition (Section 13.9). Second, the base composition and codon bias in chromosomal islands often differ significantly from those of the genome proper. Third, chromosomal islands often belong to the pan genome, being found in some but not all strains of a species. In addition, some chromosomal islands carry an integrase gene, suggesting similarity

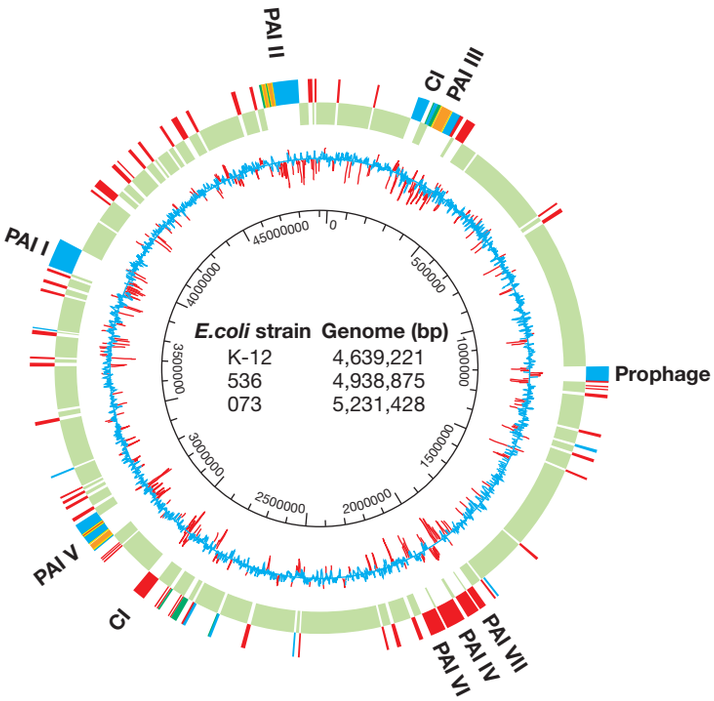


Figure 13.23 Chromosomal islands and pathogenicity islands in *Escherichia coli*. Genome comparisons of nonpathogenic *E. coli* strain K-12 and pathogenic *E. coli* strains 536 and 073 (both of which cause urinary tract infections) reveal that many differences in gene content are associated with chromosomal islands (CI) and pathogenicity islands (PAI). The inner ring represents genome position (in base pairs). The middle ring shows the frequency with which G + C base pairs occur across the genome, and anomalous regions are colored red. The outer ring compares gene content between genomes: green, genes common to all strains; red, genes present only in pathogenic strains; blue, genes found only in strain 536; orange, genes of strain 536 present in a different location in strain 073. Note the correlation between chromosomal islands and skewed GC content. Some very small inserts are deleted for clarity. Prophage, DNA from a temperate bacteriophage. Data adapted from Brzuszkiewicz, E., et al. 2006. *Proc. Natl. Acad. Sci. (USA) 103*: 12879.

with mobile elements such as conjugative transposons (Section 13.9). Finally, in a few cases, the transfer of chromosomal islands has even been demonstrated experimentally in the laboratory.

Chromosomal islands that encode *virulence factors*, molecules that facilitate disease and are found in pathogenic bacteria (Chapter 25), are called **pathogenicity islands**. The pathogenicity islands of uropathogenic strains of *E. coli* have been particularly well studied (Figure 13.23). Only a few strains of *E. coli* cause bladder and kidney infections, but those that do—the uropathogenic strains—contain pathogenicity islands that encode a variety of virulence factors including adhesins that facilitate binding to host tissues and a capsule that helps cells evade immune surveillance. Pathogenicity islands are also present in certain pathogenic strains of the gram-positive bacterium *Staphylococcus aureus*, which can cause serious skin infections (▶ Section 31.9). These pathogenicity islands can be moved between cells by transduction, a major mechanism of horizontal gene transfer (◀ Section 9.7).

Chromosomal islands in pathogenic bacteria have drawn the most attention from microbiologists because in many cases, genes encoding disease-associated functions have been linked to an island. However, chromosomal islands are also known whose genes encode proteins that degrade pollutants such as aromatic hydrocarbons and herbicides (▶ Section 22.4). In addition, many of the genes essential for the symbiotic relationship of species of the nitrogen-fixing bacterium *Rhizobium* with the root nodules of plants (▶ Section 23.4) are carried in chromosomal islands. Perhaps the most unique chromosomal island is the magnetosome island of the bacterium *Magnetospirillum*; this DNA fragment carries genes that encode the formation of magnetosomes, intracellular magnetic particles that orient the cell in a magnetic field and influence the direction of its movement (◀ Section 2.12).

In the final part of this chapter we consider how phylogenetics has revolutionized microbial systematics and has begun to crystallize our concept of a microbial species.

Check Your Understanding

- What is the difference between core genome and pan genome of a given species?
- What is a chromosomal island and how can one be identified?
- What factors likely influence the size of microbial genomes?

III • Microbial Phylogeny and Systematics

Microbial systematics blends phenotypic and genotypic information about microbes to yield a working blueprint of how the microbial world evolved and how it is organized. Phylogenetic trees are an important part of this blueprint and are used to show the evolutionary connections between microbes and to describe new taxa.

Systematics is the study of the diversity of organisms and their relationships. It links **phylogeny** with **taxonomy**, the science in which organisms are characterized, named, and classified according to defined criteria. Taxonomists describe microorganisms using a *polyphasic approach* that evaluates *phenotypic*, *genotypic*, and *phylogenetic* information. Phenotypic analyses examine the morphological, metabolic, physiological, and chemical characteristics of the cell. Genotypic analyses consider characteristics of the genome. These two analyses are used to categorize organisms based on similarities and are complemented by phylogenetic analyses, which seek to place organisms within an evolutionary framework using molecular sequence data (see Figure 13.9). We begin by exploring how molecular sequences can provide phylogenetic insight.

13.11 Molecular Phylogeny: Making Sense of Molecular Sequences

Molecular sequences provide a record of past evolutionary events and can be used to build **phylogenetic trees**, which are diagrams that depict evolutionary history. As we have seen in this chapter, mutations accumulate in DNA sequences over time. These mutations occur at random and are a major source of the natural variation that makes evolution possible (Section 13.6). Hence, the number of

nucleotide sequence differences between any two homologous genes will be a function of the number of mutations that have accumulated since they shared a common ancestor. As a result, differences in DNA sequences can be used to infer evolutionary relationships, and the technology for doing this is well developed.

Obtaining DNA Sequences

Obtaining DNA from a microorganism is relatively easy if the organism can be cultivated in isolation in the laboratory. Once the cultivar's genomic DNA is isolated, either the genome can be sequenced directly or the genomic DNA can be used to amplify one or more specific genes, using the polymerase chain reaction (PCR, ◀ Section 12.1) and primers specific for the gene(s) of interest. Advances in DNA sequencing technology (◀ Section 10.2) have made genome sequencing a standard tool employed in analyses of microbial phylogeny. However, sequence analysis of small subunit (SSU) ribosomal RNA (rRNA) genes (**Figure 13.24**), which encode the rRNA molecule found within the small subunit of the ribosome

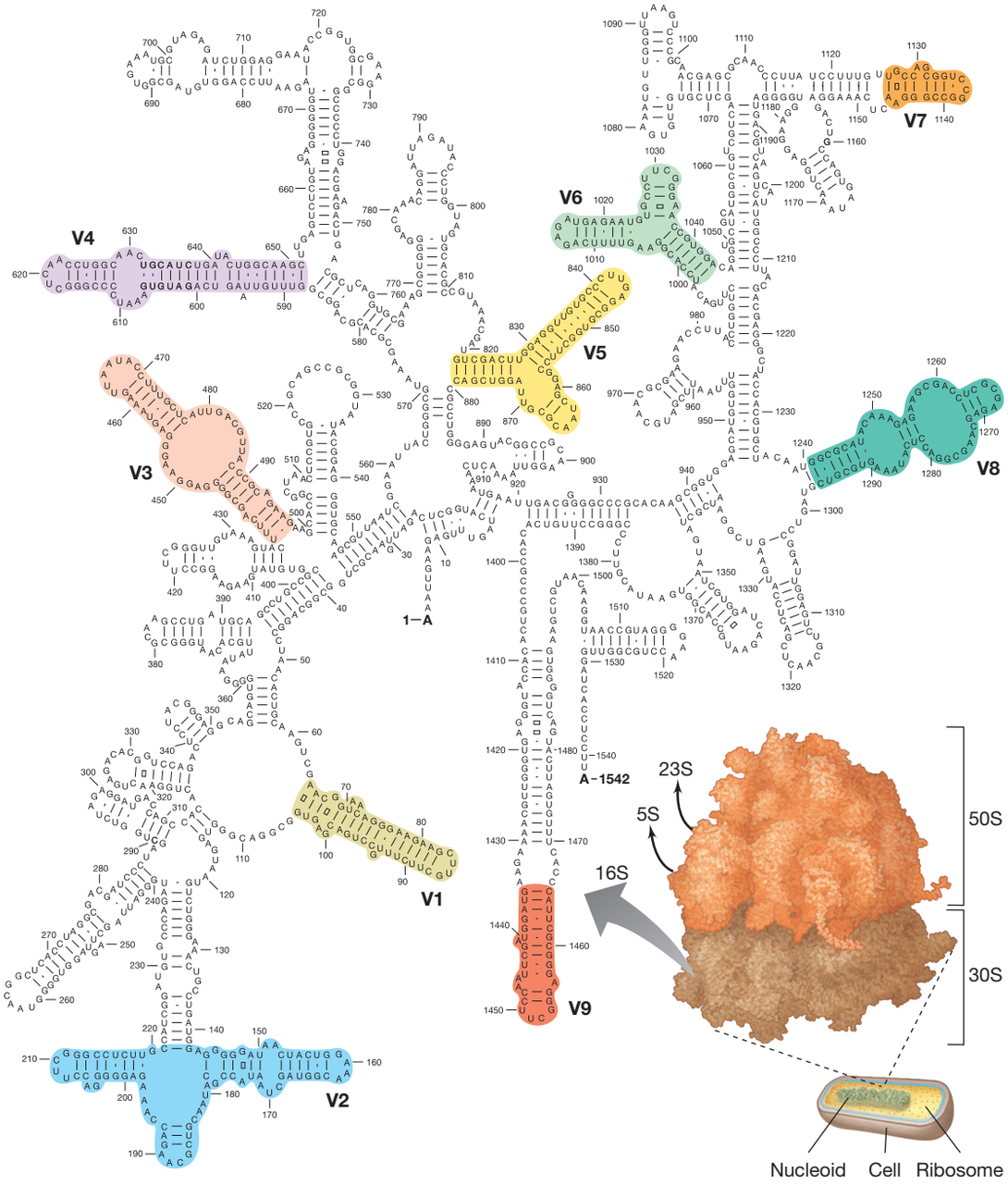


Figure 13.24 Ribosomal RNA (rRNA). Primary and secondary structure of 16S rRNA from *Escherichia coli* (*Bacteria*). The 16S rRNA from *Archaea* is similar in secondary structure (folding) to that of *Bacteria* but has numerous differences in primary structure (sequence). The molecule is composed of conserved and variable regions (V1–V9). The approximate positions of the variable regions are indicated in color. Inset: the 70S ribosome of *Bacteria* is composed of 30S and 50S subunits;16S rRNA is part of the 30S subunit whereas 5S and 23S rRNAs are parts of the 50S subunit.

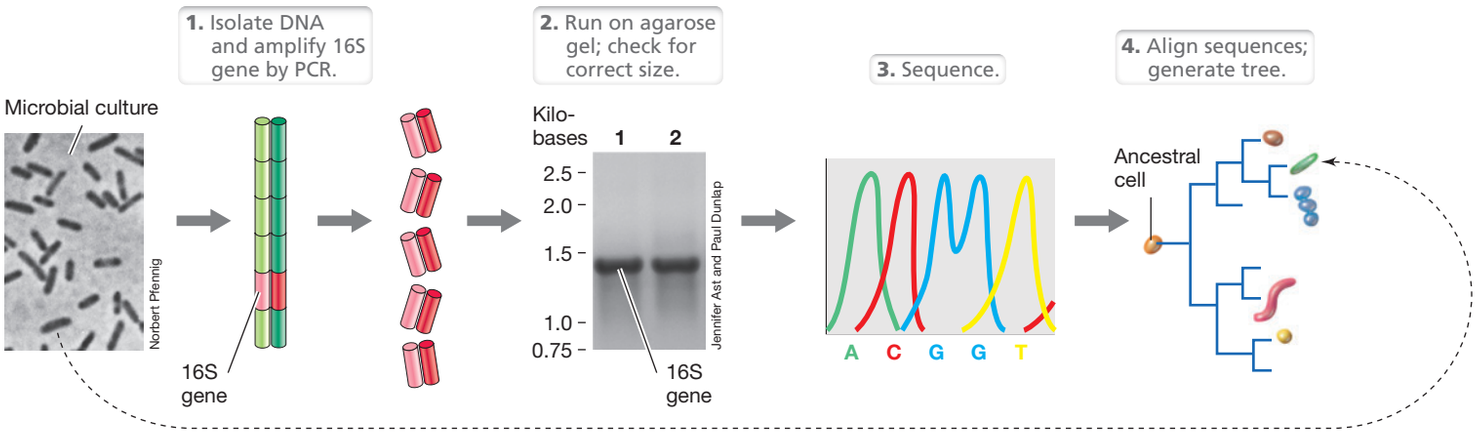


Figure 13.25 PCR amplification of the 16S rRNA gene from an unknown microbial culture. Following DNA isolation, primers complementary to the ends of the 16S rRNA (Figure 13.24) are used to PCR-amplify the 16S rRNA gene from the genomic DNA of the bacterium. The PCR product is then run on an agarose gel (lane 1) versus a standard (lane 2). The bands of amplified DNA are approximately 1465 nucleotides in length (positions that DNA of different lengths would run in the gel are indicated at the left). The band from the unknown culture is then excised from the gel, purified, and sequenced. The sequence is then aligned with 16S rRNA sequences from other organisms and a phylogenetic tree constructed.

(◀ Section 6.10), remains a cornerstone of molecular phylogeny in microbiology because **SSU rRNA** genes are highly conserved, present in all cellular organisms, and easily sequenced and analyzed (see Figure 13.9).

PCR primers exist for many highly conserved genes, such as the SSU rRNA gene, and can be designed in such a way as to have different levels of phylogenetic specificity. That is, primers can be designed that target distinct species (or all species) within a genus, all genera within a phylum, or all phyla within a domain, and there are even "universal" PCR primers that will amplify the SSU rRNA gene from any organism. PCR products are visualized by agarose gel electrophoresis, excised from the gel, extracted and purified from the agarose, and then sequenced, often using the same oligonucleotides as primers for the sequencing reactions (**Figure 13.25**). Instead of starting with a microbial culture, it is also possible to amplify SSU rRNA genes from DNA extracted directly from an environmental sample or to sequence environmental DNA directly without amplification (metagenomics, ◀ Section 10.7 and ▶ Section 19.6). These latter approaches are widely used to characterize microorganisms that are difficult or not yet possible to grow in laboratory culture. Once sequences are obtained, they must be aligned and analyzed, issues we turn to now.

Mastering Microbiology
Art Activity: Figure 13.16 PCR amplification of the 16S rRNA gene

Sequence Alignment

Phylogeny can be inferred only from genes that have homology, that is, *genes that have been inherited from a common ancestor.* Thus, homology is a binary trait; sequences are either homologous or they are not. The concept of homology is often confused with that of sequence similarity. The latter is a continuous trait defined as a percentage of nucleotide positions shared between any two sequences. Sequence similarity is used to infer homology, but a similarity value can be calculated between any two sequences regardless of their function or evolutionary relationship. Thus, the terms similarity and homology are not interchangeable. Genes that have homology can be either orthologs, if they have the same function and originate from a single ancestral gene in a common ancestor, or paralogs, if they have evolved to have different functions as a result of gene duplication (Section 13.8 and Figures 13.16 and 13.17). Phylogenetic analyses typically focus on analyses of orthologous genes because of their common phylogeny and function.

Phylogenetic analyses estimate evolutionary changes from the number of sequence differences across a set of homologous nucleotide positions. Some mutations introduce nucleotide insertions or deletions, and these cause gene sequences to differ in length, making it necessary to *align* nucleotide positions prior to phylogenetic analysis of gene sequences. The purpose of **sequence alignment** is to add gaps to molecular sequences in order to establish positional homology; that is, to be sure that each position in the sequence was inherited from a common ancestor of all organisms under consideration (**Figure 13.26**). Proper sequence alignment is critical to phylogenetic

Sequences before alignment

```
1  GGA CCT AAA TTT ATA CCC
2  GGA AAA GGG CCC AAA CGC
3  GGA GGG CCT TTT ATA CCC
```

Sequence differences

	1	2	3
1	–	–	–
2	11	–	–
3	6	11	–

Sequences after alignment

```
1  GGA --- --- CCT AAA TTT ATA CCC
2  GGA AAA GGG CCC --- --- AAA CGC
3  GGA --- GGG CCT --- TTT ATA CCC
```

(a)

	1	2	3
1	–	–	–
2	3	–	–
3	0	3	–

(b)

Figure 13.26 Alignment of DNA sequences. *(a)* Sequences for a hypothetical region of a gene are shown for three species before alignment and after alignment. A sequence alignment should display homologous positions in vertical columns. Sequence alignment is achieved by adding gaps, indicated by hyphens, to maximize local sequence similarity between the species in the alignment. *(b)* The distance matrices show the number of sequence differences that would be inferred for each species pair both before and after alignment.

analysis because the assignment of mismatches and gaps caused by deletions is in effect an explicit hypothesis of how the sequences have diverged from a common ancestral sequence.

Phylogenetic Trees: Composition and Construction

A phylogenetic tree is a diagram that depicts the evolutionary history of the organisms on the tree and bears some resemblance to a family tree. Most microbes have not left fossils and so their ancestors are unknown, but ancestral relationships can be inferred from the DNA sequences of organisms that are alive today. Organisms that share a recent ancestor are likely to share other characteristics as well, and thus phylogenetic trees allow us to make predictions about an organism's characteristics. Phylogenetic trees are also of great use in taxonomy and species identification, as we will discuss later (Section 13.12).

A phylogenetic tree is composed of *nodes* and *branches* (**Figure 13.27**). The tips of the branches in a phylogenetic tree represent species that exist today. The nodes represent a past stage of evolution where an ancestor diverged into two new lineages. The branch length represents the number of changes that have occurred along that branch. In a phylogenetic tree, only the position of nodes and the branch lengths are informative; rotation around nodes has no effect on the tree's topology (Figure 13.27).

There is only one correct phylogenetic tree that most accurately depicts the evolutionary history of a group of gene sequences, but inferring this tree from sequence data can be a challenging task. The complexity of the problem is revealed by considering the total number of different trees that can be formed from a random set of

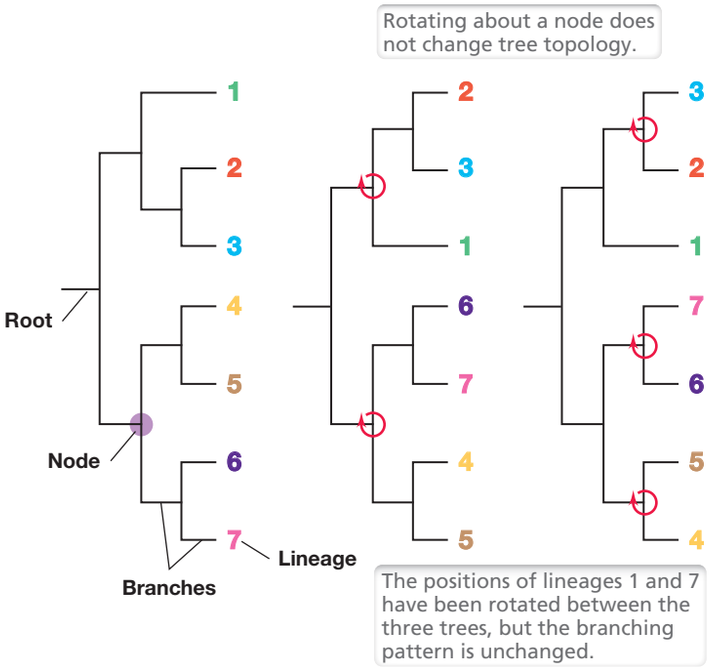


Figure 13.27 Phylogenetic trees and their interpretation. Three equivalent versions of the same phylogenetic tree are shown. The only difference between the trees is that their nodes have been rotated at the points indicated by red circles. The vertical position of species is different between the trees, but the pattern of ancestry (the nodes shared by each species) remains unchanged.

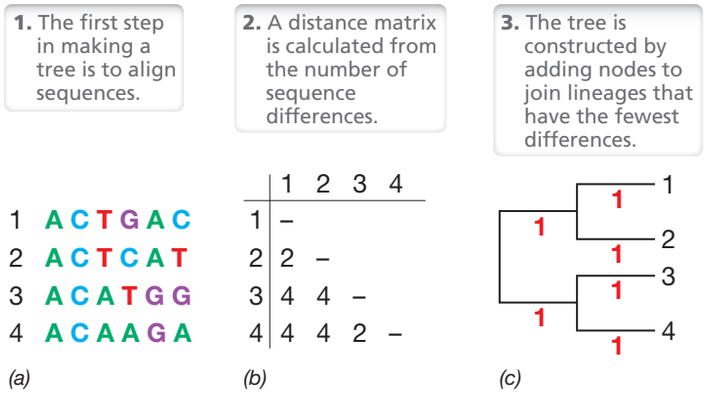


Figure 13.28 Building phylogenetic trees. The number of nucleotide differences between gene sequences can be used to build a phylogenetic tree. In the sequence alignment *(a)* we can count the number of differences between each pair of sequences to build a distance matrix *(b)*. This distance matrix can be used to build a tree *(c)* where the cumulative lengths of the horizontal branches (labeled with a red "1") between any two species in the tree are proportional to the number of nucleotide differences between these species.

UNIT 3

sequences. For example, only three possible trees can be drawn for any four arbitrary sequences. But if the number of sequences is doubled to eight, now 10,395 trees are possible. This complexity continues to expand exponentially such that 2×10^{182} different trees can be drawn to represent 100 arbitrary sequences. Phylogenetic analysis uses molecular sequence data in an attempt to identify the one correct tree that accurately represents the evolutionary history of a set of sequences.

A variety of methods are available for inferring phylogenetic trees from molecular sequence data. The structure of a phylogenetic tree is inferred by applying either an *algorithm* or some set of *optimality criteria*. An algorithm is a programmed series of steps designed to construct a single tree (**Figure 13.28**). Algorithms used to build phylogenetic trees include the *unweighted pair group method with arithmetic mean* (UPGMA) and the *neighbor-joining method*. Alternatively, phylogenetic methods that employ optimality criteria include *parsimony*, *maximum likelihood*, and *Bayesian* analyses. These latter methods evaluate many possible trees and select the one tree that has the best optimality score, that is, they select the tree that best fits the sequence data given a discrete model of molecular evolution. Optimality scores are calculated on the basis of evolutionary models that describe how molecular sequences change over time. For example, evolutionary models can account for variation in substitution rates and base frequencies between sequence positions.

Limitations of Phylogenetic Trees

Molecular phylogeny provides powerful insights into evolutionary history, but it is important to consider the limitations of building and interpreting phylogenetic trees. For example, it can be difficult to choose the true tree based on available sequence data if several different trees fit the data equally well. *Bootstrapping*, a statistical method in which phylogenetic information is resampled at random, is an approach used to deal with uncertainty in phylogenetic trees. Bootstrap values indicate the *percentage of the time* that a given node

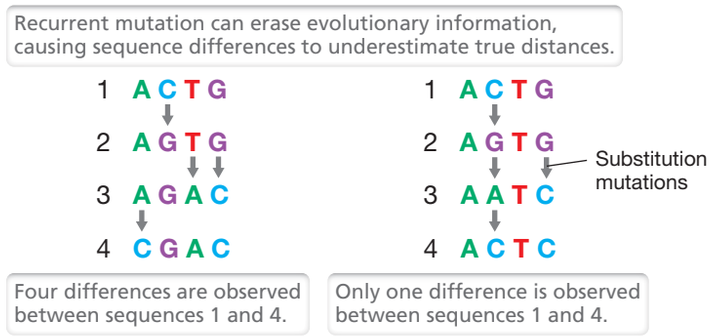
in a phylogenetic tree is supported by the sequence data. High bootstrap values indicate that a node in the tree is likely to be correct, while low bootstrap values indicate that the placement of a node cannot be accurately determined given the available sequence data.

Homoplasy, also known as *convergent evolution*, occurs when organisms share a trait that was not inherited from a common ancestor. An example is the evolution of wings in insects and birds. These traits evolved separately and do not indicate that a winged ancestor was shared among insects and birds. Homoplasy occurs in molecular sequences as well, when similar sequence positions result from recurrent mutation rather than inheritance from a common ancestor (**Figure 13.29*a***). The problem of homoplasy in molecular phylogeny then increases in proportion to evolutionary time (Figure 13.29*b*). As a result of homoplasy, the reconstruction of accurate phylogenetic trees gets more difficult when sequence divergence between organisms is very high.

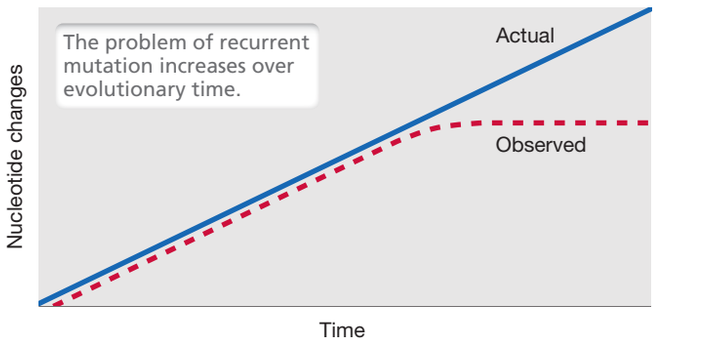
Mastering Microbiology
Art Activity: Figure 13.20 The problem of homoplasy due to recurrent mutation

The prevalence of horizontal gene transfer (Section 13.9 and Chapter 9) also creates complications when considering the evolutionary history of microorganisms. When the sequence of a gene is used to infer the phylogeny of an organism, it must be assumed that the gene is inherited in a *vertical* fashion—from mother to daughter—throughout the evolutionary history of the organism. The *horizontal* exchange of genes between unrelated organisms violates this assumption (**Figure 13.30**). Hence, it is important to consider the difference between a *gene phylogeny*, which depicts the evolutionary history of an individual gene, and an *organismal phylogeny*, which depicts the evolutionary history of the cell.

In general, genes encoding SSU rRNAs appear to be transferred horizontally at very low frequencies, and rRNA gene phylogenies agree largely with those prepared from other genes that encode genetic informational functions. Thus, SSU rRNA gene sequences are generally considered to provide an accurate record of organismal phylogeny. Nevertheless, many microbial genomes contain genes that have been acquired by horizontal gene transfer at some point in their evolutionary history, and this has important implications for microbial evolution that cannot be adequately assessed by analyzing only a single gene, even one as phylogenetically informative as that encoding SSU rRNA.



(a)



(b)

Figure 13.29 The problem of homoplasy due to recurrent mutation. It is possible for recurrent mutation to obscure the true number of mutations that have occurred since a pair of sequences have shared a common ancestor. *(a)* Two series of mutations during the evolution of a gene sequence are compared. On the left side, the number of mutations is equal to the number observed between species 1 and 4. However, if there is recurrent mutation (right side), the number of mutations observed between species 1 and 4 can be less than the number that actually occurred. *(b)* The likelihood of recurrent mutation increases as more and more mutations accumulate over time.

Check Your Understanding

- Why are small subunit ribosomal RNA (SSU rRNA) genes commonly used in microbial phylogeny?
- How does bootstrapping aid in choosing a true phylogenetic tree?
- Why is sequence alignment critical to phylogenetic analysis?

13.12 Microbial Systematics

Microbial systematics is the branch of microbiology concerned with naming and classifying microorganisms, describing their traits, and investigating their evolutionary histories. *Nomenclature*—the naming of organisms—follows the **binomial system** devised by the Swedish medical doctor and botanist Carl Linnaeus in which species are given *genus names* and *species epithets*. The names are Latin or Latinized Greek derivations in which the genus name is a noun and the species epithet an adjective describing some key property of the organism or the habitat from which the organism was obtained. Occasionally, a species epithet will be derived from the last name of a scientist who has made major contributions to the study of a particular group of organisms.

Taxonomy is the classification of organisms into groups of similar organisms. Individual microbial strains are classified into species, species classified into genera, genera into families, families into orders, orders into classes, classes into phyla, and phyla into domains (**Table 13.1**). By classifying organisms into groups and naming them, we order the natural microbial world and make it possible to communicate effectively about all aspects of particular organisms, including their behavior, ecology, physiology, pathogenesis, and evolutionary relationships.

Species are the fundamental units of biological diversity, and how we distinguish and classify species in microbiology greatly affects our ability to explain and assess the diversity of the microbial world. For the purpose of microbial systematics, a microbial species is a taxonomic category that defines a population of individuals that (1) is monophyletic (descended from a common ancestor, see next subsection), (2) is genomically coherent, (3) is phenotypically coherent, and (4) can be clearly differentiated from other species. The description of microbial species employs a *polyphasic approach*, using many different methods in combination,

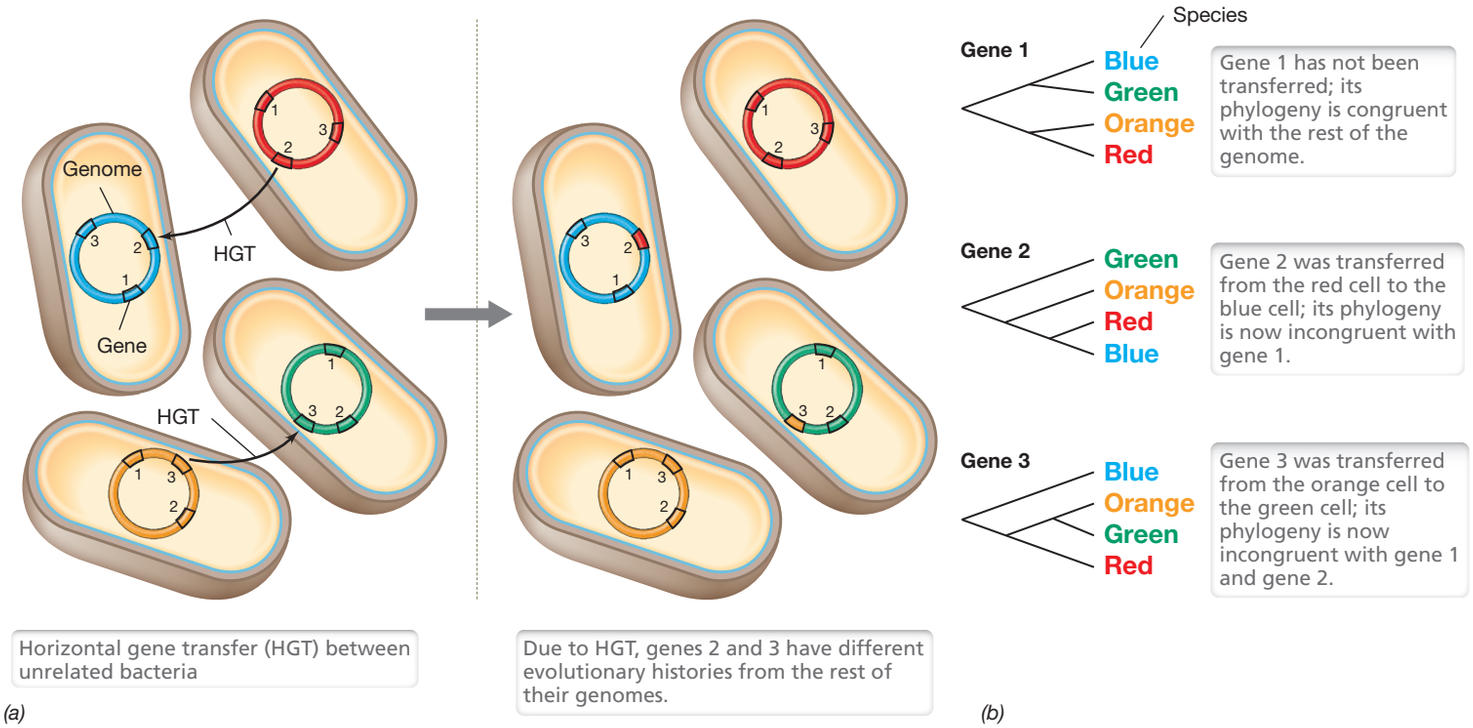


Figure 13.30 Horizontal gene transfer. The horizontal transfer of a gene will cause it to have a different evolutionary history from the rest of the genome. *(a)* Genes are transferred horizontally between distantly related microorganisms. Colors are used to match microorganisms with their genomes. *(b)* As a result of the horizontal transfer events in part *a*, different phylogenetic trees for gene 1, gene 2, and gene 3 are obtained. Only the gene tree for gene 1, which was not transferred, remains congruent with the organismal phylogeny.

to identify, describe, and name species of *Bacteria* and *Archaea*. In this section we describe methods commonly used for characterizing species of *Bacteria* and *Archaea*.

Phylogenetic Analyses

A microbial species should be **monophyletic**, that is, the strains composing the species should all share a recent common ancestor to the exclusion of other species. The phylogenetic relationships of microorganisms are typically determined by analyzing their SSU rRNA gene sequences (Section 13.11). Phylogenetic trees will show that the SSU rRNA gene sequences from strains of a distinct species will all form a coherent group that shares a recent common ancestor to the exclusion of strains of any other species. In addition, SSU rRNA genes from strains of the same species typically have greater than 98.6% sequence similarity, whereas SSU rRNA genes from different species typically have less than 97% sequence similarity (**Figure 13.31**).

SSU rRNA gene sequence similarity can also be useful in classification at higher taxonomic ranks. While there are no established cutoffs or formal criteria used to define higher taxonomic ranks, taxonomic ranks above species generally correlate with SSU rRNA gene sequence similarity. For example, the median SSU rRNA gene sequence similarity between strains of the same group is approximately 95% at the genus level, 92% for family, 89% for order, 86% for class, and 83% for phylum.

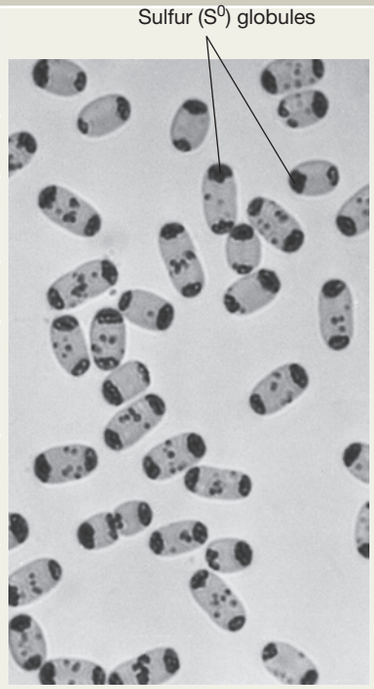
Multilocus sequence analysis (MLSA) can be used in addition to SSU rRNA gene sequence analysis to determine phylogenetic relationships. SSU rRNA gene sequences are highly conserved, and while they provide valuable phylogenetic information, they are not always useful for distinguishing closely related species. By contrast, other highly conserved genes, such as *recA*, which encodes a recombinase protein (◀ Sections 9.4 and 9.5 and Figures 9.10 and 9.12), and *gyrB*, which encodes DNA gyrase (◀ Sections 6.1 and 6.4 and Figures 6.4 and 6.16), can be useful for distinguishing bacteria at the species level, and techniques are available to examine them collectively. The DNA sequences of protein-encoding genes accumulate mutations more rapidly than rRNA genes and thus their sequences can often distinguish prokaryotic species that cannot be resolved by rRNA gene sequence analyses alone (**Figure 13.32**). MLSA multigene analyses generally employ a minimum of four genes but can include more than a thousand genes if genome data are available. These genes can be analyzed independently, or they can be linked into a single alignment and analyzed as a unit to produce a single phylogenetic tree (Figure 13.32).

Genomic Analyses

The use of *entire genomes* for the identification and description of bacteria is becoming increasingly common as DNA sequencing capacities improve and costs decline (◀ Section 10.2). A wide range of sequence analyses are performed on whole genomes, thereby providing insights into microbial physiology and microbial evolution. Comparative analyses of gene content (presence or absence of genes), *synteny* (the order of genes in the genome), and genome G + C content (Section 13.9) provide powerful insights into evolutionary relationships between strains. Whole genome sequences can

TABLE 13.1 Taxonomic hierarchy for the purple sulfur bacterium *Allochromatium warmingii*

Taxon	Name	Properties	Confirmed by
Domain	*Bacteria*	Bacterial cells; rRNA gene sequences typical of *Bacteria*	Microscopy; 16S rRNA gene sequence analysis; presence of unique biomarkers, for example, peptidoglycan
Phylum	*Proteobacteria*	rRNA gene sequence typical of *Proteobacteria*	16S rRNA gene sequence analysis
Class	*Gammaproteobacteria*	Gram-negative bacteria; rRNA sequence typical of *Gammaproteobacteria*	Gram-staining, microscopy
Order	*Chromatiales*	Phototrophic purple bacteria	Characteristic pigments ▶ Figures 14.6, 14.7, and 14.13)
Family	*Chromatiaceae*	Purple sulfur bacteria	Ability to oxidize H_2S and store S^0 within cells; microscopic observation of S^0 (see photo); 16S rRNA gene sequence
Genus	*Allochromatium*	Rod-shaped purple sulfur bacteria; <95% 16S rRNA gene sequence identity with other genera	Microscopy (see photo)
Species	*warmingii*	Cells 3.5–4.0 μm × 5–11 μm; storage of sulfur mainly in poles of cell (see photo); <97% 16S rRNA gene sequence identity with other species	Cell size measured microscopically with a micrometer; observation of polar position of S^0 globules in cells (see photo); 16S rRNA gene sequence



Cells of *A. warmingii*

also be used for metabolic reconstruction to determine the potential physiological characteristics of a given strain.

Several methods in comparative genomics and population genomics have been developed for use in systematic analyses. In particular, metrics of overall genome relatedness can be calculated by determining sequence similarity across the entire genome. **Average nucleotide identity (ANI)** is the most commonly used metric for estimating overall genome relatedness between a pair of organisms. To do this, a computer breaks a genome sequence into fragments about 1000 bp in length. Each fragment is then aligned to its orthologous sequence in a second genome and the average nucleotide identity between the two genomes calculated. Genomes from strains of the same species typically have greater than 96% ANI, and genomes from different species typically have less than 93% ANI (Figure 13.31).

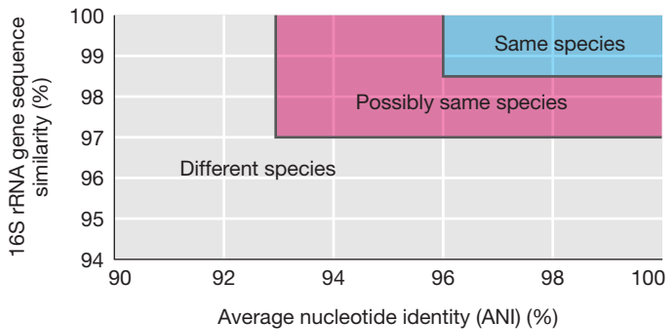


Figure 13.31 Classification of bacterial and archaeal species. Species identification is facilitated by performing pairwise comparison of 16S rRNA similarity and average nucleotide identity (ANI) between strains. Strains with >98.6% 16S rRNA similarity and >96% ANI typically belong to the same species. Strains with 97–98.6% 16S rRNA similarity and 93–96% ANI possibly belong to the same species, but further tests are needed to confirm identity. Strains with <97% 16S rRNA similarity and <93% ANI are highly unlikely to belong to the same species.

Phenotypic Analyses

The observable characteristics—the **phenotype**—of a bacterium provide many traits that can be used to differentiate species. Typically, several phenotypic traits are determined routinely when describing a new microorganism. These phenotypic results are then compared to the phenotypes of organisms that have been described previously. The phenotypic traits that are determined will depend on the type of organism being described. For example, in applied situations, such as clinical diagnostic microbiology, where timely identification is important, a well-defined subset of traits can be tested to rapidly discriminate between different types of microorganisms. Table 13.2 lists general categories and examples of some phenotypic traits used in identifications and species descriptions.

Phenotypic characteristics of a given organism can be highly dependent on growth conditions, and the phenotype of an organism observed in the laboratory may not be the same as that expressed in the natural environment. Thus, care must be taken in using phenotypic characteristics in systematic analyses. In addition, the value of different phenotypic characteristics for reaching a firm systematics conclusion can vary with the taxonomic groups being examined.

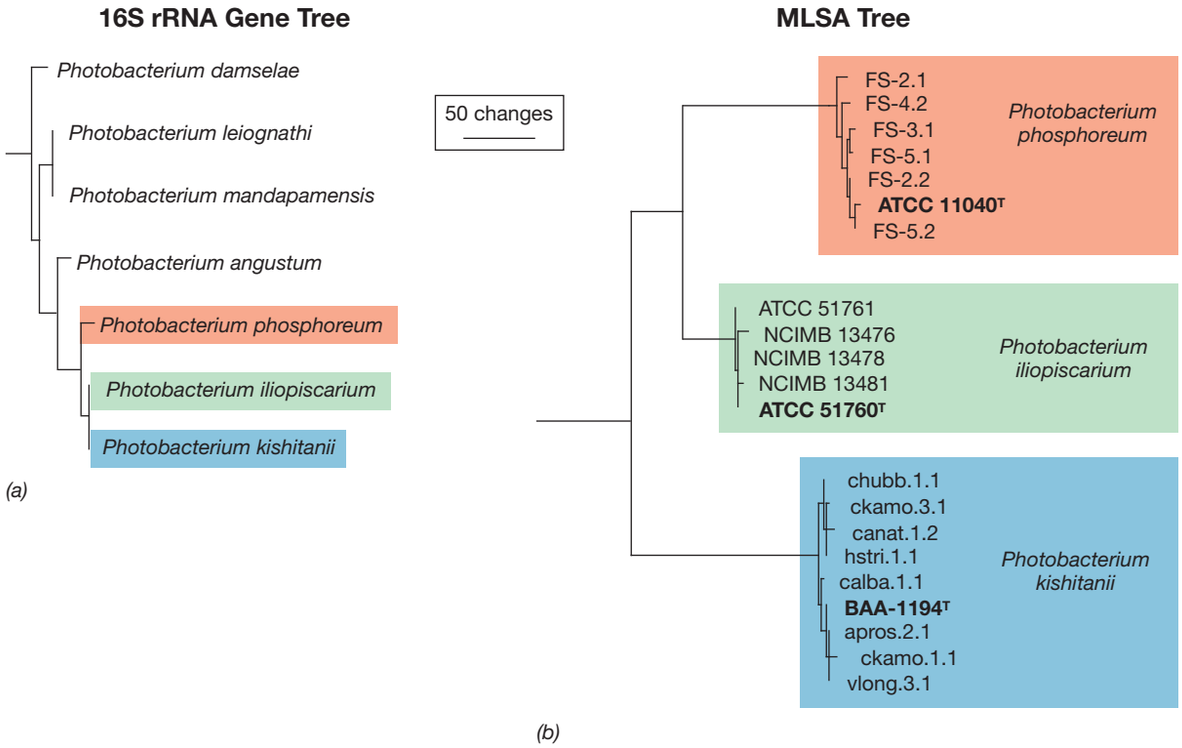


Figure 13.32 Multilocus sequence analysis (MLSA). Phylogenies are shown for species in the genus *Photobacterium*. *(a)* 16S rRNA gene tree, showing the species to be poorly resolved. *(b)* MLSA based on the combined analysis of the 16S rRNA gene and *gyrB* and *luxABFE* genes in 21 isolates of three *Photobacterium* species. MLSA clearly resolves the strains into three distinct species, *P. phosphoreum*, *P. iliopiscarium*, and *P. kishitanii*. The scale bar indicates the branch length equal to a total of 50 nucleotide changes. The type strain of each species is listed in bold. (All abbreviations are part of strain designations.) Phylogenetic analyses courtesy of Tory Hendy and Paul V. Dunlap, University of Michigan.

The Description of New Species

When a newly discovered strain of *Bacteria* or *Archaea* is thought to be unique, a decision must be made as to whether it is sufficiently different from established taxa to be described as a new taxon. The creation of new taxa of *Bacteria* and *Archaea* must follow the rules described in the *International Code of Nomenclature of Prokaryotes* (the Prokaryotic Code). To achieve formal validation of taxonomic standing as a new genus or species, a detailed description of the organism's characteristics and distinguishing traits, along with its proposed name, must be published in a specialized peer-reviewed journal, and viable cultures of the organism must be deposited in at least two international culture collections. Most new taxa are described in the *International Journal of Systematic and Evolutionary Microbiology* (*IJSEM*), the publication of record for the nomenclature of *Bacteria* and *Archaea*. Two websites provide listings of valid, approved bacterial names: List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net), and Prokaryotic Nomenclature Up-to-Date (http://www.dsmz.de).

Microbial culture collections play an important role in protecting microbial biodiversity, just as museums do in preserving plant and animal specimens for future study. Culture collections store microorganisms as *viable cultures*, typically frozen or in a freeze-dried state, and distribute cultures to qualified scientists who wish to study them. These storage methods maintain the cells indefinitely in a living state and prevent genetic changes that might occur if the organisms were continually subcultured. Culture collections also serve as repositories for *type strains*. When a new species of bacteria is described in a scientific journal, a strain is designated as the **type strain** and serves as the nomenclatural type of the taxon for future taxonomic comparison with other strains of that species. Major international culture collections exist in the United States (ATCC), Germany (DSMZ), Japan (JCM Riken), and several other countries. Each collection has a website linked to its acronym from which its holdings can be searched.

It is possible to use molecular and genomic techniques (Chapters 10 and 19) to characterize the phenotypic and genotypic characteristics of microorganisms without the need for the cultivation of an isolated strain. However, in the absence of an isolate that can be deposited in two international culture collections, it is not yet possible to validly name a new species of microorganism under the Prokaryotic Code. However, when an organism is well characterized but not yet obtained in pure culture, a provisional taxonomic name can be applied. The moniker *Candidatus* is appended to candidate taxonomic ranks. For example, "*Candidatus* Pelagibacter ubique" is a globally widespread and well-characterized marine bacterium that is difficult to grow in laboratory media (▶ Section 20.12) and so has not been formally named under the Prokaryotic Code. By contrast, the bacterium "*Candidatus* Heliomonas lunata" can be grown in laboratory culture but the culture is not pure, so this bacterium also retains *Candidatus* status.

TABLE 13.2 Some phenotypic characteristics of taxonomic value

Category	Characteristics
Morphology	Colony morphology; Gram reaction; cell size and shape; pattern of flagellation; presence of spores, inclusion bodies (e.g., PHB,[a] glycogen, or polyphosphate granules, gas vesicles, magnetosomes); capsules, S-layers, or slime layers; stalks or appendages; fruiting body formation
Motility	Nonmotile; gliding motility; swimming (flagellar) motility; swarming; motile by gas vesicles
Metabolism	Mechanism of energy conservation (phototroph, chemoorganotroph, chemolithotroph); utilization of individual carbon, nitrogen, or sulfur compounds; fermentation of sugars; nitrogen fixation; growth factor requirements
Physiology	Temperature, pH, and salt ranges for growth; response to oxygen (aerobic, facultative, anaerobic); presence of catalase or oxidase; production of extracellular enzymes
Cell lipid chemistry	Fatty acids; polar lipids; respiratory quinones
Cell wall chemistry	Presence or absence of peptidoglycan; amino acid composition of cross-links; presence or absence of cross-link interbridge
Other traits	Pigments; luminescence; antibiotic sensitivity; serotype; production of unique compounds, for example, antibiotics

[a]PHB, poly-β-hydroxybutyric acid (◀ Section 2.7).

The classification system most widely accepted by microbiologists is that of *Bergey's Manual of Systematics of Archaea and Bacteria*, a major taxonomic treatment of *Bacteria* and *Archaea* that has served microbiologists since 1923 as a compendium of information on all recognized prokaryotic species. Each chapter, written by experts, contains tables, photos, figures, and other systematic information useful for identification purposes. A second major source describing the physiology, ecology, phylogeny, enrichment, isolation, and cultivation of *Bacteria* and *Archaea* is *The Prokaryotes*. This work is available online by subscription through university libraries. Collectively, *Bergey's Manual* and *The Prokaryotes* offer microbiologists both the concepts and the details of the biology of *Bacteria* and *Archaea* as we know them today and are the primary resources for microbiologists characterizing newly isolated organisms.

How Many Species of *Bacteria* and *Archaea* Exist?

The result of nearly 4 billion years of evolution is the microbial world we see today (Figure 13.9). Microbial taxonomists agree that no firm estimate of the number of bacterial and archaeal species can be given at present. However, they also agree that in the final analysis, this number will be very large. The diversity of bacterial and archaeal species on Earth is unquestionably higher than that of all plant and animal species combined, and the total species numbers of *Bacteria* and *Archaea* are likely several orders of magnitude higher than the 14,000 species that have been characterized to date.

Every environment on Earth contains a diverse community of microorganisms. For example, analyses of environmental SSU rRNA gene sequences (▶ Section 19.6) suggest that over 14,000 different species can coexist in a single gram of soil! Since 1977, more than 3.3 million SSU rRNA sequences have been deposited in international databases such as GenBank and used to characterize the vast diversity of the microbial world. These SSU rRNA sequences encompass more than 225,000 *operational taxonomic units* (OTUs), species-like units of diversity which are defined at a 97% sequence similarity threshold (Figure 13.31). The Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) contains an ever-growing collection of these sequences and provides computational programs for their analysis and for the construction of phylogenetic trees.

While we do not yet know the extent of biodiversity of microbial life, we do know that nearly all plant and animal species have microbiomes (Chapter 23) that contain countless numbers of unique microbes. Thus, microorganisms are not only the oldest but also unquestionably the most diverse forms of life on our planet. We will see this diversity in action over the next five chapters.

Check Your Understanding

- What are some key criteria used to determine whether two strains belong to the same species?
- What is average nucleotide identity and how is it calculated?
- What is the process for describing and naming a new species of bacteria?

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CHAPTER REVIEW

I • Early Earth and the Origin and Diversification of Life

13.1 Planet Earth is about 4.5 billion years old. After its formation, Earth was hot and sterile. Gradual cooling of the planet allowed formation of liquid water, a requirement for the origin of life. The earliest evidence of life comes from isotopic analysis of ancient sedimentary rocks and zircon minerals indicating that life was present on Earth by 3.7–4.1 billion years ago.

Q What is LUCA, and what is a plausible explanation for the origin of cellular life?

13.2 In rocks 3.5 billion years old or younger, microbial formations called stromatolites are abundant and show extensive microbial diversification. The evolution of

oxygenic photosynthesis caused O_2 to accumulate 2.4 billion years ago, eventually leading to the formation of banded iron formations, the ozone shield, and an oxygenated atmosphere, which set the stage for rapid diversification of metabolic types and the evolution of multicellularity.

Q Why was the origin of cyanobacteria of such importance to the evolution of life on Earth?

13.3 Ribosomal RNA genes have been used to construct a universal tree of life revealing that life on Earth evolved in three major directions, forming the domains *Bacteria*, *Archaea*, and *Eukarya*. The universal tree of life shows that the domains *Bacteria* and *Archaea* diverged billions of years ago, and that *Eukarya* split from *Archaea* later in the history of life.

Q What is the three-domain concept of the tree of life? How was the tree of life constructed?

13.4 The eukaryotic cell developed from endosymbiotic events. The modern eukaryotic cell is a chimera with genes and characteristics from both *Bacteria* and *Archaea*. 16S rRNA sequence analyses indicate the ancestors of mitochondria are found in the phylum *Proteobacteria* and those of chloroplasts are found in the phylum *Cyanobacteria*.

Q What is the endosymbiotic theory for the origin of mitochondria and chloroplasts? What evidence supports this theory?

13.5 Viruses lack universally conserved genes and so cannot be placed on the universal tree of life. Viruses might have evolved many times from rogue mobile genetic elements such as gene transfer agents, or they might have a single origin as remnants of pre-cellular life from the RNA world.

Q All viruses protect their nucleic acid using a capsid shell composed of protein. Provide a hypothesis to explain the evolution of capsid proteins.

II • Mechanisms of Microbial Evolution

13.6 Evolution is defined as a change in allele frequencies in a population of organisms over time, resulting in descent with modification. New alleles are created through the processes of mutation and recombination. Mutations occur at random and most mutations are neutral or deleterious, but some are beneficial. Natural selection and genetic drift are two mechanisms that cause allele frequencies to change in a population over time.

Q What is fitness? To what degree does fitness depend on the environment in which organisms live?

13.7 Experimental evolution uses microbial systems to investigate evolution in the laboratory. Mutations occur at random, but those that improve fitness will increase in frequency in a population of cells. Environmental conditions dictate whether mutations cause a change in fitness. Mutations may cause a gain, loss, change, or no change in function.

Q Do *Rhodobacter* pigment mutations occur at the same frequency in the light and in the dark? In the LTEE, do you expect that citrate-using mutants would still have occurred if citrate had never been present in the growth medium?

13.8 A gene family contains a group of homologs that have all descended from the same ancestral gene. Homologs are orthologs if they have same function or paralogs if they have evolved different functions. Paralogs often evolve as a result of gene duplication. Deletion mutations are common in microbial genomes, and they cause the rapid elimination of genetic material that is unnecessary or redundant in function.

Q Explain how deletion mutations can increase fitness. Explain how duplication mutations can promote the evolution of new gene functions.

13.9 Horizontal gene transfer allows genes to be exchanged between cells irrespective of species boundaries. The mobilome is the portion of the genome that contains mobile genetic elements that facilitate horizontal gene exchange and chromosomal rearrangements. The mobilome includes plasmids, viruses, transposons, integrons, and insertion sequences.

Q How can comparative genomics help identify horizontal gene transfer?

13.10 Microbial genomes are dynamic, and genome size and gene content can vary considerably between strains of a species. The core genome is defined as the set of all genes shared by a species, while the pan genome is defined as the core genome plus genes whose presence varies among strains of a species. Many bacteria contain chromosomal islands, large clusters of genes acquired by horizontal gene transfer, that encode specialized functions such as virulence (pathogenicity islands).

Q How can microbial genomes vary extensively between strains of a single microbial species?

III • Microbial Phylogeny and Systematics

13.11 Molecular sequences accumulate random mutations over time, and molecular phylogenetic analysis examines differences in molecular sequences to determine the evolutionary history of life. A phylogenetic tree is a diagram that depicts the evolutionary history of a set of genes or organisms.

Q How can horizontal gene transfer (HGT) lead to phylogenetic incongruence? How can this be overcome?

13.12 Systematics is the study of the diversity and relationships of living organisms. Species of *Bacteria* and *Archaea* are classified using phylogenetic, genomic, and phenotypic information. Strains of the same species are monophyletic, typically have >98.6% SSU rRNA sequence similarity and >96% average nucleotide identity across their genomes, and are expected to share a coherent set of phenotypic traits.

Q Is it possible to provide a formal name for a microorganism that has not been cultivated in isolation? How might you attempt to describe and name such a species?

APPLICATION QUESTIONS

1. Compare and contrast the physical and chemical conditions on Earth at the time life first arose with conditions today. From a physiological standpoint, discuss at least two reasons why *animals* could not have existed on early Earth. In what ways has microbial metabolism altered Earth's biosphere? How might life on Earth be different if oxygenic photosynthesis had not evolved?
2. For the following sequences, construct the phylogenetic tree that best depicts their evolutionary relationships (assume that the sequences are aligned).

   Taxon 1: GTTCCCTA

   Taxon 2: GTTCGCTA

   Taxon 3: GATAGCCA

   Taxon 4: GTTCGCTA
3. Imagine that you are given several bacterial strains from a non-native plant species in your country. How could you infer their evolutionary history in relation to bacterial strains from the same plant species found at their native habitats?

CHAPTER GLOSSARY

Allele a sequence variant of a given gene

Archaea a domain of life consisting of microorganisms that have prokaryotic cell structure and are distinct from *Bacteria*

Average nucleotide identity (ANI) a genome-wide measure of genetic similarity between two microorganisms based on aligning all orthologous gene pairs across the two genomes and determining the percentage of matching nucleotides (see also *orthologs*)

Bacteria a domain of life consisting of microorganisms that have prokaryotic cell structure and are distinct from *Archaea*

Banded iron formation iron oxide–rich ancient sedimentary rocks containing zones of oxidized iron (Fe^{3+}) formed by oxidation of Fe^{2+}

Binomial system the system devised by the Swedish scientist Carl Linnaeus for naming living organisms in which an organism is given a genus name and a species epithet

Chromosomal island a chromosome region of foreign origin containing a cluster of genes that work together to confer upon the cell a particular trait such as virulence or symbiosis

Core genome those genes shared by all strains of a species

Endosymbiotic theory the idea that mitochondria and chloroplasts originated from *Bacteria*

Eukarya a domain of life consisting of organisms that have eukaryotic cell structure; includes algae, protists, fungi, slime molds, plants, and animals

Evolution a change over time in gene sequence and frequency within a population of organisms, resulting in descent with modification

Evolutionary selection a process that results in a change in allele frequencies in a population when individuals that are favored in a given environment are able to produce more offspring and make a greater contribution to the gene content of future generations

Fitness the capacity of an organism to survive and reproduce in a given environment as compared to that of competing organisms

Gene family genes related in sequence to each other because of common evolutionary origin

Genetic drift a process that results in a change in allele frequencies in a population as a result of random changes in the number of offspring from each individual over time

Homologs genes that were inherited from a shared ancestor; includes both orthologs and paralogs

Homoplasy when two organisms have the same trait as a result of recurrent mutation or convergent evolution

Horizontal gene transfer the unidirectional transfer of genes between cells through a process uncoupled from reproduction

Mobilome the mobile genetic elements in a genome

Molecular clock a DNA sequence, such as a gene for rRNA, that can be used as a comparative temporal measure of evolutionary divergence

Monophyletic in phylogeny, a group descended from a single shared ancestor

Multilocus sequence analysis (MLSA) phylogenetic analysis of microorganisms performed using multiple different genes

Mutation a heritable change in the base sequence of the genome of an organism

Orthologs genes that are homologous (that is, they are descended from a common ancestor) and share the same function (see also *paralogs*)

Pan genome the totality of the genes present in the different strains of a species

Paralogs genes that are homologous (that is, they are descended from a common ancestor) but which have diverged to have different functions; typically, paralogous genes are the result of gene duplication (see also *orthologs*)

Pathogenicity island a bacterial chromosome region of foreign origin that contains clustered genes for virulence

Phenotype the observable characteristics of an organism

Phylogenetic tree a diagram that depicts the evolutionary history of an organism; consists of nodes and branches

Phylogeny evolutionary history of organisms

Phylum a major lineage of organisms in one of the three domains of life

Recombination a resorting or rearrangement of DNA fragments resulting in a new sequence

Ribosomal RNA (rRNA) the types of RNA found in the ribosome; some participate actively in protein synthesis

Sequence alignment the insertion of gaps into a set of molecular sequences organized in rows so that homologous positions are organized in vertical columns. Alignment is necessary prior to phylogenetic analysis because deletion and insertion mutations cause variations in the length of molecular sequences

16S rRNA the type of SSU rRNA found in *Bacteria* and *Archaea*; its eukaryotic counterpart is 18S rRNA (see also *SSU rRNA*)

Species defined in microbiology as a collection of strains that all share the same major properties and differ in one or more significant properties from other collections of strains; defined phylogenetically as a monophyletic, exclusive group based on DNA sequence

SSU rRNA (small subunit rRNA) the rRNA molecule found in the small subunit of the ribosome, comprised of 16S rRNA, which is present in the 30S ribosomal subunit of *Bacteria* and *Archaea*, or its orthologous counterpart 18S rRNA, which is present in the 40S ribosomal subunit of *Eukarya*. SSU rRNA genes are conserved in all forms of cellular life, and this gene is often used in phylogenetic analysis of microorganisms

Stromatolite a laminated microbial mat that can become fossilized and is typically built from layers of filamentous microorganisms

Systematics the study of the diversity of organisms and their relationships; includes taxonomy and phylogeny

Taxonomy the science of identification, classification, and nomenclature

Type strain the strain chosen to represent the nomenclatural type of a species; type strains are deposited in microbial culture collections and are used to represent the typical characteristics of their species

Universal tree of life a phylogenetic tree that shows the positions of representatives of all domains of cellular life

14 Metabolic Diversity of Microorganisms

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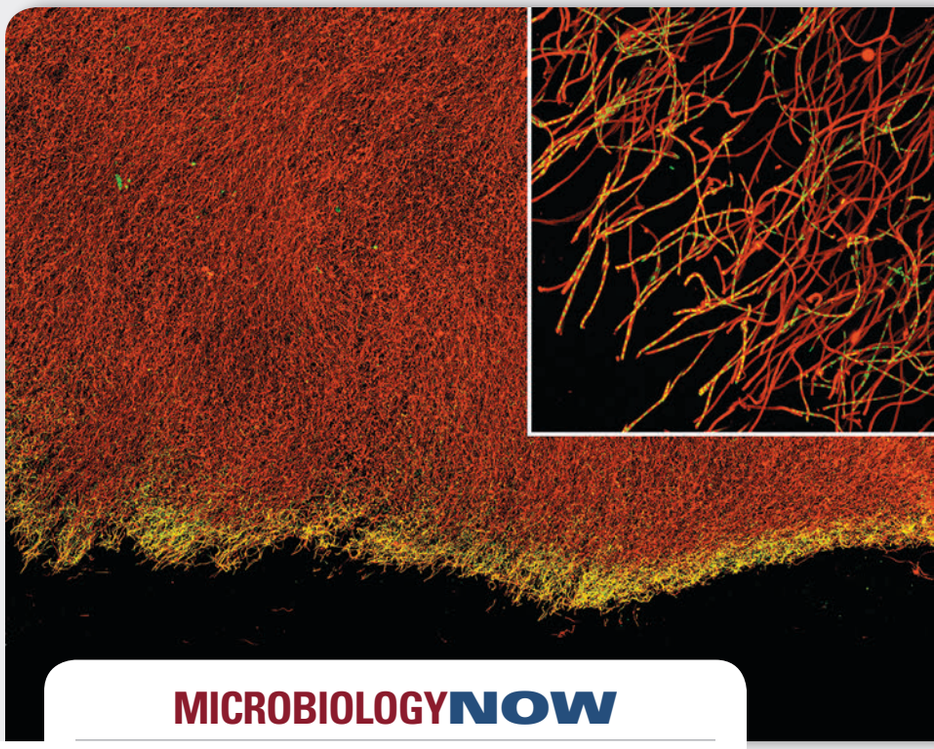


MICROBIOLOGYNOW

Ferreting Out the Peculiar Life of Iron Bacteria

Life requires the movement of electrons from one place to another, and microbes have found many different ways to accomplish this. Many iron-oxidizing bacteria are autotrophic chemolithotrophs that conserve energy by oxidizing Fe^{2+} and transporting electrons to an electron acceptor such as O_2. Fe^{2+} is present in anoxic groundwater, and the "iron bacteria" grow where this ferrous-rich water reaches the oxic surface. However, aerobic iron bacteria face an unusual challenge because when reduced iron meets O_2, it spontaneously oxidizes to form rust. To survive, iron bacteria must outcompete this abiotic process. Another challenge is that iron bacteria form iron oxide minerals as waste products, and as they grow, they risk becoming entombed within a metallic crust of their own making.

Iron bacteria have evolved elegant strategies for overcoming these obstacles. They have specialized mechanisms for donating electrons to extracellular electron acceptors, and this prevents iron oxides from forming within cells. In addition, they catalyze iron biomineralization, forming stalks and sheaths that are extruded away from the cell. The photos here show cells of the iron bacterium *Leptothrix ochracea* forming a microbial mat in a spring in Spruce Point, Maine (USA). In these fluorescence micrographs, the cells (about 5 μm long) are stained yellow and occur only at the tips of hollow iron oxide sheaths, stained red. The aerobic oxidation of iron provides only a very small amount of energy and because of this, iron bacteria leave long empty iron oxide sheaths as they grow. The cells sense and constantly move toward O_2, meaning that sheath production functions almost as a form of motility that allows *L. ochracea* to position itself effectively in response to gradients of Fe^{2+} and O_2.

Microbes are often defined by their metabolic properties, and to understand bacteria like *L. ochracea*, we must first understand their metabolism.

Source: Chan, C.S., et al. 2016. The architecture of iron microbial mats reflects the adaptation of chemolithotrophic iron oxidation in freshwater and marine environments. *Front. Microbiol.* 7: 796.

A major theme of microbiology is the great *phylogenetic* diversity of microbial life on Earth. We will explore this microbial diversity in the following four chapters. In this chapter we focus on the *metabolic* diversity of microorganisms, with special emphasis on the processes and mechanisms that underlie this diversity. Metabolism provides one framework we can use to make sense of microbial diversity. In the following chapters, we will see that microbial diversity can be understood in terms of metabolic diversity, ecological diversity, and phylogenetic diversity.

I • Introduction to Metabolic Diversity

All cells conserve energy, obtain reducing power, and achieve redox balance by carrying out oxidation–reduction reactions. Metabolic pathways are modular and often reversible, facilitating the evolution of metabolic diversity through horizontal gene transfer.

While microbial metabolism is amazingly diverse, all microbes follow the same basic metabolic principles. These principles were introduced in Chapter 3, and we revisit them now and expand upon them to see how they apply to diverse metabolic types. In all forms of metabolism, energy is conserved and reducing power is obtained during catabolic reactions (◀ Figure 3.1), and cells grow by coupling this energy to anabolic (biosynthetic) reactions such as CO_2 fixation. We begin our review of metabolic diversity by reviewing the mechanisms of energy conservation and their relationship to redox reactions.

14.1 Foundational Principles of Metabolic Diversity: Energy and Redox

The diversity of metabolic types in the microbial world is tremendous, and these diverse organisms are essential to sustain our biosphere. In part, this diversity is driven by the modularity of metabolic reactions. Metabolic modularity facilitates the evolution of metabolic diversity by allowing the formation of new pathways from the horizontal transfer and modification of existing enzymes and pathways. For example, the components of electron transport allow for a tremendous diversity of respirations, most of which do not involve O_2 and are thus anaerobic respirations (**Figure 14.1**). By understanding the principles that underlie metabolism, we can move beyond the memorization of pathways as unique entities and instead learn to predict the metabolic needs of any organism we encounter in the microbial world.

All organisms need to **conserve energy** by converting chemical or light energy into ATP. They also need a source of **reducing power**, a source of highly electronegative electrons for performing redox reactions. Finally, they need to achieve **redox balance** by regenerating oxidized electron carriers (such as NAD^+) through the use of an external electron acceptor (in respiration), or by recycling electrons back onto a metabolic intermediate and excreting them as fermentation products (in fermentation).

Conservation of Energy

All cells conserve energy by coupling a flow of electrons to the synthesis of ATP. Electron flow in the cell consists of a series of *redox*

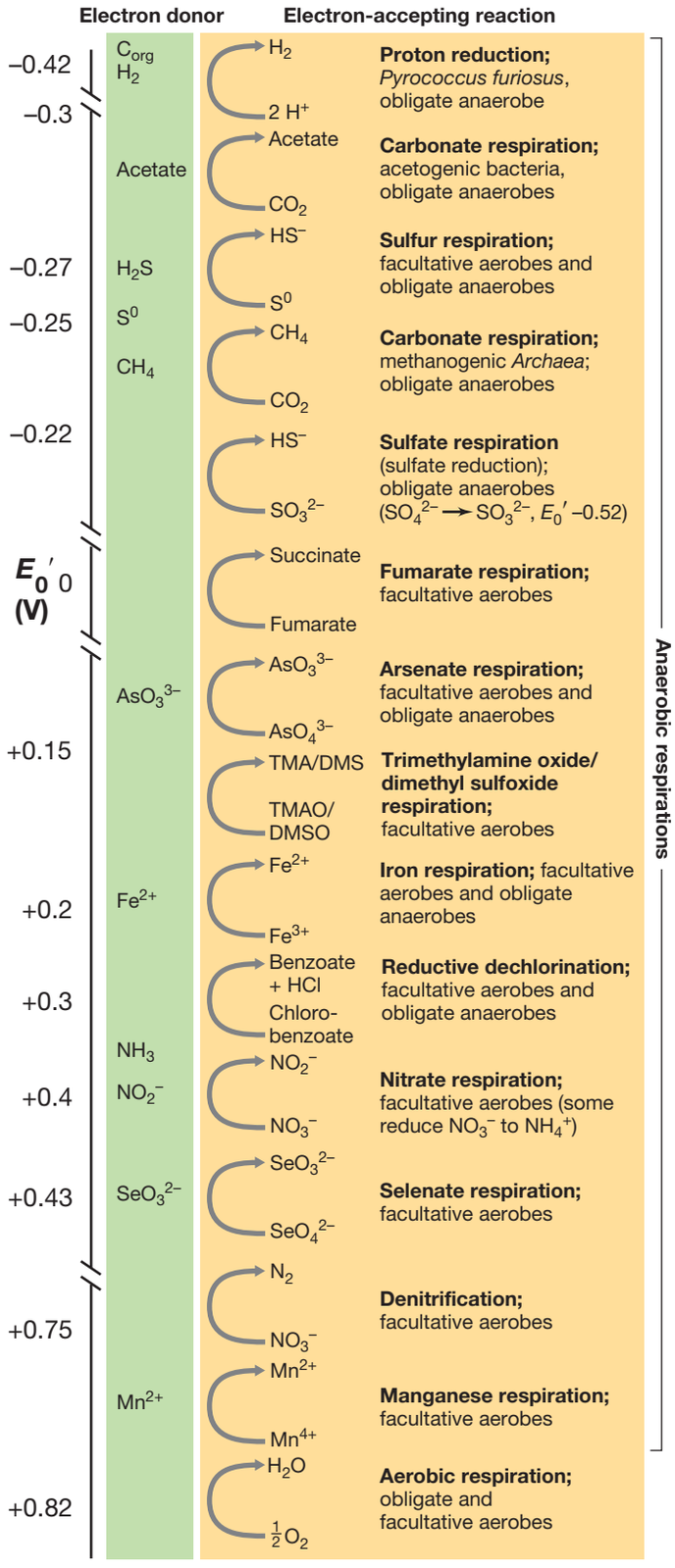


Figure 14.1 Diversity of respiration reactions. The redox couples (right, yellow box) are arranged in order from most electronegative E_0' (top) to most electropositive E_0' (bottom), assuming neutral pH. An exergonic reaction results when an electron donor (left, green box) donates electrons to an electron acceptor from a more electropositive (lower) redox couple. See Figure 3.4 and Table 3.1 to compare how the energy yields of some of these anaerobic respirations vary. The E_0' of the Fe^{3+}/Fe^{2+} couple at pH 2 is +0.77 V. NH_3 is shown to indicate its relative E_0' value as a donor. C_{org}, organic carbon compounds (glucose, ethanol, etc.).

reactions. Recall from Chapter 3 that redox reactions are reactions in which electrons are transferred from one substance (in one half reaction that *donates* electrons) to another substance (in a second half reaction that *accepts* electrons). The tendency of a substance to donate or accept electrons is defined by its reduction potential (E_0', Figure 14.1; ◀ Figure 3.4 and Table 3.1). The farther apart the two half reactions are in terms of the E_0' of their redox couples, the greater the amount of energy available (◀ Figure 3.4).

Based on these simple principles governing redox reactions, a wide variety of organic or inorganic electron donors can be coupled to a wide variety of external electron acceptors (through respiration, Figure 14.1) or internal metabolic products (through fermentation), generating the tremendous diversity of metabolic options that exist in the microbial world. These reactions can support growth, provided sufficient energy is released for the production of ATP (−31.8 kJ/mol). Ultimately, the flow of electrons through redox reactions provides the energy for ATP synthesis by one of three mechanisms: *substrate-level phosphorylation*, *oxidative phosphorylation*, or *photophosphorylation* (Chapter 3). Chemotrophs conserve energy by the former two mechanisms while phototrophs use the latter. We will consider phototrophs in detail in Sections 14.3–14.6 and focus first on chemotrophic metabolism.

Chemotrophic Metabolism

Chemotrophic organisms can conserve energy through either fermentation or respiration. **Fermentation** does not require an external electron acceptor and ATP is generated primarily by substrate-level phosphorylation, with electron balance obtained by reducing metabolic intermediates that are then excreted as fermentation products (◀ Section 3.7). **Respiration**, by contrast, requires an external electron acceptor, and ATP is generated by oxidative phosphorylation resulting from electron transport reactions that generate a proton motive force (pmf); the enzyme complex ATP synthase harnesses this force to make ATP (◀ Section 3.9). Chemolithotrophs are chemotrophic organisms that use *inorganic* electron donors in respiration reactions (**Table 14.1**). Note in Table 14.1 how the energy yield of each reaction shown is determined by the $\Delta E_0'$ of its redox couples.

Respiration can occur under both oxic and anoxic conditions. **Anaerobic respirations** are those whose electron acceptors are other than O_2. From Figure 14.1 we can see that almost any half reaction can serve as an electron acceptor if coupled with a sufficiently electronegative electron donor. Because the O_2/H_2O couple is so electropositive, more energy is typically available from aerobic respiration than from anaerobic respiration. Therefore, for any given electron donor, aerobic organisms will always conserve more energy—and will therefore outcompete—anaerobic organisms. However, because O_2 is such a good electron acceptor, and because it is poorly soluble in water, it is consumed rapidly in poorly mixed environments. Thus, anoxic habitats and anaerobic organisms are widespread in nature.

Reducing Power and Redox Balance

The conservation of energy on its own is not sufficient to support life. Cells also need a source of reducing power for biosynthesis and need to achieve redox balance by balancing electron flow within the cell. The reducing power required for biosynthetic reactions is typically in the form of low-potential electron carriers such as NAD(P)H ($E_0' = -0.32$ V) or reduced ferredoxin (Fd_{red}, $E_0' = -0.37$ to -0.50 V). In the case of most chemoorganotrophs, this reducing power is readily generated during the oxidation of reduced organic molecules (◀ Sections 3.6 and 3.7). The challenge for chemoorganotrophs is managing their redox balance; that is, they need a way to export electrons from the cell in order to regenerate the oxidized $NAD(P)^+$ or Fd_{ox} they need for catabolic reactions. Chemoorganotrophs achieve redox balance by donating electrons to external electron acceptors (in respiration) or metabolic intermediates (in fermentation) that are reduced and excreted from the cell. In contrast, the problem for many chemolithotrophs and some phototrophs (◀ Section 3.11) is a lack of reducing power. Their electron donors (such as S^0, H_2S, NH_3, and the like) are unable to reduce the $NAD(P)^+$ or Fd_{ox} they need for catabolic and biosynthetic reactions. Hence, these organisms must generate reducing power by coupling the endergonic reduction of these electron carriers to some other exergonic reaction.

Three mechanisms of energy coupling are known that can increase reducing power or achieve redox balance at the expense of energy. The first is by coupling a reaction directly to ATP hydrolysis; this requires a substantial energy expenditure by the cell and so is used

TABLE 14.1 Energy yields from the oxidation of various inorganic electron donors[a]

Electron donor	Chemolithotrophic reaction	Group of chemolithotrophs	E_0' of couple (V)	$\Delta G^{0\prime}$ (kJ/reaction)	Number of electrons/reaction	$\Delta G^{0\prime}$ (kJ/2 e^-)
Phosphite[b]	$4\,HPO_3^{2-} + SO_4^{2-} + H^+ \rightarrow 4\,HPO_4^{2-} + HS^-$	Phosphite oxidizers	−0.69	−364	8	−91
Hydrogen[b]	$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$	Hydrogen oxidizers	−0.42	−237.2	2	−237.2
Sulfide[b]	$HS^- + H^+ + \frac{1}{2}O_2 \rightarrow S^0 + H_2O$	Sulfide oxidizers	−0.27	−209.4	2	−209.4
Sulfur[b]	$S^0 + 1\frac{1}{2}O_2 + H_2O \rightarrow SO_4^{2-} + 2\,H^+$	Sulfur oxidizers	−0.20	−587.1	6	−195.7
Ammonia	$NH_3 + 1\frac{1}{2}O_2 \rightarrow NO_2^- + H^+ + H_2O$	Ammonia oxidizers	+0.34	−287.6	6	−95.9
Nitrite[b]	$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^-$	Nitrite oxidizers	+0.43	−74.1	2	−74.1
Ferrous iron[b]	$Fe^{2+} + H^+ + \frac{1}{4}O_2 \rightarrow Fe^{3+} + \frac{1}{2}H_2O$	Iron oxidizers	+0.77	−32.9	1	−65.8

[a]Data are from G_i^0 values in Table 3.1 (or references therein) and Figure 3.4, and from bioenergetics calculations as described in Sections 3.2 and 3.3; E_0' values for Fe^{2+} are for pH 2, and others are for pH 7. At pH 7, the E_0' for the Fe^{3+}/Fe^{2+} couple is about +0.2 V.
[b]Except for phosphite, all reactions are shown coupled to O_2 as electron acceptor. The only known phosphite oxidizer couples to SO_4^{2-} as electron acceptor. H_2 and most sulfur compounds can be oxidized anaerobically using one or more electron acceptors, and Fe^{2+} can be oxidized at neutral pH with NO_3^- as electron acceptor. For other chemolithotrophic reactions of sulfur compounds, see Table 14.2.

primarily by organisms that perform highly exergonic catabolic reactions. The second is **reverse electron transport** (◀ Section 3.11) wherein the endergonic reduction of $NAD(P)^+$ or Fd_{ox} is driven by dissipation of the pmf. For example, during electron transport, an NADH:quinone oxidoreductase (such as Complex I, ◀ Figure 3.19) can donate electrons to an oxidized quinone, an exergonic reaction that contributes to the formation of the pmf. However, during reverse electron transport, this enzyme runs in the reverse direction and transfers electrons from a reduced quinone to NAD^+, an endergonic reaction driven by the pmf.

The third, and most energy-efficient, mechanism of energy coupling is flavin-based **electron bifurcation** (Figure 14.2). In electron bifurcation, the endergonic reduction of a low-potential electron acceptor such as Fd_{ox} is driven by the exergonic reduction of a higher-potential electron acceptor such as NAD^+ (Figure 14.2). Electron-bifurcating enzymes contain a flavin coenzyme. The flavin accepts two electrons at a time and donates one of these electrons in an exergonic reaction to a higher-potential electron acceptor (such as NAD^+) in order to drive the endergonic reduction of a lower-potential electron acceptor (such as Fd_{ox}) by the other electron (Figure 14.2). For example, Figure 14.2 depicts a bifurcation reaction in which 2 NADH ($2 \times 2\ e^-$, $E_0' = -0.32$ V) are oxidized to reduce one low-potential Fd_{ox} ($2 \times 1\ e^-$, $E_0' = -0.42$ V) and one crotonyl-CoA ($2 \times 1\ e^-$, $E_0' = -0.01$ V). Some electron-bifurcating systems are even reversible, resulting in electron *confurcation* (see Sections 14.12 and 14.17).

Flavin-based electron-bifurcating enzymes have two important functions. (1) They allow the cell to make a highly electronegative intermediate (such as Fd_{red}) that can drive difficult endergonic reductions and can also be used to conserve energy through pmf formation (see Sections 14.12, 14.15–14.17, and 14.19). (2) They can increase fermentative energy yields by allowing the cell to oxidize NADH to NAD^+ through H_2 production from Fd_{red} (see Sections 14.17, 14.19). Many obligate anaerobes use flavin-based electron bifurcation reactions, particularly those having very low energy yields such as fermenters, sulfate reducers, acetogens, and methanogens. We will learn about these processes later in this chapter.

Assimilative and Dissimilative Processes

While chemoorganotrophs often get nutrients from organic materials, many chemotrophs and phototrophs can assimilate nutrients by

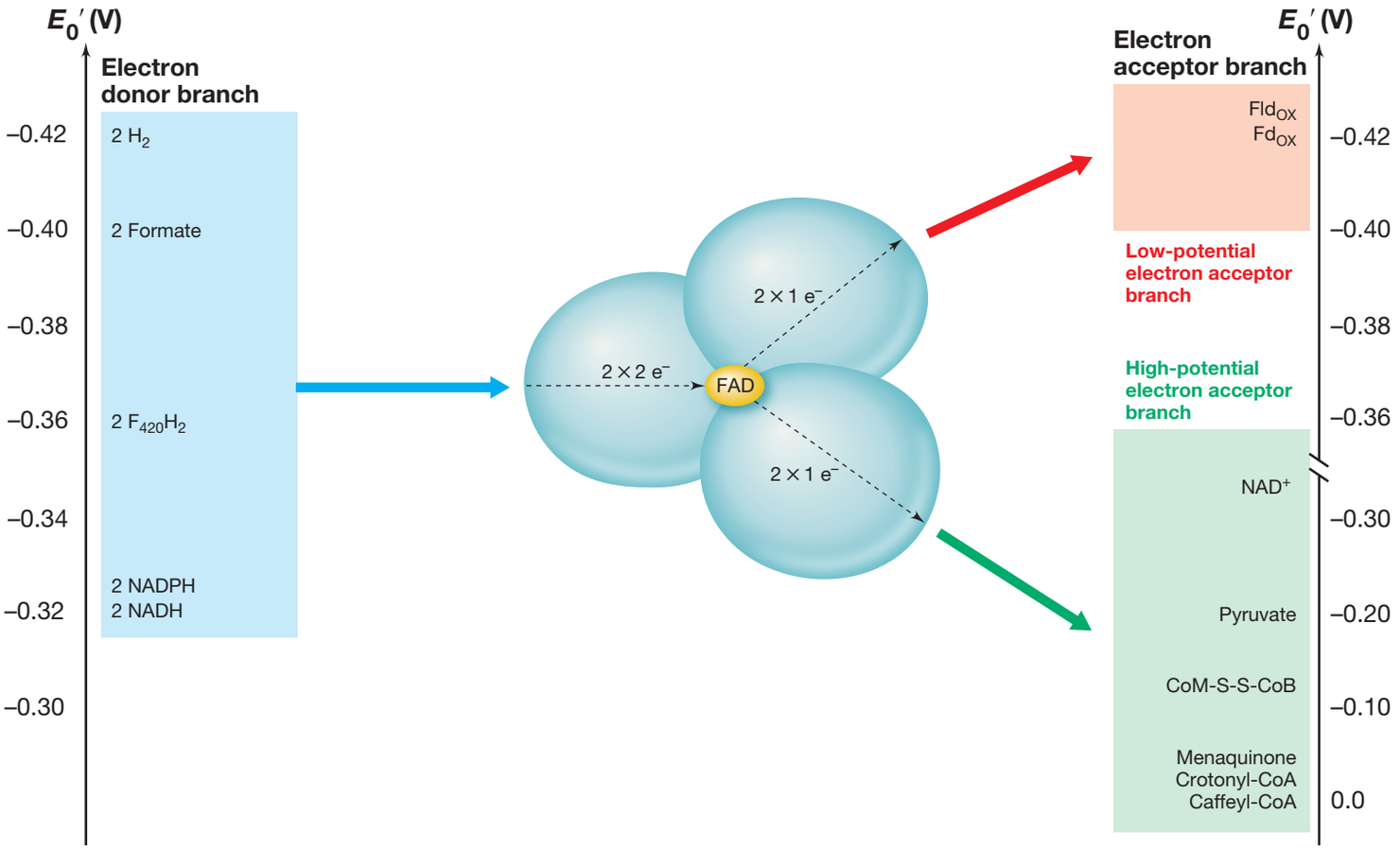


Figure 14.2 The reaction scheme for flavin-based electron bifurcation. Many obligate anaerobes require reducing power in the form of reduced ferredoxin (Fd_{red}) or flavodoxin (Fld_{red}) to perform difficult reactions (such as CO_2 fixation), but lack electron donors sufficiently electronegative to reduce ferredoxin. In flavin-based electron bifurcation, two electrons from an electron donor (such as H_2) are transferred to a flavin (FAD), and one electron is used to reduce a favorable electron acceptor (such as NAD^+), making it possible to drive the second electron to an unfavorable electron acceptor (such as Fd_{ox}). The reaction can be reversed yielding *electron confurcation*, for example by organisms that need to achieve redox balance by driving the endergonic formation of H_2 from NADH.

reducing inorganic molecules such as N_2, NO_3^-, SO_4^{2-}, and CO_2. *Assimilative* processes are those processes used to assimilate inorganic nutrients into cell material. By contrast, *dissimilative* processes are those processes used to conserve energy.

Assimilative and dissimilative processes differ markedly. In assimilative metabolism, *energy is consumed* as nutrients are assimilated into cellular material, and so assimilatory reactions are performed only to acquire those nutrients needed to satisfy the biosynthetic needs of growing cells. Since assimilatory reactions contribute to biosynthesis, they require energy in the form of ATP and reducing power. By contrast, in dissimilative metabolism, *energy is conserved*, and this means that a large amount of the electron acceptor must be reduced and then excreted from the cell. Most microbes can perform a variety of assimilative reduction reactions, whereas dissimilative reduction reactions are the result of anaerobic respiration.

The most important assimilative process in the biosphere is CO_2 fixation performed by autotrophic organisms. A diversity of pathways for fixing CO_2 into cellular material exist, as we will see next, and by exploring how these pathways differ we will shed light on the evolutionary forces that have produced the vast metabolic diversity within the microbial world.

Check Your Understanding

- In a coupled reaction, how can you tell the electron donor half reaction from the electron acceptor half reaction?
- How does aerobic respiration differ from anaerobic respiration, and why does aerobic respiration repress anaerobic respiration?
- Describe the major differences between assimilative and dissimilative processes.

14.2 Autotrophic Pathways

An **autotroph** is an organism that can assimilate CO_2 into cell material. Many microbes are autotrophic, including virtually all phototrophs and chemolithotrophs. In all autotrophs, CO_2 fixation supplies carbon for the biosynthesis of cellular materials, and in phototrophs, CO_2 is also the ultimate electron acceptor for photosynthesis.

The evolutionary origin of CO_2 fixation likely occurred very soon after the origin of life when Earth was still anoxic. As a result, most CO_2 fixation pathways contain enzymes inhibited by O_2. Pathways of CO_2 fixation often share some enzymes with catabolic pathways, revealing the modularity of enzyme systems. Indeed, the presence of shared metabolites between pathways for catabolism and CO_2 fixation create opportunities for some organisms to grow as **mixotrophs**, meaning that rather than growing as either a heterotroph or an autotroph, they exist on a continuum between heterotrophy and autotrophy depending on the opportunities available to them in their environments. We will also see, as is the case with the Calvin cycle, that enzymes that provide a strong evolutionary benefit are often shared between microorganisms by horizontal gene transfer.

The Calvin Cycle

The **Calvin cycle** (◀ Section 3.12) is the most widespread and globally important pathway for CO_2 fixation. The Calvin cycle is used by all oxygenic phototrophs (including cyanobacteria, algae, and plants), most purple bacteria, and most aerobic chemolithotrophic bacteria (◀ Section 3.11). This wide distribution indicates that many microbes acquired Calvin cycle genes from horizontal gene transfer. The key enzyme of the Calvin cycle is the enzyme **RuBisCO**, which reduces CO_2 to the level of glyceraldehyde 3-phosphate (◀ Section 3.12). The Calvin cycle requires 12 NAD(P)H and 18 ATP to synthesize one molecule of fructose 6-phosphate from 6 CO_2 (◀ Figure 3.27).

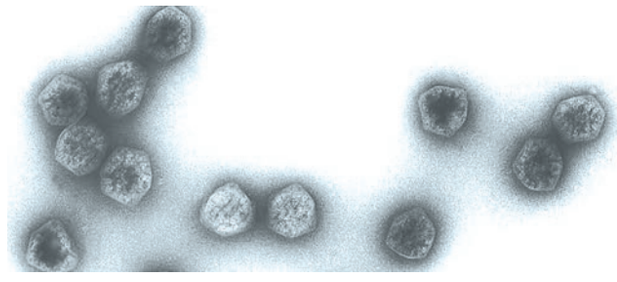
Many bacteria that perform the Calvin cycle have **carboxysomes** (**Figure 14.3**), which are proteinaceous microcompartments and are the site of RuBisCO activity. RuBisCO has very low affinity for CO_2 and is inhibited by O_2. This inhibition occurs because O_2 competes with CO_2 for access to the RuBisCO active site, resulting in a waste of reducing power and decreasing autotrophic efficiency. Carboxysomes improve the efficiency of RuBisCO dramatically because they concentrate CO_2 and exclude O_2 at the site of RuBisCO activity. Even with carboxysomes, RuBisCO is a relatively inefficient and sluggish enzyme and so cells performing the Calvin cycle must contain very large amounts of RuBisCO protein. As a result, RuBisCO is thought to be the single most abundant protein on the Earth.

The evolutionary origins of the Calvin cycle remain unclear, but the low affinity for CO_2 and high sensitivity to O_2 of its key enzyme, RuBisCO, indicates that the cycle originated prior to the Great Oxidation Event (◀ Section 13.2). Carboxysomes were unnecessary at that time because the Earth's atmosphere had much higher levels of CO_2 and lower levels of O_2 than it has today. Indeed, carboxysomes are completely absent from chloroplasts and this infers that the cyanobacterial ancestor of chloroplasts possessed the Calvin cycle but lacked carboxysomes. Molecular sequence analysis indicates that carboxysomes originated in nonphototrophic bacteria and were acquired by cyanobacteria during horizontal gene transfer events well after the origin of chloroplasts. There is intense interest in discovering a way to bioengineer carboxysomes into the chloroplasts of plants because such a modification would increase photosynthetic efficiency and plant growth.

Although the Calvin cycle is the most widespread and important pathway of CO_2 fixation in the biosphere, many autotrophic *Bacteria* and *Archaea* employ alternative pathways for fixing CO_2.

The Reverse Citric Acid Cycle

Not all phototrophic organisms rely on the Calvin cycle for CO_2 fixation. The **reverse citric acid cycle** (also called the *reductive tricarboxylic acid cycle* or *rTCA cycle*) is a pathway of CO_2 fixation used



Jessup M. Shively

Figure 14.3 Crystalline Calvin cycle enzymes: Carboxysomes. Electron micrograph of carboxysomes purified from the chemolithotrophic sulfur oxidizer *Halothiobacillus neapolitanus*. The structures are about 100 nm in diameter. Carboxysomes are present in a wide variety of obligately autotrophic aerobic *Bacteria*.

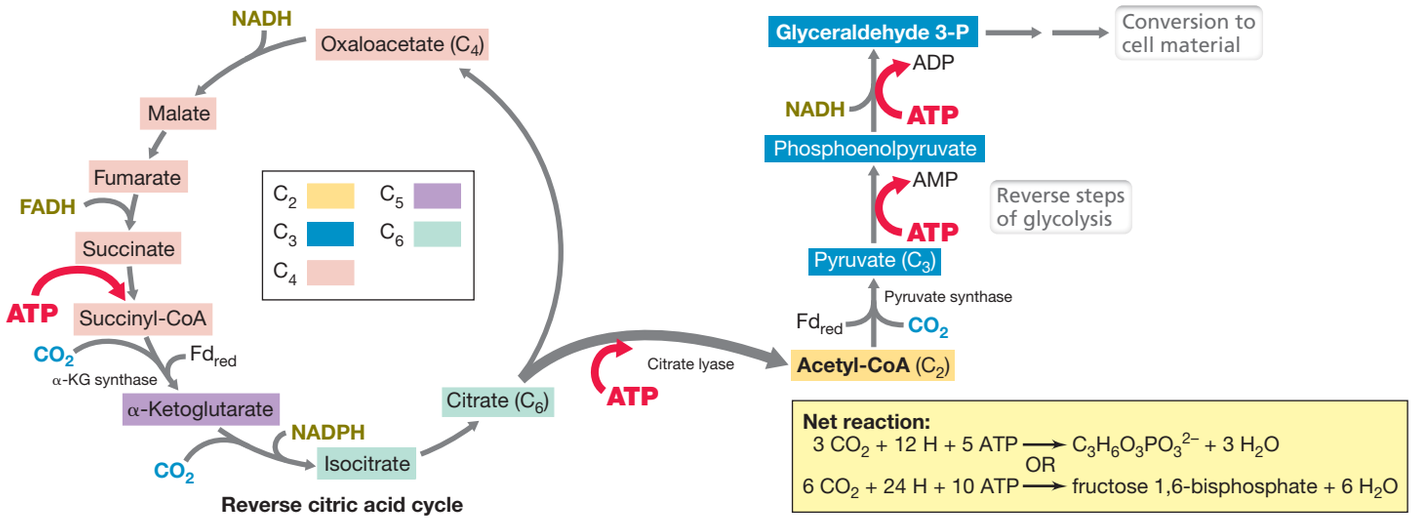


Figure 14.4 Reverse citric acid cycle. The reverse citric acid cycle is the mechanism of CO_2 fixation in green sulfur bacteria and many microaerophilic and anaerobic chemolithotrophs. Fd_{red} indicates carboxylation reactions requiring reduced ferredoxin. Starting from oxaloacetate, each turn of the cycle results in three molecules of CO_2 being incorporated and pyruvate as the product. Pyruvate is a key metabolite that can feed directly into the glycolytic pathway, generating sugars and other important biosynthetic intermediates.

by green sulfur bacteria such as *Chlorobium* (▶ Section 15.6). The rTCA cycle is also used by many anaerobic and microaerophilic chemolithotrophic *Bacteria*. In the rTCA cycle, CO_2 is reduced by a reversal of steps in the citric acid cycle (◀ Section 3.6; **Figure 14.4**). The rTCA cycle is more energy efficient than the Calvin cycle, requiring 24 H (that come from 4 NADH, 2 NADPH, 2 FADH, and 4 Fd_{red}) but only 10 ATP to fix 6 CO_2 into one molecule of fructose 1,6-bisphosphate. [Note that in the cycle (Figure 14.4), although only four ATP are shown, one ATP is converted into AMP instead of ADP and hence 5 ATP equivalents are consumed per glyceraldehyde 3-P formed. Two molecules of glyceraldehyde 3-P can be converted into fructose 1,6-bisphosphate by reversal of the aldolase step of glycolysis (◀ Figure 3.11).]

As the name implies, most of the reactions of the reverse citric acid cycle are catalyzed by reverse reactions of enzymes of the citric acid cycle. However, the cycle also requires the activity of several unique enzymes. These include in particular the enzymes α-ketoglutarate synthase and pyruvate synthase, which catalyze the reductive fixation of CO_2 using electrons supplied by Fd_{red}. In green sulfur bacteria, Fd_{red} is produced in the light reactions of photosynthesis (see Figure 14.16), whereas chemolithotrophic bacteria must produce it from reverse electron transport or flavin-based electron bifurcation (Figure 14.2). The two ferredoxin-linked reductions are (1) the carboxylation of succinyl-CoA to α-ketoglutarate, and (2) the carboxylation of acetyl-CoA to pyruvate (Figure 14.4). The rTCA cycle also replaces the enzyme *citrate synthase* from the citric acid cycle (◀ Figure 3.12) with the enzyme *citrate lyase* (an ATP-dependent enzyme that cleaves citrate into acetyl-CoA and oxaloacetate), and the enzyme *succinate dehydrogenase* from the citric acid cycle by the FADH-linked *fumarate reductase* (an enzyme that forms succinate from fumarate) in the reverse cycle (Figure 14.4).

Other Pathways of CO_2 Fixation

In addition to the Calvin cycle and the rTCA cycle, at least four other pathways of CO_2 fixation are known, and we consider them here only briefly. Green nonsulfur bacteria such as *Chloroflexus* (▶ Section 15.7) grow autotrophically with either H_2 or H_2S as electron donor. However, neither the Calvin cycle nor the reverse citric acid cycle operates in this organism. Instead, two molecules of CO_2 are reduced to glyoxylate by the *3-hydroxypropionate bi-cycle*. This cycle is so named because hydroxypropionate, a three-carbon compound, is a key intermediate and because it couples two cycles to ultimately yield pyruvate, which can then be converted into glyceraldehyde 3-P as in the rTCA cycle (Figure 14.4).

Archaea appear to lack the Calvin and rTCA cycles as well as the 3-hydroxypropionate bi-cycle. Instead, many chemolithotrophic *Archaea* use either the *3-hydroxypropionate/4-hydroxybutyrate cycle* or the *dicarboxylate/4-hydroxybutyrate cycle*. Each of these autotrophic cycles includes two connected pathways in which bicarbonate and/or CO_2 is reduced to acetyl-CoA, and they are named for their key intermediates, 3-hydroxypropionate, 4-hydroxybutyrate, and C_4 dicarboxylic acids.

The final pathway of CO_2 fixation is the **reductive acetyl-CoA pathway** (see Figure 14.34). This pathway is present in some obligately anaerobic species of *Bacteria* and *Archaea*, and because it is limited to anaerobes and found in both domains, it is thought to be of ancient origin. The reductive acetyl-CoA pathway is found in methanogens, many acetogens, and *Planctomyces* that carry out the anammox reaction (metabolisms to be described later in this chapter). The reductive acetyl-CoA pathway is the most efficient of all CO_2 fixation pathways and requires only about 1 ATP, 3 Fd_{red}, and 2 H_2 per 3 molecules of CO_2 fixed. It is also the only CO_2 fixation pathway coupled directly to energy conservation. The reductive acetyl-CoA pathway was likely one of the earliest mechanisms of

CO_2 fixation to appear on Earth and we will consider it in detail in Section 14.14.

We now pick up where we left off in Chapter 3 and explore the process of photosynthesis and the amazing diversity it supports.

Check Your Understanding

- What is the purpose of carboxysomes in cyanobacteria, and why are they not present in the chloroplasts of plants and algae?
- Make a hypothesis to explain why the rTCA cycle is present in diverse unrelated species of bacteria.
- What evidence supports the hypothesis that the reductive acetyl-CoA cycle was one of the first to appear on Earth?

II • Phototrophy

Phototrophs, which can be anoxygenic or oxygenic, use pigments to transfer light energy to photosynthetic reaction centers. Here they excite an electron to become a strong electron donor for photophosphorylation.

Phototrophy—the use of light energy—is widespread in the microbial world. Phototrophic organisms appeared early in the history of life, they are tremendously diverse, and they have transformed the biosphere. Ultimately, the origin of cyanobacteria billions of years ago converted Earth from an anoxic to an oxic world and set the stage for an explosion of eukaryotic microbial diversity that eventually gave rise to plants and animals (◀ Sections 1.5 and 13.2). In this section, we examine the properties and energy-conserving strategies of phototrophic microorganisms.

14.3 Photosynthesis and Chlorophylls

The most important biological process on Earth is **photosynthesis**, the use of light energy to drive biosynthesis. **Phototrophs** are organisms that convert light energy into chemical energy. Photosynthetic organisms are phototrophs that are also **autotrophs**. Hence, photosynthetic organisms harness light energy to drive the reduction of CO_2 to organic compounds; this lifestyle is called *photoautotrophy*. It is important to note however that *not all phototrophs are autotrophic*. Some phototrophs use organic carbon as their carbon source; this lifestyle is called *photoheterotrophy*.

Phototrophy originated within the *Bacteria*, and a wide diversity of bacterial species can harvest energy from light. No less than seven different phototrophic systems have evolved within *Bacteria* and these systems are found in diverse phyla. These include the *Acidobacteria* (genus *Chloracidobacterium*), *Chlorobi* (green sulfur bacteria), *Chloroflexi* (green nonsulfur bacteria, also known as filamentous anoxygenic phototrophs), *Cyanobacteria*, *Firmicutes* (heliobacteria), *Gemmatimonadetes* (genus *Gemmatimonas*), and *Proteobacteria* (purple bacteria). Ultimately, phototrophy also evolved within the *Eukarya* as a result of the endosymbiotic origin of chloroplasts from cyanobacterial relatives (◀ Section 13.4). These phototrophic systems all differ in characteristic ways, but they all reveal similar underlying principles that we will review in the sections that follow.

Photosynthesis supporting autotrophic growth is comprised of two distinct sets of reactions that operate in parallel: the *light reactions* and the *light-independent reactions*. The term "light reactions" is used to describe those reactions that convert light energy into chemical energy in the form of the proton motive force and ATP. By contrast, the light-independent reactions are those of CO_2 fixation (Section 14.2). CO_2 fixation requires both ATP and reducing power (such as NAD(P)H or Fd_{red}); hence in addition to light, photosynthetic organisms need a source of electrons to fix CO_2 into cellular carbon.

Water (H_2O) is the electron donor for photosynthesis in cyanobacteria, algae, and green plants. When electrons are removed from H_2O, molecular oxygen (O_2) is produced as a waste product. Consequently, the term **oxygenic photosynthesis** describes phototrophic organisms that consume H_2O and produce O_2 as a waste product (**Figure 14.5**). However, phototrophic organisms can use many different electron donors other than H_2O and these organisms

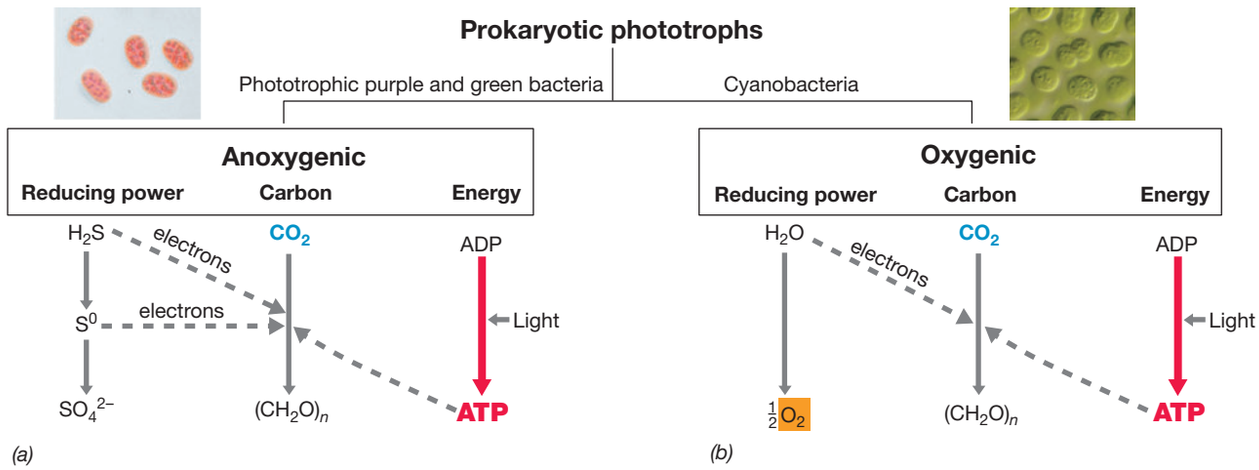


Figure 14.5 Patterns of photosynthesis and examples of each. Energy and reducing power synthesis in *(a)* anoxygenic and *(b)* oxygenic phototrophs. Note that oxygenic phototrophs produce O_2, while anoxygenic phototrophs do not. Photos: Left, photomicrograph of cells of a typical anoxygenic phototroph, the purple sulfur bacterium *Chromatium*; cells are about 5 μm in diameter. Note the sulfur globules inside the cells produced from the oxidation of H_2S. Right, photomicrograph of cells of the cyanobacterium *Halothece*; cells are about 5 μm in diameter.

do not produce O_2 as a waste product. For example, in green and purple sulfur bacteria the donor could be a reduced sulfur compound such as hydrogen sulfide (H_2S), or even molecular hydrogen (H_2). The term **anoxygenic photosynthesis** describes photosynthetic organisms that do not produce O_2 (Figure 14.5).

Photosynthesis requires light-sensitive pigments, the *chlorophylls*—present in plants, algae, and the cyanobacteria—and *bacteriochlorophylls*, present in anoxygenic phototrophs. Absorption of light energy by chlorophylls and bacteriochlorophylls begins the process of photosynthetic energy conversion, and the net result is chemical energy, ATP.

Chlorophyll and Bacteriochlorophyll

Chlorophyll and **bacteriochlorophyll** are tetrapyrroles that are related to the parent structure of the cytochromes. But unlike cytochromes, chlorophylls contain *magnesium* instead of *iron* at the center of the ring. Chlorophylls also contain specific substituents on the tetrapyrrole ring and a hydrophobic alcohol that helps anchor the chlorophyll into photosynthetic membranes. The structure of chlorophyll *a*, the principal chlorophyll of oxygenic phototrophs, is shown in **Figure 14.6*a***. Chlorophyll *a* is green because it *absorbs* red and blue light and *transmits* green light; its absorption spectrum shows strong absorbance near 680 nm and 430 nm (Figure 14.6*b*). Several structurally distinct chlorophylls are known, each distinguished by its unique *absorption spectrum*. Cyanobacteria contain chlorophyll *a* (a few species contain chlorophyll *d* in place of *a*), while their relatives the prochlorophytes contain chlorophylls *a* and *b*.

Anoxygenic phototrophs produce one or more bacteriochlorophylls. Bacteriochlorophyll *a* (Figure 14.6 and **Figure 14.7**), present in most purple bacteria (▶ Sections 15.4 and 15.5), absorbs maximally between 800 and 925 nm. This range of wavelengths results from the fact that different species of purple bacteria synthesize photocomplexes of slightly different protein structure, and this affects the absorption maxima of bacteriochlorophyll *a*. Other bacteriochlorophylls, whose distribution runs along phylogenetic lines, absorb in other regions of the visible and near-infrared spectrum (Figure 14.7). This diversity in photosynthetic pigments allows phototrophs, collectively, to absorb energy across a wide range of the electromagnetic spectrum. By employing different pigments with distinct absorption properties, different phototrophs can coexist in the same habitat, each absorbing wavelengths of light that others cannot. Thus, pigment diversity has *ecological* significance for the successful coexistence of different phototrophs in the same habitat.

Reaction Centers and Antenna Pigments

In oxygenic phototrophs and in purple anoxygenic phototrophs, chlorophyll/bacteriochlorophyll molecules do not exist freely in the cell but are attached to proteins and housed within membranes to form *photocomplexes* consisting of anywhere from 50 to 300 chlorophyll/bacteriochlorophyll molecules. A small number of these pigment molecules are present within photosynthetic **reaction centers** (**Figure 14.8**), the complex macromolecular structures that participate directly in the reactions that lead to energy conservation. Photosynthetic reaction centers are surrounded by larger numbers of light-harvesting chlorophylls/bacteriochlorophylls (shown as LH1 and LH2 in Figure 14.8). These so-called **antenna pigments** function to absorb light and funnel some of the energy to the reaction center (Figure 14.8). At the low light intensities that are often found in nature, this arrangement for concentrating energy allows reaction centers to receive light energy that would otherwise be missed.

Photosynthetic Membranes, Chloroplasts, and Chlorosomes

The chlorophyll pigments and all the other components of the light-gathering apparatus exist within membranes in the cell. The

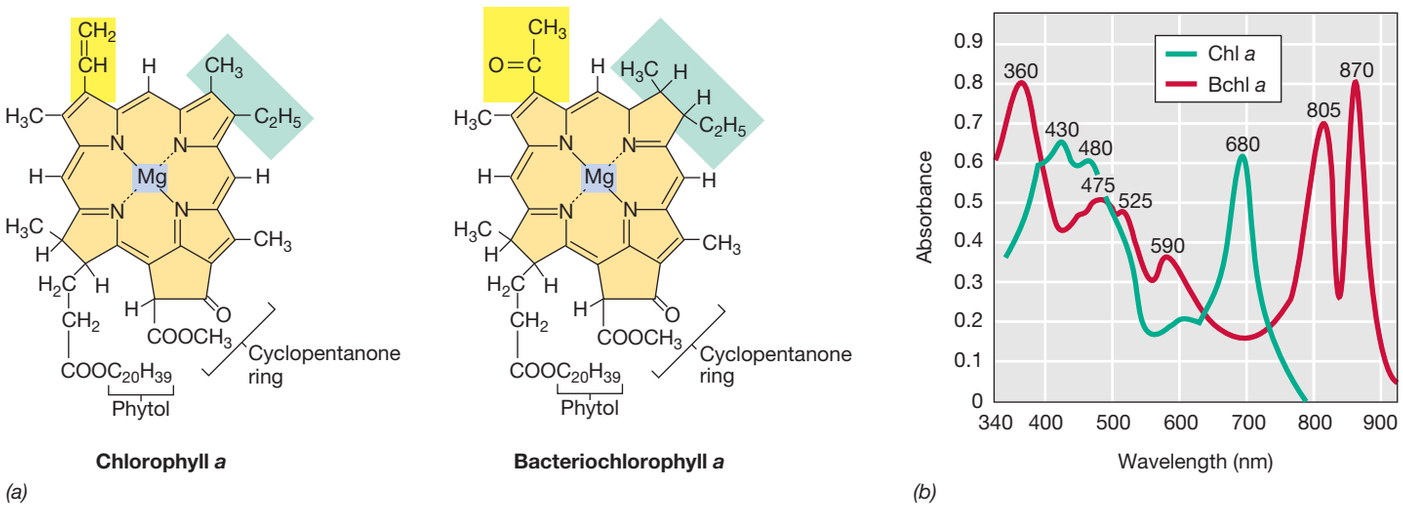
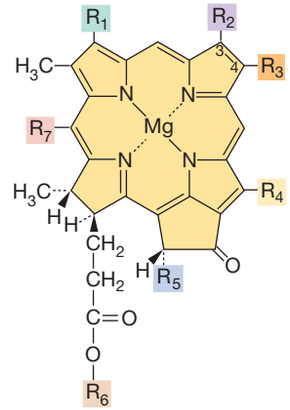


Figure 14.6 Structures and spectra of chlorophyll *a* and bacteriochlorophyll *a*. *(a)* The two molecules are identical except for those portions contrasted in yellow and green. *(b)* Absorption spectrum (green curve) of cells of the green alga *Chlamydomonas*. The peaks at 680 and 430 nm are due to chlorophyll *a*, and the peak at 480 nm is due to carotenoids. Absorption spectrum (red curve) of cells of the phototrophic purple bacterium *Rhodopseudomonas palustris*. Peaks at 870, 805, 590, and 360 nm are due to bacteriochlorophyll *a*, and peaks at 525 and 475 nm are due to carotenoids.

Pigment/Absorption maxima (in vivo)	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Bchl *a* (purple bacteria)/ 805, 830–890 nm	$-\overset{\overset{O}{\|}}{C}-CH_3$	$-CH_3$[a]	$-CH_2-CH_3$	$-CH_3$	$-\overset{\overset{O}{\|}}{C}-O-CH_3$	P/Gg[b]	$-H$
Bchl *b* (purple bacteria)/ 835–850, 1020–1040 nm	$-\overset{\overset{O}{\|}}{C}-CH_3$	$-CH_3$[c]	$=\underset{\underset{H}{\|}}{C}-CH_3$	$-CH_3$	$-\overset{\overset{O}{\|}}{C}-O-CH_3$	P	$-H$
Bchl *c* (green sulfur bacteria)/745–755 nm	$-\underset{\underset{OH}{\|}}{\overset{\overset{H}{\|}}{C}}-CH_3$	$-CH_3$	$-C_2H_5$ $-C_3H_7$[d] $-C_4H_9$	$-C_2H_5$ $-CH_3$	$-H$	F	$-CH_3$
Bchl c_s (green nonsulfur bacteria)/740 nm	$-\underset{\underset{OH}{\|}}{\overset{\overset{H}{\|}}{C}}-CH_3$	$-CH_3$	$-C_2H_5$	$-CH_3$	$-H$	S	$-CH_3$
Bchl *d* (green sulfur bacteria)/705–740 nm	$-\underset{\underset{OH}{\|}}{\overset{\overset{H}{\|}}{C}}-CH_3$	$-CH_3$	$-C_2H_5$ $-C_3H_7$ $-C_4H_9$	$-C_2H_5$ $-CH_3$	$-H$	F	$-H$
Bchl *e* (green sulfur bacteria)/719–726 nm	$-\underset{\underset{OH}{\|}}{\overset{\overset{H}{\|}}{C}}-CH_3$	$-\overset{\overset{O}{\|}}{C}-H$	$-C_2H_5$ $-C_3H_7$ $-C_4H_9$	$-C_2H_5$	$-H$	F	$-CH_3$
Bchl *g* (heliobacteria)/ 670, 788 nm	$-\overset{\overset{H}{\|}}{C}=CH_2$	$-CH_3$[a]	$-C_2H_5$	$-CH_3$	$-\overset{\overset{O}{\|}}{C}-O-CH_3$	F	$-H$



[a]No double bond between C_3 and C_4; additional H atoms are in positions C_3 and C_4.

[b]P, Phytyl ester ($C_{20}H_{39}O-$); F, farnesyl ester ($C_{15}H_{25}O-$); Gg, geranylgeraniol ester ($C_{10}H_{17}O-$); S, stearyl alcohol ($C_{18}H_{37}O-$).

[c]No double bond between C_3 and C_4; an additional H atom is in position C_3.

[d]Bacteriochlorophylls *c*, *d*, and *e* consist of isomeric mixtures with the different substituents on R_3 as shown.

Figure 14.7 Structure of all known bacteriochlorophylls (Bchl). The different substituents present in the positions R_1 to R_7 in the structure at the right are listed. In vivo absorption maxima are the physiologically relevant absorption peaks. The spectrum of bacteriochlorophylls extracted from cells and dissolved in organic solvents is often quite different.

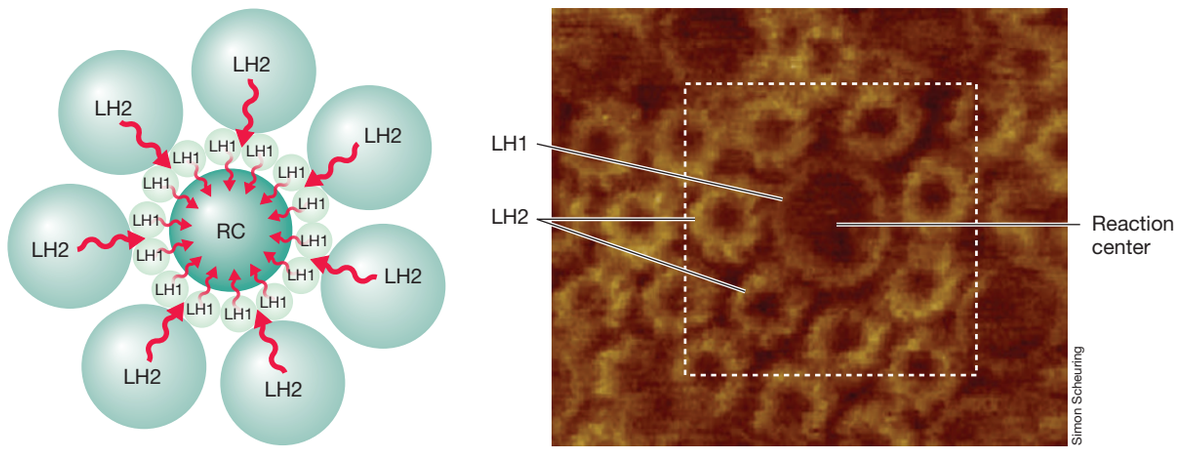


Figure 14.8 Arrangement of light-harvesting chlorophylls/bacteriochlorophylls and reaction centers within a photosynthetic membrane. *(a)* Light energy absorbed by light-harvesting (LH) molecules (light green) is transferred to the reaction centers (RC) where photosynthetic electron transport reactions begin. Pigment molecules are secured within the membrane by specific pigment-binding proteins. *(b)* Atomic force micrograph of photocomplexes of the purple bacterium *Phaeospirillum molischianum*. This organism has two types of light-harvesting complexes, LH1 and LH2. LH2 complexes transfer energy to LH1 complexes, and these transfer energy to the reaction center (see Figure 14.14*b*).

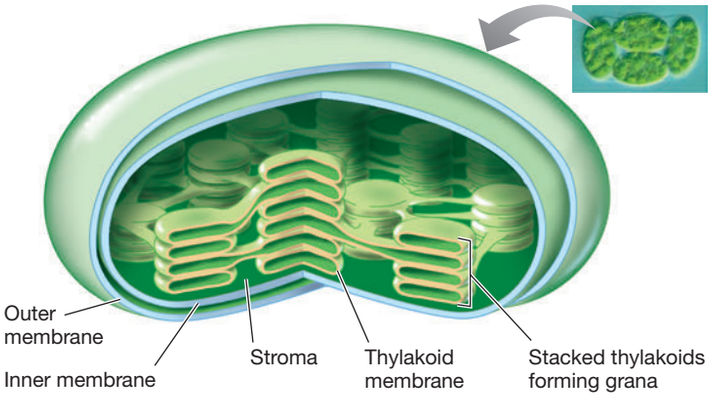


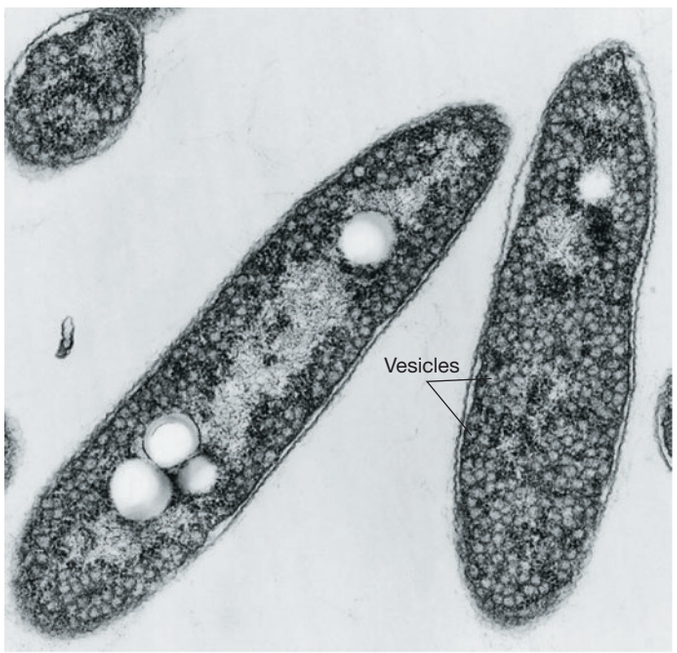
Figure 14.9 The chloroplast. Details of chloroplast structure, showing how the convolutions of the thylakoid membranes define an inner space called the stroma and form membrane stacks called grana. Inset: Photomicrograph of cells of the green alga *Makinoella*. Each of the four cells in a cluster contains several chloroplasts.

location of these photosynthetic membranes differs between prokaryotic and eukaryotic phototrophs. In eukaryotic phototrophs, photosynthesis takes place in intracellular organelles, the *chloroplasts*, which contain sheetlike photosynthetic membrane systems called **thylakoids** (Figure 14.9); stacks of thylakoids within the chloroplast form *grana*. The thylakoids are arranged so that the chloroplast is divided into two regions, the matrix space that surrounds the thylakoids, called the *stroma*, and the inner space within the thylakoid array, called the *lumen*. This arrangement makes possible the generation of a light-driven proton motive force used to synthesize ATP (see Section 14.5).

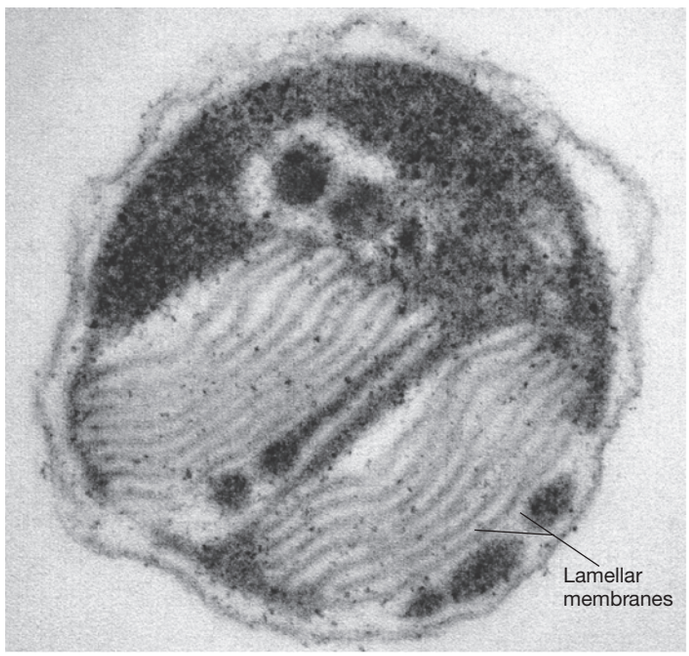
Chloroplasts are absent from prokaryotic phototrophs. In purple bacteria, the photosynthetic pigments are integrated into internal membrane systems that arise from invagination of the cytoplasmic membrane. Membrane vesicles called *chromatophores* or membrane stacks called *lamellae* are common membrane arrangements in purple bacteria (Figure 14.10). In cyanobacteria, photosynthetic pigments reside in lamellar membranes (Figure 14.10*b*) that are also called *thylakoids* because of their resemblance to the thylakoids in the chloroplasts of algae (Figure 14.9).

The ultimate structure for capturing energy from low light intensities is the **chlorosome** (Figure 14.11). Chlorosomes are present in the green sulfur bacteria (*Chlorobium*, ▶ Section 15.6), green nonsulfur bacteria (*Chloroflexus*, ▶ Section 15.7), and phototrophic *Acidobacteria* (*Chloracidobacterium*, ▶ Section 15.8), all of which are anoxygenic phototrophs. Chlorosomes function as giant antenna systems, but unlike the antennae of purple bacteria or cyanobacteria, bacteriochlorophyll molecules in the chlorosome are not attached to proteins. Chlorosomes contain bacteriochlorophyll *c*, *d*, or *e* (Figure 14.7) arranged in dense arrays running along the long axis of the structure. Light energy absorbed by these antenna pigments is transferred to bacteriochlorophyll *a* in the reaction center in the cytoplasmic membrane through a small protein called the *FMO protein* (Figure 14.11).

Chlorosomes allow these phototrophs to grow at extremely low light intensities, and hence these phototrophs are typically found in the deepest waters of lakes, inland seas, and other anoxic aquatic habitats where light levels are too low to support other phototrophs. Green nonsulfur bacteria and *Chloracidobacterium* are major components of microbial mats, thick biofilms that form in



(a)



(b)

Figure 14.10 Membranes in anoxygenic phototrophs. *(a)* Chromatophores. Transmission electron micrograph of a section through a cell of the purple bacterium *Rhodobacter* showing vesicular photosynthetic membranes. The vesicles are continuous with and arise by invagination of the cytoplasmic membrane. A cell is about 1 μm wide. *(b)* Lamellar membranes in the purple bacterium *Ectothiorhodospira*. A cell is about 1.5 μm wide. These membranes are also continuous with and arise from invagination of the cytoplasmic membrane, but instead of forming vesicles, they form membrane stacks.

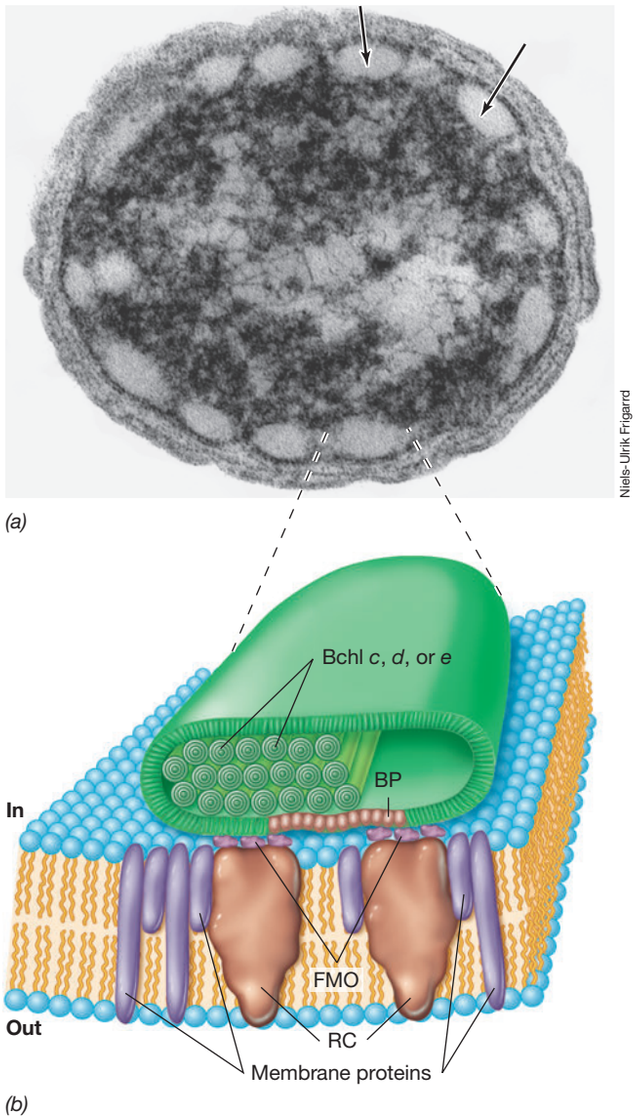


Figure 14.11 The chlorosome of green sulfur and green nonsulfur bacteria. *(a)* Transmission electron micrograph of a cross section of a cell of the green sulfur bacterium *Chlorobaculum tepidum*. Note the chlorosomes (arrows). *(b)* Model of chlorosome structure. The chlorosome (green) lies appressed to the inside surface of the cytoplasmic membrane. Antenna bacteriochlorophyll (Bchl) molecules are arranged in tubelike arrays inside the chlorosome, and energy is transferred from these to reaction center (RC) Bchl *a* in the cytoplasmic membrane through a protein called FMO. Base plate (BP) proteins function as connectors between the chlorosome and the cytoplasmic membrane.

hot springs and highly saline environments (▶ Section 20.5). Microbial mats experience a steep light gradient, with light levels even a few millimeters into the mat approaching darkness. Green sulfur bacteria are common near the sediments of sulfidic aquatic systems and can even carry out photosynthesis in the darkness of the deep ocean by using infrared radiation emitted from hydrothermal vents. Hence, chlorosomes allow phototrophs that produce them to carry out photosynthesis with only the minimal light intensities available.

Check Your Understanding

- What is the fundamental difference between an oxygenic and an anoxygenic phototroph?
- What is the purpose of chlorophyll and bacteriochlorophyll molecules? In what ways do they resemble cytochromes and in what ways do they differ?
- Why can phototrophic green bacteria grow at light intensities that will not support purple bacteria or cyanobacteria?

14.4 Carotenoids and Phycobilins

Although chlorophyll/bacteriochlorophyll is required for photosynthesis, phototrophic organisms contain other pigments as well. These pigments include, in particular, the *carotenoids* and *phycobilins*.

Carotenoids

The most widespread accessory pigments in phototrophs are the **carotenoids**. Carotenoids are hydrophobic pigments that are firmly embedded in the photosynthetic membrane. **Figure 14.12** shows the structure of a common carotenoid, β-*carotene*. Carotenoids are typically yellow, red, brown, or green and absorb light in the blue region of the spectrum. Some of the major carotenoids of anoxygenic phototrophs are shown in **Figure 14.13**. Because they tend to mask the color of bacteriochlorophylls, carotenoids are responsible for the brilliant colors of red, purple, pink, green, yellow, or brown that are observed in different species of anoxygenic phototrophs (▶ Figure 15.12).

Carotenoids are closely associated with chlorophyll or bacteriochlorophyll in photosynthetic complexes, and some of the energy absorbed by carotenoids can be transferred to the reaction center. However, carotenoids function primarily as photoprotective agents. Bright light can be harmful to cells because it can catalyze photooxidation reactions that can produce toxic forms of oxygen, such as singlet oxygen ($^1O_2$). Like superoxide and other forms of toxic oxygen (◀ Section 4.16), singlet oxygen can spontaneously oxidize photocomplexes, rendering them nonfunctional. Carotenoids quench toxic oxygen species by absorbing much of this harmful light and in this way prevent these dangerous photooxidations. Because phototrophic organisms by their very nature must live in the light, the photoprotection conferred by carotenoids is clearly advantageous.

Phycobiliproteins and Phycobilisomes

Cyanobacteria and the chloroplasts of red algae (which are descendants of cyanobacteria, ▶ Section 18.1) contain pigments called **phycobiliproteins**, which are the main light-harvesting systems of these phototrophs. Phycobiliproteins consist of red or blue-green

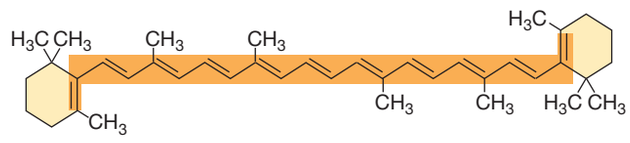


Figure 14.12 Structure of β-carotene, a typical carotenoid. The conjugated double-bond system is highlighted in orange.

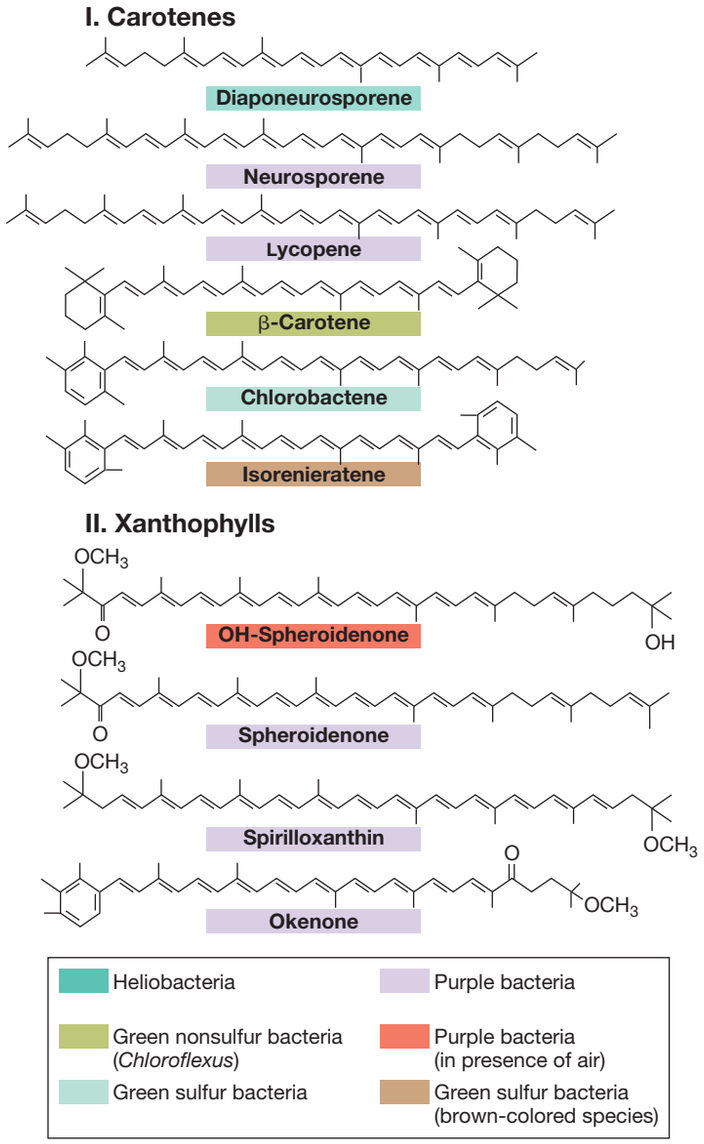


Figure 14.13 Structures of some common carotenoids found in anoxygenic phototrophs. Carotenes are hydrocarbon carotenoids, and xanthophylls are oxygenated carotenoids. Compare the structure of β-carotene shown in Figure 14.12 with how it is drawn here. For simplicity in the structures shown here, methyl (CH_3) groups are designated by bond only.

linear tetrapyrroles, called *bilins*, bound to proteins, and give cyanobacteria and red algae their characteristic colors (**Figure 14.14**). The red phycobiliprotein, called *phycoerythrin*, absorbs most strongly at wavelengths around 550 nm, whereas the blue phycobiliprotein, *phycocyanin* (Figure 14.14*b*), absorbs most strongly at 620 nm. A third phycobiliprotein, called *allophycocyanin* (Figure 14.14*b*) absorbs at about 650 nm.

Phycobiliproteins assemble into aggregates called **phycobilisomes** that attach to cyanobacterial thylakoids (Figure 14.14*c*). Phycobilisomes are arranged such that the allophycocyanin molecules are in direct contact with the photosynthetic membrane. Allophycocyanin is surrounded by phycocyanin or phycoerythrin (or both, depending on the organism). Phycocyanin and phycoerythrin absorb light of shorter wavelengths (higher energy) and transfer some energy to allophycocyanin, which is positioned closest to the reaction center chlorophyll and transfers energy to it (Figure 14.14*b*). Thus, in a fashion similar to how antenna bacteriochlorophyll systems function in anoxygenic phototrophs (Figure 14.8), energy transfer proceeds "downhill" from phycobilisomes to the reaction center. Phycobilisomes thus facilitate energy transfer to cyanobacterial reaction centers, allowing cyanobacteria to grow at lower light intensities than would otherwise be possible.

Check Your Understanding

- In which phototrophs are carotenoids found? Phycobiliproteins?
- How does the structure of a phycobilin compare with that of a chlorophyll?
- Phycocyanin is blue-green. What color of light does it absorb?

14.5 Anoxygenic Photosynthesis

In the photosynthetic light reactions, electrons travel through an electron transport chain whose components are arranged in a photosynthetic membrane in order of their increasingly more electropositive reduction potential (E_0'), similar to the electron transport chains used in respiration (◀ Sections 3.9–3.11). This electron transport generates a proton motive force that drives ATP synthesis (◀ Section 3.11). Key parts of this process include photosynthetic reaction centers and photosynthetic membranes (Section 14.3).

Photosynthetic reaction centers are complex macromolecular structures localized within photosynthetic membranes. They are composed of multiple protein subunits and cofactors (including chlorophylls or bacteriochlorophylls), and they interact with both antenna pigments and components of the electron transport chain. Photosynthetic antenna pigments funnel light energy to the reaction center to excite a pair of chlorophylls or bacteriochlorophylls (referred to as the *special pair*), thereby generating highly electronegative electrons that can be donated to a subsequent electron transport chain. Several different types of reaction centers have been described in anoxygenic phototrophs, but all fall into one of two classes; they are either of a *quinone type* (Q-type) or *iron–sulfur type* (FeS-type) depending on the electron acceptor in the reaction center.

Photosynthetic Electron Flow in Purple Bacteria

Purple bacteria use a Q-type reaction center that contains three polypeptides, designated L, M, and H. These proteins along with a molecule of cytochrome *c* are firmly embedded in the photosynthetic membrane (Figure 14.10) and wind through the membrane several times (**Figure 14.15**). The L, M, and H polypeptides bind four molecules of bacteriochlorophyll *a*, two of which are the special pair and another two that transfer electrons within the reaction center, two molecules of bacteriopheophytin *a* (bacteriochlorophyll *a* minus its magnesium atom), two molecules of quinone (◀ Section 3.8), and one carotenoid molecule (Figure 14.15).

Photosynthetic light reactions begin when light energy absorbed by antenna pigments is transferred to the special pair of bacteriochlorophyll *a* molecules (Figure 14.15*a*, ◀ Figure 3.25). This excites the special pair, converting them from a relatively weak to a very

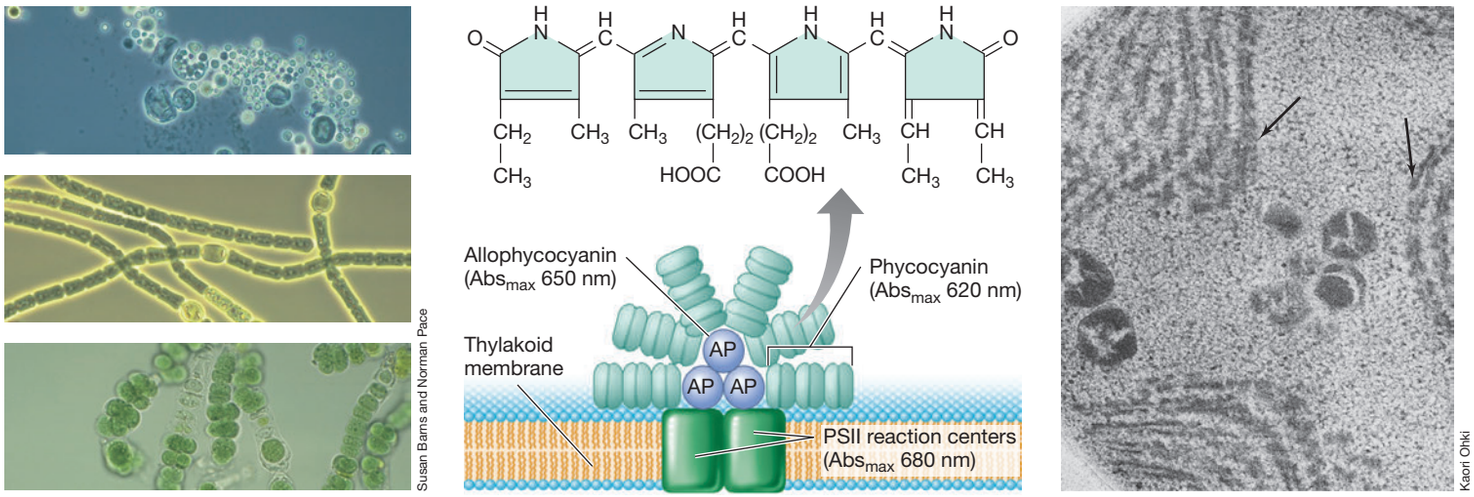


Figure 14.14 Phycobiliproteins and phycobilisomes. *(a)* Light photomicrographs of cells of the cyanobacteria (top to bottom) *Dermocarpa*, *Anabaena*, and *Fischerella*, showing the typical blue-green color of cells due to phycobiliproteins. *(b)* Structure of phycocyanin (top) and a phycobilisome. Phycocyanin absorbs at higher energies (shorter wavelengths) than allophycocyanin. Chlorophyll *a* absorbs at longer wavelengths (lower energies) than allophycocyanin. Energy flow is thus phycocyanin → allophycocyanin → chlorophyll *a* of PSII (see Figure 14.16). *(c)* Electron micrograph of a thin section of the cyanobacterium *Synechocystis*. Note the darkly staining phycobilisomes (arrows) attached to the lamellar membranes.

strong electron donor (very electronegative E_0', ◀ Sections 3.3 and 3.11). Once this strong donor has been produced, the remaining steps in photosynthetic electron flow are highly reminiscent of those we have seen before in respiration (◀ Sections 3.9 and 3.10); that is, electrons flow through a membrane from carriers of low E_0' to those of high E_0' and in the process generate a proton motive force that is used to synthesize ATP (◀ Section 3.11).

Before excitation, the purple bacterial reaction center, which is called *P870*, has an E_0' of about +0.5 V; after excitation, it has a potential of about −1.0 V (**Figure 14.16**). An excited electron within P870 proceeds to reduce a molecule of bacteriochlorophyll *a* within the reaction center (Figures 14.15 and 14.16). This transition takes place incredibly fast, taking only about three-trillionths (3×10^{-12}) of a second. Once reduced, bacteriochlorophyll *a* proceeds to reduce bacteriopheophytin *a*, and the latter reduces quinone molecules within the membrane (Figure 14.16). These transitions are also very fast, taking less than one-billionth of a second. From the quinone, electrons are transported through the membrane more slowly (on a

Mastering Microbiology
Art Activity: Figure 14.12*a* Electron flow in anoxygenic photosynthesis

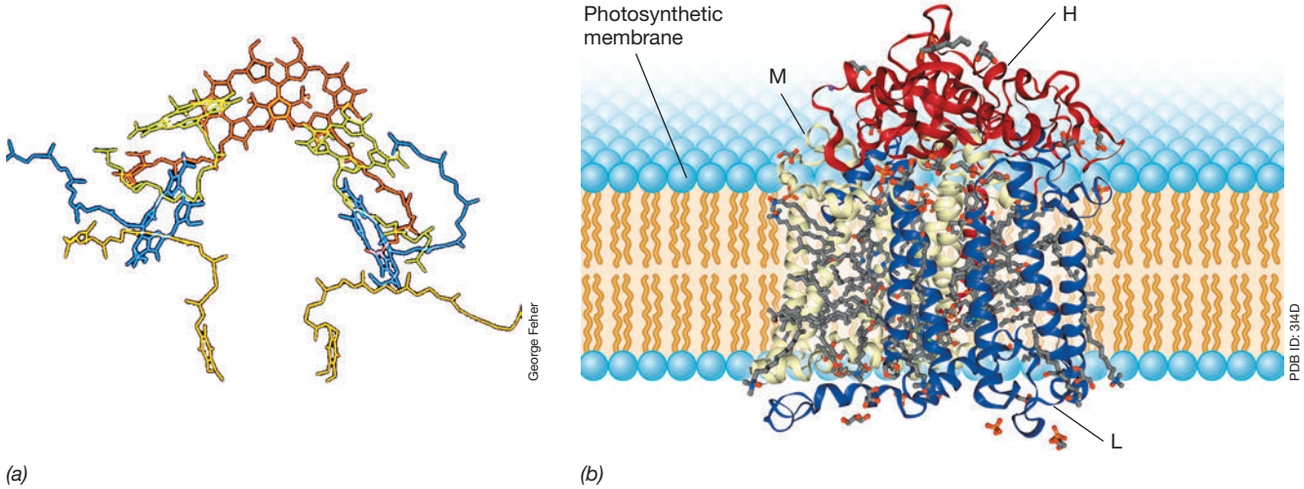


Figure 14.15 Structure of the reaction center of a purple phototrophic bacterium. *(a)* Arrangement of pigment molecules in the reaction center. The "special pair" of bacteriochlorophyll molecules (orange) overlap and occur at the top of the reaction center structural diagram; adjacent to and below the special pair are a pair of accessory bacteriochlorophylls (yellow near the top). Bacteriopheophytin molecules (blue) are arranged below the bacteriochlorophylls, and quinones (yellow at the bottom) are present at the bottom of the structural model. Compare the structure of these molecules in the reaction center to their role in electron transfer (see Figure 14.16). *(b)* Molecular model of the protein structure of the reaction center. The molecular structures (C in gray, O in red, N in blue) of pigments described in part *a* are depicted in context with ribbon diagrams of reaction center protein subunits: H (red), M (white), and L (blue). The reaction center pigment–protein complex is integrated into the lipid bilayer.

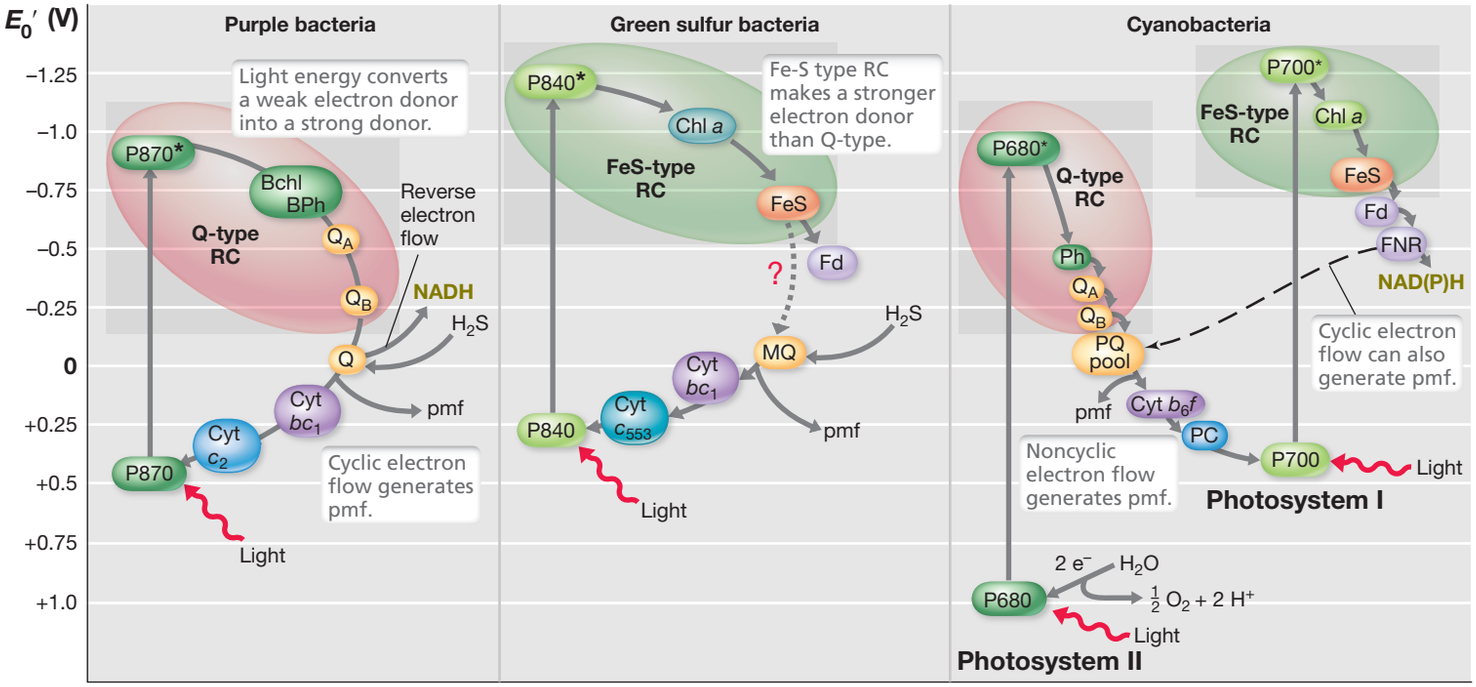


Figure 14.16 A comparison of electron flow in anoxygenic and oxygenic phototrophs. Purple bacteria and green sulfur bacteria have Q-type and FeS-type reaction centers (RC), respectively, while oxygenic phototrophs have both types. The FeS-type reaction center of green sulfur bacteria produces highly electronegative electrons that can reduce ferredoxin (Fd), allowing for CO_2 fixation through the reverse citric acid cycle (Figure 14.4). Q-type reaction centers cannot reduce Fd, which makes reverse electron transport necessary to generate the NADH needed to fix CO_2 by the Calvin cycle (◀ Figures 3.26 and 3.27). Cyclic electron flow occurs in purple bacteria but not in green sulfur bacteria. Electron flow in oxygenic phototrophs is noncyclic, resulting in the characteristic "Z-scheme" pattern of electron flow. If photosystem II is blocked, oxygenic phototrophs can perform cyclic photosynthesis at photosystem I (dashed line in the cyanobacteria panel, see also Figure 14.17). Bchl, bacteriochlorophyll; BPh, bacteriopheophytin; Chl, chlorophyll; Cyt, cytochrome; FeS, iron–sulfur protein; FNR, ferredoxin–NADP oxidoreductase; P870, P840, P680, and P700, reaction centers; PC, plastocyanin; Ph, pheophytin; PQ, plastoquinone; Q, quinone; Q_A, Q_B, intermediate quinones; MQ, menaquinone.

millisecond scale) through a series of iron–sulfur proteins and cytochromes used to generate the proton motive force (Figure 14.16), eventually returning to the reaction center. This overall process is referred to as *cyclic photophosphorylation* because the electrons cycle in a closed loop, thereby generating the proton motive force needed to synthesize ATP (◀ Section 3.11).

Overview of Electron Flow in Anoxygenic Phototrophs

Thus far, we have focused on electron flow in purple bacteria. Although analogous membrane-associated reactions drive photophosphorylation in other phototrophs, there are significant differences in the details. Q-type reaction centers that contain quinone molecules are found in purple bacteria (▶ Sections 15.4 and 15.5), green nonsulfur bacteria (▶ Section 15.7), and *Gemmatimonadetes* (genus *Gemmatimonas*, ▶ Section 15.8), but FeS-type reaction centers that lack quinones and contain instead FeS clusters are found in the green sulfur bacteria (▶ Section 15.6), *Acidobacteria* (genus *Chloracidobacterium*, ▶ Section 15.8), and heliobacteria (▶ Section 15.8). The Q-type and FeS-type reaction centers have important structural differences. For example, whereas Q-type reaction centers contain bacteriochlorophyll, FeS-type reaction centers contain both bacteriochlorophyll and chlorophyll *a* (green sulfur bacteria) or a modified form of chlorophyll *a* (hydroxychlorophyll *a* in heliobacteria). The structural differences between Q-type and FeS-type reaction centers cause major differences in electron flow (Figure 14.16).

The bacteriochlorophyll molecules of FeS-type reaction centers have an excited state that is significantly *more* electronegative than Q-type reaction centers (Figure 14.16). In purple bacteria, the Q-type reaction center donates electrons directly to a quinone (Figure 14.16). However, these quinones (E_0' about 0 V) are insufficiently electronegative to provide the reducing power (E_0' of −0.32 V for NAD(P)H and −0.42 V for ferredoxin) needed for CO_2 fixation and other biosynthetic reactions (Section 14.2). In contrast, FeS-type reaction centers donate electrons directly to low-potential FeS-proteins able to reduce ferredoxin. Green sulfur bacteria then use this Fd_{red} when they fix CO_2 using the rTCA cycle (Section 14.2 and Figure 14.4). Electrons from ferredoxin can also pass to ferredoxin–NAD^+ oxidoreductase for the production of NAD(P)H needed for biosynthesis.

Phototrophs having a Q-type reaction center perform cyclic photophosphorylation providing cells with the ability to produce ATP as long as light is present, but these organisms still require a source of reducing power in order to perform the biosynthetic reactions required for growth. Since Q-type reaction centers produce electrons that are insufficiently electronegative to reduce $NAD(P)^+$, these phototrophs require reverse electron transport (Section 14.1 and ◀ Section 3.11) to produce the NAD(P)H they need to carry out CO_2 fixation and other biosynthetic reactions.

It remains unclear whether electron transfer in green sulfur bacteria is cyclic or noncyclic. It has been proposed that the FeS-type reaction centers in these phototrophs can transfer electrons directly to menaquinone,

thereby generating a proton motive force resulting in cyclic photophosphorylation as seen in purple bacteria (Figure 14.16). However, little evidence for cyclic photophosphorylation has been observed in green sulfur bacteria. Alternatively, these phototrophs may employ noncyclic electron flow whereby electrons from external electron donors, such as H_2S, enter at the level of the menaquinone pool. These electrons would be transferred through the reaction center and then to ferredoxin where they would ultimately be channeled into biosynthetic reactions without the need for reverse electron transport (Figure 14.16). In heliobacteria, FeS-type phototrophs that are not autotrophic, cyclic photophosphorylation has also not been demonstrated. However, in this group of strictly photoheterotrophic bacteria, reducing power needs are less extensive than for photoautotrophs and thus it is more likely that cyclic flow occurs to generate sufficient ATP for biosynthesis.

Mastering Microbiology
Art Activity: Figure 14.14 Electron flow in oxygenic photosynthesis, the "Z" scheme

We move on now to consider photosynthesis in oxygenic phototrophs such as the cyanobacteria. These organisms contain *both* FeS-type and Q-type reaction centers that likely evolved from those of green and purple bacteria, respectively. These two photocomplexes function in parallel to both generate ATP and reducing power, the latter from H_2O (Figure 14.16). We explore how this fascinating process occurs now.

Check Your Understanding

- What parallels exist in the processes of photophosphorylation and oxidative phosphorylation?
- Why is reverse electron transport needed by phototrophs having Q-type reaction centers and not needed by those having FeS-type reaction centers?
- What is the difference between cyclic and noncyclic photophosphorylation?

14.6 Oxygenic Photosynthesis

In contrast to photosynthetic electron flow in *anoxygenic* phototrophs, which have either FeS-type or Q-type photosynthetic reaction centers, *oxygenic* phototrophs have both types of reaction centers. In oxygenic phototrophs, electrons flow through two distinct photosystems called *photosystem I* (*PSI*, or *P700*), which has an FeS-type reaction center, and *photosystem II* (*PSII*, or *P680*), which has a Q-type reaction center. PSI and PSII interact in the "Z scheme" of photosynthesis, so named because the pathway resembles the letter Z turned on its side (Figure 14.16). As in anoxygenic photosynthesis, the light reactions in oxygenic photosynthesis occur in photocomplexes embedded in specialized photosynthetic membranes. In eukaryotic cells, the membranes are in the chloroplast (Figure 14.9), whereas in cyanobacteria, the membranes are arranged in stacks within the cytoplasm (Figure 14.14*c*).

Electron Flow and ATP Synthesis in Oxygenic Photosynthesis

PSII performs the first—and most distinctive—step in oxygenic photosynthesis, the splitting of water into oxygen and electrons (**Figure 14.17**). Upon absorbing light energy, the P680 chlorophyll *a* molecule in PSII is excited to a very electronegative reduction potential that allows it to donate an electron to pheophytin *a* (chlorophyll *a* lacking its magnesium atom), a molecule with an E_0' of about −0.5 V. This creates a charge separation that causes P680 to become so strongly electropositive that it can accept electrons from H_2O, an extremely weak electron donor. The oxidation of water by PSII occurs at the *water-oxidizing complex* and is catalyzed by a Mn_4Ca

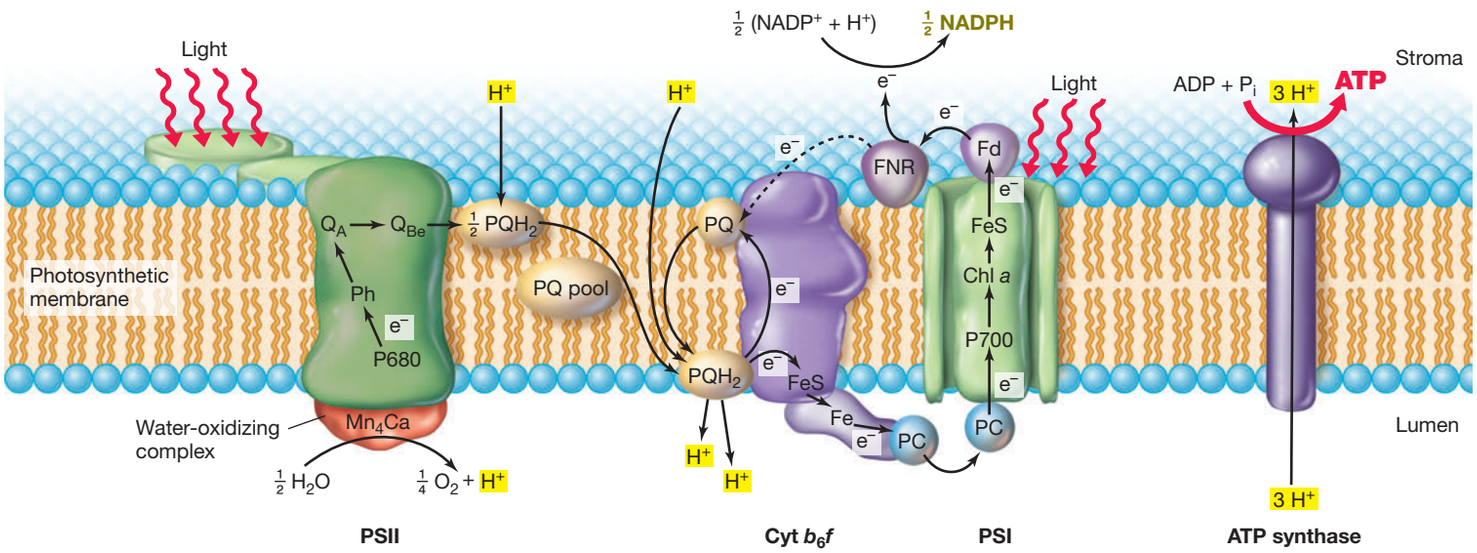


Figure 14.17 Electron transport in oxygenic photosynthesis. Photosystem II (PSII) is activated by photons, causing H_2O to be oxidized on the Mn_4Ca cluster of the water-oxidizing complex. Electrons are transferred from PSII to the plastoquinone pool (PQ/PQH_2). Protons are exchanged across the membrane when plastoquinone is oxidized by cytochrome b_6f. Per two molecules of water oxidized to one O_2, a total of 12 protons are released to the lumen to fuel ATP synthase. Electrons are then transferred to plastocyanin (PC), which carries them to photosystem I (PSI). Upon activation by light, PSI reduces ferredoxin (Fd), with sequential reduction of ferredoxin:NADP oxidoreductase (FNR), and then $NADP^+$. The ATP and NADPH produced by the light reactions are used in CO_2 fixation by the Calvin cycle (◀ Section 3.12 and Section 14.2). Cyclic photophosphorylation occurs when FNR donates electrons to cytochrome b_6f instead of to $NADP^+$. During cyclic photophosphorylation, more ATP and less NADPH are produced than during noncyclic photophosphorylation.

cluster (Figure 14.17), which binds 2 molecules of H_2O. P680 removes one electron from the Mn_4Ca cluster of the water-oxidizing complex for each photon absorbed. In this way 4 electrons are sequentially removed from the 2 H_2O molecules bound to the Mn_4Ca cluster, resulting in the production of O_2 and 4 H^+. Each electron transferred to pheophytin then travels through a series of quinones (Q_A and Q_B) within the PSII photocomplex. Finally, two electrons from the PSII photocomplex are used to reduce plastoquinone (PQ) to PQH_2, a step that allows for the generation of the proton motive force.

The proton motive force is generated in oxygenic photosynthesis by electron transport through quinones and cytochromes of increasingly positive reduction potential. These electron transport reactions are similar to those encountered during our discussion of aerobic respiration (◀ Section 3.9). Electrons from PQH_2 are transferred through cytochrome b_6f and through a copper-containing protein called *plastocyanin* before being donated to the PSI reaction center (Figures 14.16 and 14.17). The absorption of light by P700 of PSI allows it to accept electrons donated from plastocyanin. Electrons travel through several intermediates in PSI terminating with the reduction of $NADP^+$ to NADPH (Figures 14.16 and 14.17). The electrons from NADPH are used in CO_2 fixation (by the Calvin cycle, ◀ Section 3.12 and Section 14.2) and other biosynthetic reactions, thereby regenerating $NADP^+$ and making CO_2 the ultimate electron acceptor for oxygenic photosynthesis. Two protons are generated for each water molecule that is split by PSII and four protons are translocated across the membrane for every two electrons transferred through the electron transport chain, resulting in a total of 12 protons translocated for every molecule of O_2 produced. This proton motive force is then used by ATP synthase to produce ATP.

Oxygenic photosynthesis results in *noncyclic photophosphorylation* because electrons do not cycle back to reduce the oxidized P680, but instead are used in the reduction of $NADP^+$. However, when the cell requires less NADPH for biosynthesis, oxygenic phototrophs can perform *cyclic photophosphorylation*. This occurs when, instead of reducing $NADP^+$, electrons from PSI are returned to the electron transport chain that connects PSII to PSI. When this happens, the recycled electrons can be used to generate a proton motive force that supports additional ATP synthesis (dashed line in the cyanobacteria panel of Figure 14.16; Figure 14.17).

Anoxygenic Photosynthesis in Oxygenic Phototrophs

Photosystems I and II normally function in tandem in oxygenic photosynthesis (Figures 14.16 and 14.17). However, if PSII activity is blocked, some oxygenic phototrophs can perform photosynthesis using only PSI. Under these conditions, cyclic photophosphorylation (Figure 14.16) occurs exclusively and reducing power for CO_2 reduction comes from sources other than water. In effect, this is anoxygenic photosynthesis occurring in oxygenic phototrophs.

Many cyanobacteria can use H_2S as an electron donor under these conditions, and many green algae can use H_2. When H_2S is used, it is oxidized to elemental sulfur (S^0), and sulfur granules similar to those produced by green sulfur bacteria (Figure 14.5) are deposited outside the cyanobacterial cells. **Figure 14.18** shows this in the filamentous cyanobacterium *Oscillatoria limnetica*. This organism lives in anoxic salt ponds where it oxidizes sulfide and carries out anoxygenic photosynthesis along with green and purple bacteria.

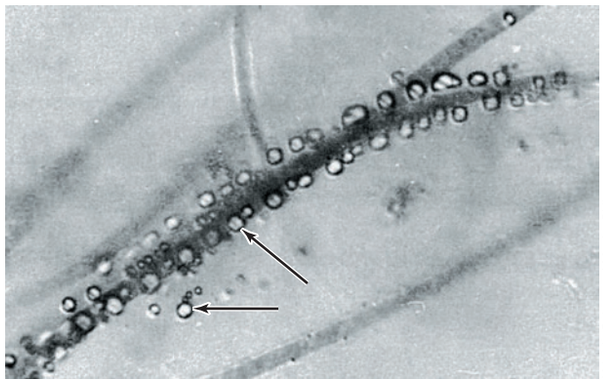

Yehuda Cohen and Moshe Shilo

Figure 14.18 Oxidation of H_2S by *Oscillatoria limnetica*. Note the globules of S^0 (arrows), the oxidation product of H_2S, formed outside the cells. *O. limnetica* carries out oxygenic photosynthesis, but cells revert to the anoxygenic process in the presence of H_2S.

From an evolutionary standpoint, the process of photophosphorylation in both oxygenic and anoxygenic phototrophs is one of many indications of their close relationship. Indeed, the structure of the Q-type reaction centers is homologous with PSII, and the structure of the FeS-type reaction centers is homologous with PSI. Because the evidence is strong that purple and green bacteria preceded cyanobacteria on Earth by perhaps as many as 0.5 billion years (◀ Section 13.2), it is clear that anoxygenic photosynthesis was the first form of photosynthesis on Earth. The key evolutionary event that ultimately led to the evolution of oxygenic photosynthesis in cyanobacteria was to acquire both types of reaction centers by horizontal gene transfer. The ancestors of cyanobacteria were certainly anoxygenic phototrophs, but the presence of redundant reaction centers facilitated their evolutionary diversification, as often results from gene duplication events (◀ Section 13.8). Ultimately, this process of functional diversification and experimentation led to the origin of oxygenic photosynthesis when chance events caused PSII to acquire the ability to use H_2O as an electron donor. The evolutionary origin of oxygenic photosynthesis was a seminal event in Earth's history since it not only oxygenated the planet (◀ Figure 13.1) but also allowed photoautotrophs to tap an inexhaustible supply of electrons.

In Part III we turn our attention to chemotrophic organisms that conserve energy through the respiration of inorganic electron donors.

Check Your Understanding

- Differentiate between cyclic and noncyclic electron flow in oxygenic photosynthesis.
- What is the key role of light energy in the initial step of the photosynthetic light reactions?
- What evidence is there that anoxygenic and oxygenic photosynthesis are related processes?

III • Respiratory Processes Defined by Electron Donor

Chemolithotrophs use inorganic electron donors such as H_2S, Fe^{2+}, or NH_3 in electron transport, generating ATP by oxidative phosphorylation. However, these electropositive donors force these organisms to generate NADH through endergonic reactions.

As we learned in Chapter 3 and reviewed in Section 14.1, energy is conserved in respiration by redox reactions that transfer electrons from an initial electron donor to a final electron acceptor. A tremendous diversity of respirations exist, and the microbes that carry them out are typically characterized by the nature of their electron donors and/or electron acceptors. In this part of the chapter, we focus on diversity from the standpoint of electron donors.

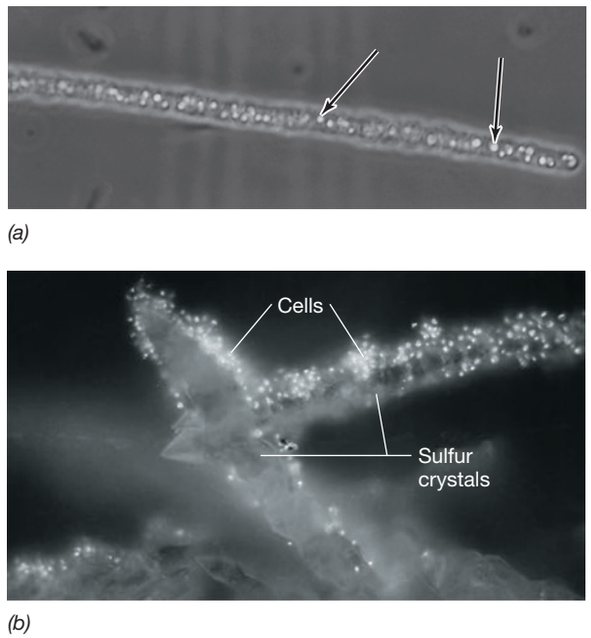
14.7 Oxidation of Sulfur Compounds

Many reduced sulfur compounds can be electron donors for the colorless sulfur bacteria, called *colorless* to distinguish them from the pigmented green and purple sulfur bacteria discussed earlier in this chapter (Figure 14.5 and Section 14.5). Historically, the concept of chemolithotrophy emerged in the late nineteenth century from studies of the sulfur bacteria by the Russian microbiologist Sergei Winogradsky (◀ Section 1.13) and was a radically new idea at the time. However, as our understanding of metabolic diversity has improved, it has become clear that chemolithotrophy, and in particular sulfur chemolithotrophy, is a major metabolic lifestyle of many *Bacteria* and *Archaea*.

Energetics of Sulfur Oxidation

The most common sulfur compounds used as electron donors are hydrogen sulfide (H_2S), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}) (Table 14.1, **Table 14.2**, and see Table 14.4). In most cases, the final oxidation product is sulfate (SO_4^{2-}). Sulfide oxidation occurs in stages, with the first oxidation step yielding elemental sulfur, S^0. Some sulfide-oxidizing bacteria, such as *Beggiatoa*, deposit this elemental sulfur inside the cell (**Figure 14.19*a***), where the sulfur exists as a potential energy (electron) reserve. When the supply of sulfide has been depleted, additional energy can then be conserved from the oxidation of sulfur to sulfate. When S^0 is present externally, the organism must attach itself to the sulfur particle because elemental sulfur is rather insoluble (Figure 14.19*b*).

One product of the oxidation of reduced sulfur compounds is protons (Tables 14.1 and 14.2). Consequently, one result of sulfur chemolithotrophy is acidification of the environment. Because of this, many sulfur bacteria have evolved to be acid-tolerant or even acidophilic. *Acidithiobacillus thiooxidans*, for example, grows best at a pH between 2 and 3.



(a)

(b)

Figure 14.19 Sulfur bacteria. *(a)* Internal sulfur granules in *Beggiatoa* (arrows). *(b)* Attachment of cells of the sulfur-oxidizing archaeon *Sulfolobus acidocaldarius* to a crystal of elemental sulfur. Cells were visualized by fluorescence microscopy after being stained with the dye acridine orange, which does not stain sulfur.

Biochemistry of Sulfur Oxidation: The Sox System

There are diverse pathways for conserving energy from the oxidation of sulfur compounds. One of the best characterized is the *Sox* (for sulfur *ox*idation) *system* (**Figure 14.20**), which has been detailed in the bacterium *Paracoccus pantotrophus* and is widespread among sulfur-oxidizing bacteria. The Sox system contains over 15 genes encoding various cytochromes and other proteins necessary for the oxidation of reduced sulfur compounds directly to sulfate. Elements of the Sox system are found in diverse sulfur chemolithotrophs and also in some phototrophic sulfur bacteria, organisms that oxidize sulfide to obtain reducing power for CO_2 fixation rather than for energy conservation. The fact that this biochemical system is distributed among bacteria that oxidize sulfide for very different reasons is a good indication that the genes that encode Sox have been

TABLE 14.2 Comparison of the energetics of oxidation of some common reduced sulfur compounds

Chemolithotrophic reaction	Electrons	Stoichiometry[a]	Energetics (kJ/electron)[a]
Sulfide to sulfate	8	$H_2S + 2\,O_2 \rightarrow SO_4^{2-} + 2\,H^+$	$\Delta G^{0\prime} = -798.2$ kJ/reaction (-99.75 kJ/e$^-$)
Sulfite to sulfate	2	$SO_3^{2-} + \frac{1}{2}\,O_2 \rightarrow SO_4^{2-}$	$\Delta G^{0\prime} = -258$ kJ/reaction (-129 kJ/e$^-$)
Thiosulfate to sulfate	8	$S_2O_3^{2-} + H_2O + 2\,O_2 \rightarrow 2\,SO_4^{2-} + 2\,H^+$	$\Delta G^{0\prime} = -818.3$ kJ/reaction (-102 kJ/e$^-$)

[a]All reactions are balanced, both atomically and electrically. See Table 3.3 and Section 3.3 for details of calculations. For the reaction and energetics of the oxidation of sulfide to sulfur and sulfur to sulfate, see Table 14.1.

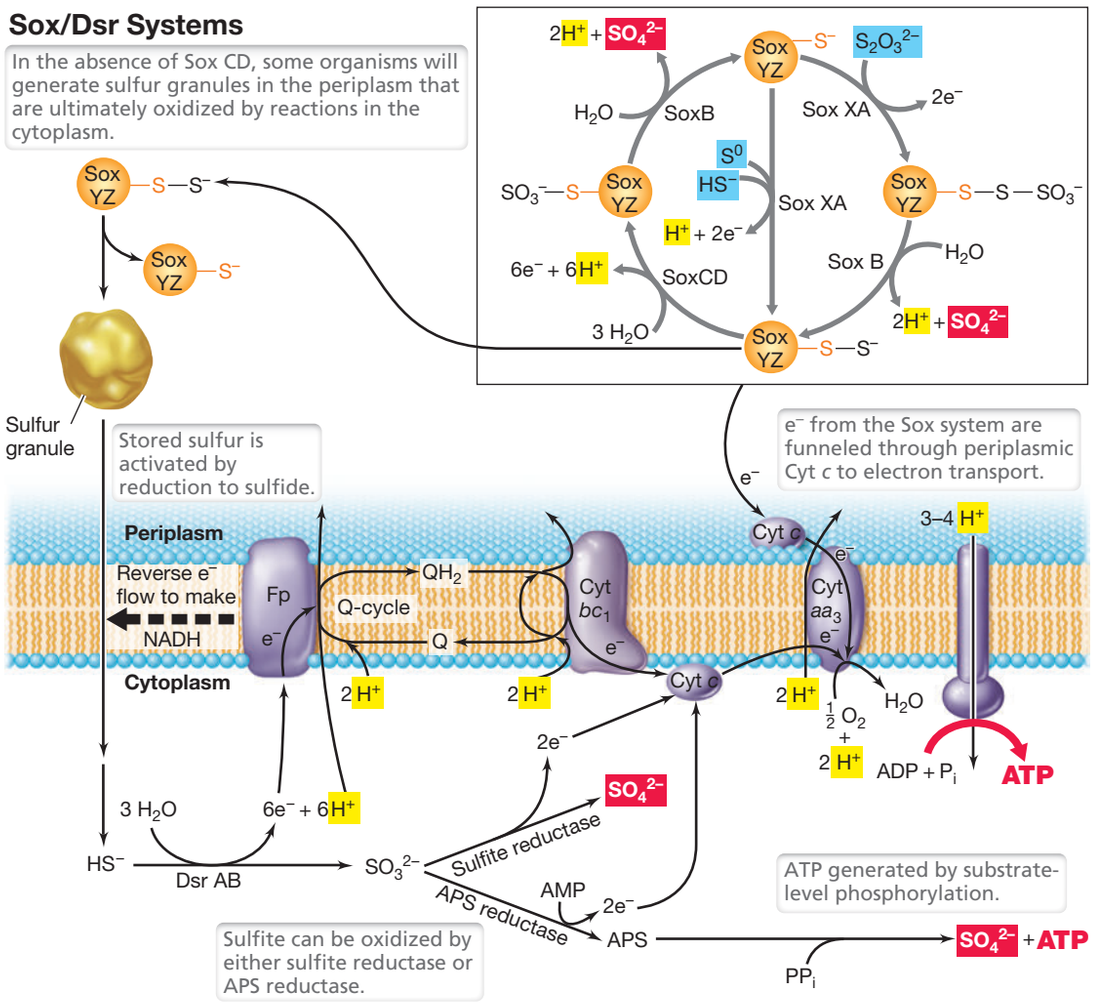


Figure 14.20 Oxidation of reduced sulfur compounds by sulfur chemolithotrophs. There are several different pathways for conserving energy through the oxidation of sulfide (H_2S), thiosulfate ($S_2O_3^-$), and elemental sulfur (S^0). In the Sox system (*sulfur oxidation*), SoxXA attaches a reduced sulfur compound to the carrier protein SoxYZ. The protein SoxCD, sulfur dehydrogenase, catalyzes removal of 6 e^- from the bound sulfur atom, and is a key enzyme for bacteria that use the complete Sox system for sulfur oxidation (such as *Paracoccus pantotrophus*). Sulfate (SO_4^{2-}) is released by the action of SoxB. In contrast, bacteria that form sulfur granules, such as *Beggiatoa* (Figure 14.19*a*), lack SoxCD and instead oxidize sulfur compounds using the enzymes DsrAB, dissimilatory sulfite reductase, and APS reductase (see Section 14.12). In sulfur oxidation, these enzymes are run backwards to oxidize sulfur compounds. In certain sulfur oxidizers, APS reductase is replaced by sulfite reductase. Reactions of the Sox cycle take place in the periplasm and electrons enter the electron transport chain through the activity of a periplasmic *c*-type cytochrome (Cyt *c*), while reactions of Sox/Dsr systems take place instead in the cytoplasm and electrons can enter electron transport at either the level of flavoproteins (Fp) or *c*-type cytochromes.

transferred between species by horizontal gene flow (◀ Section 13.9 and Chapter 9).

There are four key proteins in the Sox system: SoxXA, SoxYZ, SoxB, and SoxCD. All of these proteins are present in the periplasm (◀ Section 2.4). The pathway begins when the enzyme SoxXA forms a heterodisulfide bond between the sulfur compound to be oxidized (which can be HS^-, S^0, or $S_2O_3^{2-}$) and the carrier protein, SoxYZ (Figure 14.20). The sulfur compound remains bound to the carrier throughout the pathway, being ultimately released as sulfate through the activity of SoxB. The enzyme SoxCD (sulfur dehydrogenase) is the key enzyme that mediates the removal of 6 electrons from the sulfur compound bound to the carrier (Figure 14.20). Electrons from the Sox system are funneled into the electron transport chain (see later), while the protons generated in the periplasm are released to and acidify the external environment.

Other Aspects of Chemolithotrophic Sulfur Oxidation

Sulfur-oxidizing microbes that store sulfur granules within the cell (see Figure 14.19*a*) also use components of the Sox system but lack the key enzyme sulfur dehydrogenase (SoxCD). In the absence of SoxCD, a sulfur atom bound to SoxYZ is added to a growing sulfur granule in the periplasm (Figure 14.20). The sulfur in the granule can be reductively activated and transported to the cytoplasm where it is eventually oxidized to sulfite (SO_3^{2-}) by the reverse activity of DsrAB, an enzyme homologous to the enzyme sulfite reductase found in sulfate-reducing bacteria (Section 14.12). The sulfite is then oxidized to sulfate plus two electrons through one of two different pathways. The most widespread system employs the reverse activity of the cytoplasmic enzyme *sulfite reductase*. This enzyme oxidizes sulfite and transfers the electrons to the electron transport chain. By contrast, some sulfur chemolithotrophs oxidize SO_3^{2-} to SO_4^{2-} via a reversal of the activity of the enzyme adenosine phosphosulfate (APS) reductase (an enzyme essential for the metabolism of sulfate-reducing bacteria, see Section 14.12). The oxidation of SO_3^{2-} to SO_4^{2-} yields an energy-rich phosphate bond by substrate-level phosphorylation when AMP is converted to ATP (Figure 14.20).

Electrons from the oxidation of reduced sulfur compounds eventually reach the electron transport chain, as shown in Figure 14.20. Though the exact details remain unclear, electrons likely enter at the flavoprotein or cytochrome *c* ($E_0' = +0.3$ V) levels and are transported through the chain to O_2, generating a proton motive force that triggers ATP synthase activity (Figure 14.20). Electrons for CO_2 fixation come from reverse electron transport (Section 14.1), eventually yielding NADH, and autotrophy is driven by reactions of the Calvin cycle or some other autotrophic pathway (Section 14.2).

Although the sulfur chemolithotrophs are primarily an aerobic group, some species can grow by anaerobic respiration using nitrate as an electron acceptor. The sulfur bacterium *Thiobacillus denitrificans* is a classic example, reducing nitrate to dinitrogen gas (the process of denitrification, Section 14.11).

We now consider iron oxidation, a process that at acidic pH poses the greatest energy challenge of all chemolithotrophic energy metabolisms.

Check Your Understanding

- How many electrons are available from the oxidation of H_2S if S^0 or SO_4^{2-} is the final product?
- In terms of intermediates, how does the Sox system differ from other sulfide-oxidizing systems?
- Why are many sulfur- and sulfide-oxidizing bacteria acidophilic?

14.8 Iron (Fe^{2+}) Oxidation

The aerobic oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) supports growth of the chemolithotrophic "iron bacteria" (▶ Section 15.14 and see MicrobiologyNow on page 460). At acidic pH, only a small amount of energy is available from this reaction (Table 14.1), and for this reason the iron bacteria must oxidize large amounts of iron in order to produce only tiny amounts of cell material. The ferric iron produced becomes hydrated to form insoluble ferric hydroxide ($Fe^{3+} + 3\,H_2O \rightarrow Fe(OH)_3 + 3\,H^+$) and other iron precipitates in aquatic environments (**Figure 14.21**), and this drives down the pH. This inevitable chemical reaction probably explains why many iron-oxidizing bacteria have evolved to be strongly acidophilic.

Iron-Oxidizing Bacteria

The best-known iron bacteria, *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, can both grow autotrophically using ferrous iron (Figure 14.21) as electron donor at pH values as low as 1; growth is optimal at pH 2–3. These bacteria are common in acid-polluted environments such as coal-mining runoff waters (Figure 14.21*a*). *Ferroplasma*, a species of *Archaea*, is an extremely acidophilic iron oxidizer and can grow at pH values below 0 (▶ Section 17.3). We discuss the role of all of these organisms in acid mine pollution and mineral oxidation in ▶ Sections 21.6, 22.1, and 22.2.

At neutral pH, Fe^{2+} spontaneously oxidizes to Fe^{3+}, so opportunities for the iron bacteria in neutral habitats are restricted to locations where Fe^{2+} is transitioning from anoxic to oxic conditions. For example, anoxic groundwater often contains dissolved Fe^{2+}, and when it is released, as in iron-rich spring water, it becomes exposed to O_2. At such interfaces, iron bacteria oxidize Fe^{2+} to Fe^{3+} before it oxidizes spontaneously. *Gallionella ferruginea*, *Sphaerotilus natans*, and *Leptothrix discophora* are examples of bacteria that live at these interfaces. They are typically seen mixed in with the characteristic ferric iron deposits they form (▶ Figures 15.55 and 21.6).

Energy from Iron Oxidation

The bioenergetics of ferrous iron oxidation by *Acidithiobacillus ferrooxidans* and other acidophilic iron oxidizers are of considerable interest because of the very electropositive reduction potential of the Fe^{3+}/Fe^{2+} couple at acidic pH ($E_0' = +0.77$ V at pH 2). The

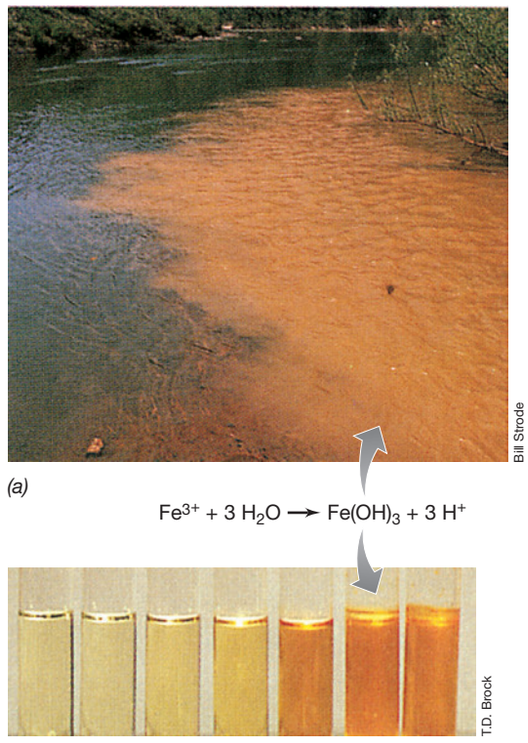


Figure 14.21 Iron-oxidizing bacteria. *(a)* Acid mine drainage, showing the confluence of a normal river and a creek draining a coal-mining area. At low pH values, Fe^{2+} does not oxidize spontaneously in air, but *Acidithiobacillus ferrooxidans* carries out the oxidation; insoluble $Fe(OH)_3$ and complex ferric salts precipitate. *(b)* Cultures of *A. ferrooxidans*. Shown is a dilution series, with no growth in the tube on the left and increasing amounts of growth from left to right. Growth is evident from the production of $Fe(OH)_3$.

respiratory chain of *A. ferrooxidans* contains cytochromes of the *c* and aa_3 types and a periplasmic copper-containing protein called *rusticyanin* (**Figure 14.22**). There is also an iron-oxidizing protein located in the outer membrane of this gram-negative bacterium.

Because the reduction potential of the Fe^{3+}/Fe^{2+} couple is so high, steps in electron transport to oxygen ($\frac{1}{2}O_2/H_2O$, $E_0' = +0.82$ V) are necessarily few. Iron oxidation begins in the outer membrane where the organism contacts either soluble Fe^{2+} or insoluble ferrous iron minerals such as FeS. Fe^{2+} is oxidized to Fe^{3+}, a one-electron transition (Table 14.1), by an outer membrane cytochrome *c* that transfers electrons into the periplasm where rusticyanin ($E_0' = +0.68$ V) is the electron acceptor. This thermodynamically slightly unfavorable reaction is thought to be pulled forward by the immediate consumption of Fe^{3+} by $Fe(OH)_3$ formation (Figure 14.22). Rusticyanin then reduces a periplasmic cytochrome *c* that transfers electrons to cytochrome aa_3, and it is the latter protein that reduces O_2 to H_2O; ATP is synthesized by ATPase in the usual fashion (Figure 14.22).

The nature of the proton motive force in *A. ferrooxidans* is of interest. In a highly acidic environment, a large gradient of protons already exists across the *A. ferrooxidans* cytoplasmic membrane (the periplasm is pH 1–2, whereas the cytoplasm is about pH 6, Figure 14.22). Although one might think that with this gradient *A. ferrooxidans* could make ATP at no energetic cost, this is not the

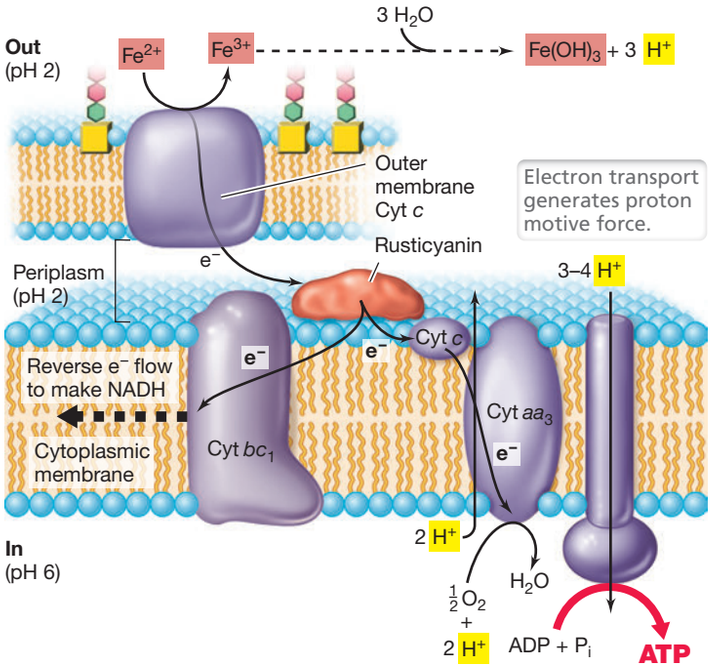


Figure 14.22 Electron flow during Fe^{2+} oxidation by the acidophile *Acidithiobacillus ferrooxidans*. The periplasmic copper-containing protein rusticyanin receives electrons from Fe^{2+} oxidized by a *c*-type cytochrome located in the outer membrane. From here, electrons travel through a short electron transport chain, resulting in the reduction of O_2 to H_2O. Reducing power comes from reverse electron flow. Note the steep pH gradient across the cytoplasmic membrane.

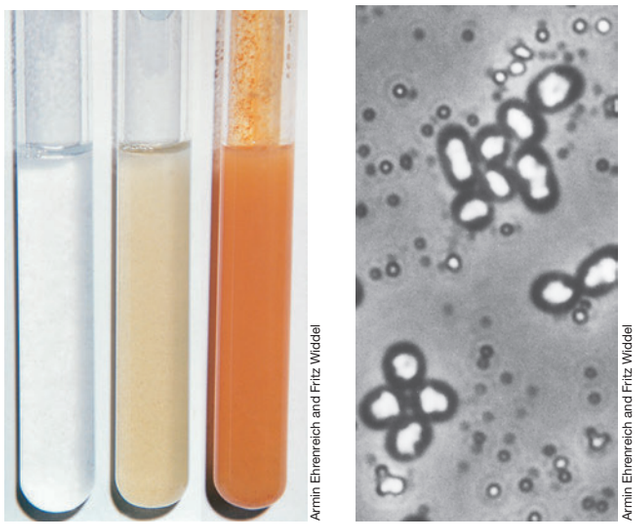


Figure 14.23 Fe^{2+} oxidation by anoxygenic phototrophic bacteria. *(a)* Oxidation in anoxic tube cultures. Left to right: Sterile medium, inoculated medium, a growing culture showing $Fe(OH)_3$. *(b)* Phase-contrast photomicrograph of an Fe^{2+}-oxidizing purple bacterium, strain L7. The bright refractile areas within cells are gas vesicles (◀ Section 2.7). The granules outside the cells are iron precipitates. Strain L7 is phylogenetically related to the purple sulfur bacterium *Chromatium*.

case; the organism cannot make ATP from this preformed proton motive force in the absence of an electron donor. This is because H^+ ions that enter the cytoplasm via ATPase must be consumed in order to maintain the internal pH within acceptable limits. Proton consumption occurs during the reduction of O_2 in the electron transport chain and this reaction requires electrons; the latter come from the oxidation of Fe^{2+} to Fe^{3+} (Figure 14.22).

Autotrophy in *A. ferrooxidans* is supported by the Calvin cycle (Section 14.2), and because of the high potential of the electron donor, much energy must be consumed in reverse electron flow reactions to obtain the reducing power (NADH or NADPH) necessary to drive CO_2 fixation (Section 14.1). NADH is formed by reduction of NAD^+ by electrons obtained from Fe^{2+} that are forced backwards through cytochrome bc_1 and the quinone pool at the expense of the proton motive force (Figure 14.22).

The relatively poor energetic yield from ferrous iron oxidation coupled with the large energetic demands of the Calvin cycle means that *A. ferrooxidans* must oxidize large amounts of Fe^{2+} to produce even a very small amount of cell material. Thus, in environments where acidophilic iron-oxidizing bacteria thrive, their presence is signaled not by the formation of high cell numbers but by the presence of the extensive ferric iron precipitates they have generated (Figure 14.21).We consider the ecology of iron bacteria in Chapters 21 and 22.

Ferrous Iron Oxidation under Anoxic Conditions

Ferrous iron can be oxidized under *anoxic* conditions by certain chemolithotrophs and phototrophic purple and green bacteria (**Figure 14.23**). In these cases, Fe^{2+} is used either as an electron donor in energy metabolism (chemolithotrophs) and/or as a reductant for CO_2 fixation (phototrophs). An important point to consider here is that at neutral pH where these organisms thrive, the E_0' of the Fe^{3+}/Fe^{2+} couple is significantly more electronegative than at acidic pH (+0.2 V versus +0.77 V, respectively, Figure 14.1). Hence, electrons from Fe^{2+} can reduce cytochrome *c* to initiate electron transport reactions. For chemolithotrophs, the electron acceptor is nitrate (NO_3^-) with either nitrite (NO_2^-) or dinitrogen gas (N_2) being the final product of this anaerobic respiration. For Fe^{2+}-oxidizing purple and green bacteria, either soluble Fe^{2+} or iron sulfide (FeS) can be used as electron donor. With FeS, both Fe^{2+} and S^{2-} are oxidized, Fe^{2+} to Fe^{3+} (one electron) and HS^- to SO_4^{2-} (eight electrons).

We now consider chemolithotrophic metabolisms of nitrogen compounds, major components of the nitrogen cycle in nature (▶ Section 21.3).

Check Your Understanding

- Why is only a very small amount of energy available from the oxidation of Fe^{2+} to Fe^{3+} at acidic pH?
- What is the function of rusticyanin and where is it found in the cell?
- How can Fe^{2+} be oxidized under anoxic conditions?

14.9 Nitrification

The reduced inorganic nitrogen compounds ammonia (NH_3) and nitrite (NO_2^-) are oxidized aerobically by the chemolithotrophic *nitrifying bacteria* in the process of **nitrification** (▶ Section 15.10). Nitrifying bacteria are widely distributed in soils, water, wastewaters, and the oceans. Nitrification consists of two different sets of

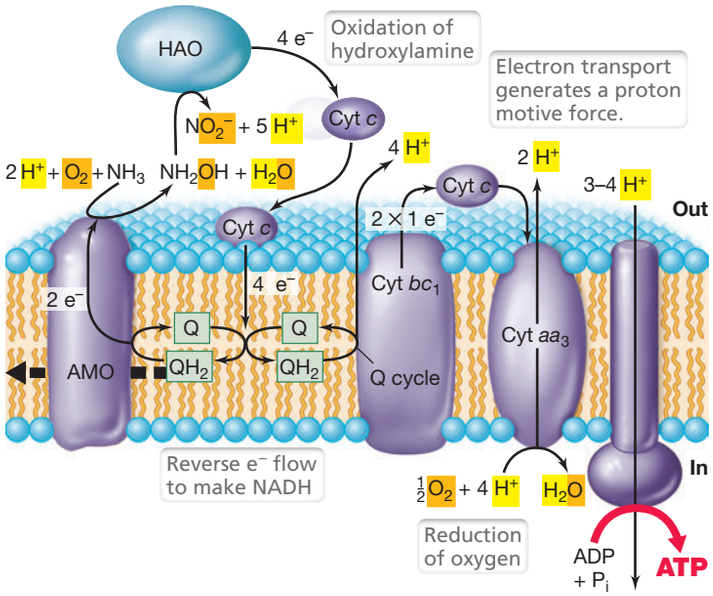


Figure 14.24 Oxidation of NH_3 and electron flow in the cytoplasmic membrane of ammonia-oxidizing bacteria. The reactants and the products of this reaction series are highlighted. The top surface of the membrane is the beginning of the periplasm. The cytochrome *c* (Cyt *c*) in the periplasm is a different form of Cyt *c* than that in the cytoplasmic membrane. AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; Q, ubiquinone.

reactions; the first set of reactions catalyze oxidation of ammonia to nitrite, and the second set catalyze oxidation of nitrite to nitrate (NO_3^-). Most nitrifying microbes are only able to catalyze one set of these reactions. For example, *Bacteria* such as *Nitrosomonas* and *Archaea* such as *Nitrosopumilus* oxidize NH_3 only to NO_2^-, and we call these organisms *ammonia oxidizers*. The full nitrification pathway is ultimately completed when other *Bacteria* such as *Nitrobacter* oxidize NO_2^- to NO_3^-, and we call these organisms *nitrite oxidizers*. As far as is known, only certain bacteria in the genus *Nitrospira* can catalyze both sets of reactions, oxidizing NH_3 all the way to NO_3^-.

Bioenergetics and Enzymology of Ammonia and Nitrite Oxidation

Mastering Microbiology
Art Activity: Figure 14.32 Oxidation of NH_3 and electron flow in ammonia-oxidizing bacteria

The bioenergetics of nitrification is based on the same principles that govern other chemolithotrophic reactions: Electrons from reduced inorganic substrates (in this case, reduced nitrogen compounds) enter an electron transport chain, and electron transport reactions establish a proton motive force that drives ATP synthesis. The complete oxidation of NH_3 to NO_3^- releases eight electrons, but the electron donors for the nitrifying bacteria are not particularly strong. For example, the E_0' of the NO_2^-/NH_3 couple (the first step in the oxidation of NH_3) is +0.34 V, and the E_0' of the NO_3^-/NO_2^- couple is even more positive, about +0.43 V. By necessity, these reduction potentials force the nitrifying bacteria to donate electrons to rather high-potential electron acceptors, and this of course limits the amount of energy that can be conserved (Section 14.1).

Mastering Microbiology
Art Activity: Figure 14.33 Oxidation of NO_2^- to NO_3^- by nitrifying bacteria

Several key enzymes participate in the oxidation of reduced nitrogen compounds. In ammonia-oxidizing bacteria such as *Nitroso-monas*, NH_3 is oxidized by *ammonia monooxygenase* (AMO; monooxygenases are discussed in Section 14.23), producing hydroxylamine (NH_2OH) and H_2O (**Figure 14.24**). A second key enzyme, *hydroxylamine oxidoreductase* (HAO), then oxidizes NH_2OH to NO_2^-, removing four electrons in the process. AMO is an integral membrane protein, whereas HAO is periplasmic (Figure 14.24). In the reaction carried out by AMO,

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \rightarrow NH_2OH + H_2O$$

two electrons and two protons are needed to reduce one molecule of oxygen (O_2) to H_2O. These electrons originate from the oxidation of hydroxylamine and are supplied to AMO from HAO (Figure 14.24). Thus, for every *four* electrons generated from the oxidation of NH_3 to NO_2^-, only *two* actually reach the terminal oxidase that interacts with O_2 to form H_2O (Figure 14.24).

Nitrite-oxidizing bacteria such as *Nitrobacter* oxidize NO_2^- to NO_3^- by the enzyme *nitrite oxidoreductase*, with electrons traveling a very short electron transport chain (because of the high potential of the NO_3^-/NO_2^- couple) to the terminal oxidase (**Figure 14.25**). Cytochromes of the *c* and *bd* types are present in the electron transport chain of nitrite oxidizers, and the activity of the *bd*-type cytochrome generates a proton motive force (Figure 14.25). As is the case with the iron bacteria (Section 14.8), only small amounts of energy are available from nitrite oxidation. Hence, minimal amounts of cell material are obtained even though large amounts of nitrite may be oxidized.

Carbon Metabolism and Ecology of Nitrifying Bacteria

Like sulfur- and iron-oxidizing chemolithotrophs (Sections 14.7 and 14.8), aerobic nitrifying *Bacteria* employ the Calvin cycle for CO_2 fixation. The ATP and reducing power requirements of the Calvin cycle place additional burdens on an energy-generating system that already has a relatively low yield; NAD(P)H to drive the Calvin cycle in nitrifiers is formed by reverse electron flow (Figures 14.24

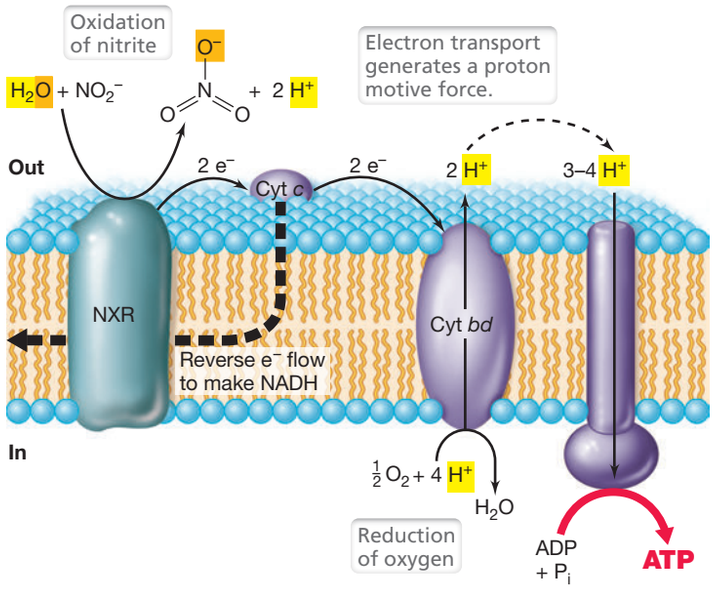


Figure 14.25 Oxidation of NO_2^- to NO_3^- by nitrifying bacteria. The reactants and products of this reaction series are highlighted to show the reaction clearly. NXR, nitrite oxidoreductase; Cyt, cytochrome.

and 14.25). The energetic constraints are particularly severe for nitrite oxidizers, and it is perhaps for this reason that most of these organisms have alternative energy-conserving mechanisms, being able to grow chemoorganotrophically on glucose and a few other organic substrates. By contrast, species of ammonia-oxidizing bacteria are either obligate chemolithotrophs and autotrophs, or are mixotrophic, both fixing CO_2 and using organic compounds (Section 14.2). Autotrophy in ammonia-oxidizing *Archaea* is supported by the 3-hydroxypropionate/4-hydroxybutyrate cycle (Section 14.2).

Nitrifying microbes play key ecological roles in the nitrogen cycle, converting ammonia in soils into nitrate, a key plant nutrient. Nitrifiers are also important in sewage and wastewater treatment, removing toxic amines and ammonia and releasing less toxic nitrogen compounds (▶ Sections 22.6 and 22.7). Nitrifiers play a similar role in the water column of lakes, where ammonia produced in the sediments from the decomposition of organic nitrogenous compounds is oxidized to nitrate, a more usable fixed nitrogen source for algae and cyanobacteria.

Although classical nitrifiers are obligate aerobes, we will see next that ammonia can be oxidized anaerobically as well, by a unique group of *Bacteria*.

Check Your Understanding

- What are the substrates for the enzyme ammonia monooxygenase?
- What is the difference between ammonia oxidation and nitrite oxidation, and in what types of organisms are these reactions found?
- Why do both ammonia- and nitrite-oxidizing bacteria require reverse electron transport?

14.10 Anaerobic Ammonia Oxidation (Anammox)

In addition to the oxygen-dependent process of ammonia oxidation by organisms such as *Nitrosomonas* and *Nitrosopumilus*, NH_3 can also be oxidized under anoxic conditions. This process is called **anammox** (for *an*aerobic *amm*onia *ox*idation) and is catalyzed by an unusual group of obligately anaerobic *Bacteria*.

Ammonia is oxidized in the anammox reaction using NO_2^- as the electron acceptor to yield N_2:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O \quad \Delta G^{0\prime} = -357\text{kJ}$$

A major anammox organism, *Brocadia anammoxidans*, is a species of the *Planctomycetes* phylum of *Bacteria* (▶ Section 16.16). *Planctomycetes* are structurally unusual *Bacteria* because their cytoplasm can contain membrane-enclosed compartments of various types (**Figure 14.26**). In cells of *B. anammoxidans*, this compartment is the *anammoxosome*, and it is within this structure that the anammox reaction occurs (Figure 14.26*c*). In addition to *Brocadia*, several other genera of anammox bacteria are known, including *Kuenenia*, *Anammoxoglobus*, *Jettenia*, and *Scalindua*, all of which are related to *Brocadia* and also contain anammoxosomes. Like aerobic ammonia oxidizers, anammox bacteria are also autotrophs, but they do not fix CO_2 using the pathways employed by aerobic ammonia oxidizers. Instead, anammox bacteria fix CO_2 by way of the reductive acetyl-CoA pathway, an autotrophic pathway widespread among some obligately anaerobic autotrophic *Bacteria* and *Archaea* (see Section 14.14).

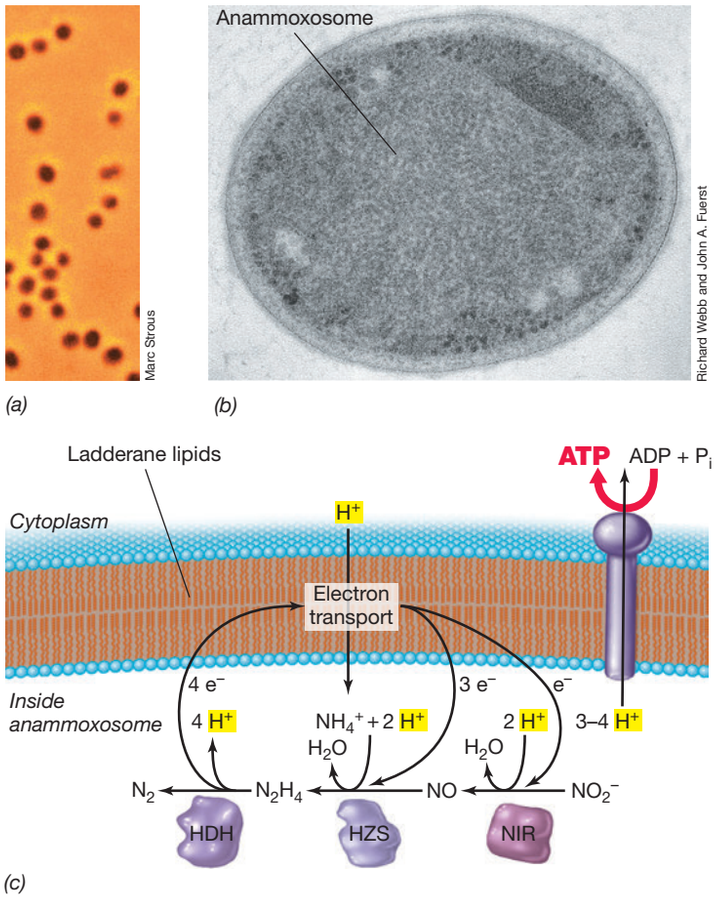


Figure 14.26 Anammox. *(a)* Phase-contrast photomicrograph of cells of *Brocadia anammoxidans*. A single cell is about 1 μm in diameter. *(b)* Transmission electron micrograph of a *Brocadia* cell; note the membrane-enclosed compartments including the large fibrillar anammoxosome. *(c)* Reactions in the anammoxosome. NIR, nitrite reductase, HZS, hydrazine synthase; HDH, hydrazine dehydrogenase.

The Anammoxosome and Its Reactions

The anammoxosome is a unit membrane–enclosed structure (Figure 14.26*b*) and in this respect is technically an organelle in the eukaryotic sense of the term. Lipids that form the anammoxosome membrane are not the typical lipids of *Bacteria* but instead consist of fatty acids constructed of multiple cyclobutane (C_4) rings bonded to glycerol by both ester and ether bonds. These *ladderane lipids*, as they are called, aggregate in the membrane to form an unusually dense membrane structure that prevents diffusion of substances from the anammoxosome into the cytoplasm.

The sturdy anammoxosome membrane is required to protect the cell from toxic intermediates produced during anammox reactions. These include, in particular, the compound *hydrazine* (N_2H_4), a very strong reductant. In the anammox reaction, NO_2^- is first reduced to nitric oxide (NO) by nitrite reductase, and then NO reacts with ammonium (NH_4^+) to yield N_2H_4 by activity of the enzyme hydrazine synthase (Figure 14.26*c*). (Note that while NH_3 is the substrate

for AMO in aerobic ammonia-oxidizing bacteria [Figure 14.24], NH_4^+ is the substrate for hydrazine synthase in the anaerobic anammox bacteria.) N_2H_4 is then oxidized to N_2 plus electrons by the enzyme hydrazine dehydrogenase, and the electrons are funneled into the electron transport chain where they are used to reduce NO_2^- and NO earlier in the pathway. In this way, anammox generates a *cyclical* series of electron transfer reactions in order to generate a proton motive force; ATP is formed from the latter by ATPases in the anammoxosome membrane (Figure 14.26*c*).

Reducing power for CO_2 fixation by anammox bacteria is derived from reverse electron transport (Section 14.1), but because electron transfer reactions are cyclic, the electrons needed for reverse electron transport derive from an independent set of reactions that oxidize NO_2^- to NO_3^- by a nitrite oxidoreductase, a reaction also present in *Nitrobacter* (Figure 14.25). Interestingly, then, NO_2^- serves two different purposes for anammox bacteria: The *reduction* of nitrite is required to generate ATP by chemiosmosis, whereas the *oxidation* of nitrite is required to generate reducing power for CO_2 fixation.

Ecology of Anammox

In nature, the source of NO_2^- for the anammox reaction is presumably aerobic ammonia-oxidizing *Bacteria* and *Archaea*. These organisms coexist with anammox bacteria in ammonia-rich habitats such as sewage and other wastewaters. The suspended particles that form in these habitats contain both oxic and anoxic zones in which ammonia oxidizers of different physiologies can coexist in close association. In mixed laboratory cultures, high levels of oxygen inhibit anammox and favor classic nitrification, and thus it is likely that in nature, the fraction of ammonia oxidation catalyzed by anammox bacteria is governed by the concentration of O_2 in the habitat.

From an environmental standpoint, anammox is a very beneficial process in the treatment of wastewaters. The anoxic removal of ammonia and amines by the formation of N_2 (Figure 14.26*c*) helps reduce the input of fixed nitrogen from wastewater treatment effluents that flow into rivers and streams, thereby maintaining higher water quality than would otherwise be possible. Also, marine anammox bacteria are likely responsible for the large amount of NH_3 that is known to disappear during mineralization processes in anoxic marine sediments. At least some ammonia-rich freshwater lake sediments also support anammox, and thus it appears that anammox can occur in any anoxic environment in which NH_3 and NO_2^- coexist.

We move on to Part IV now where chemotrophic energy metabolisms will be defined not by the electron *donor* that is used, but by the electron *acceptor* that is used to receive electrons from a wide variety of donors.

Check Your Understanding

- Name the electron donor and the electron acceptor in the anammox process.
- What does electron transport for reducing power synthesis in chemolithotrophs (e.g., anammox bacteria, nitrifiers, and sulfide oxidizers) have in common with that of purple sulfur bacteria?
- Compare autotrophy in anammox bacteria and purple sulfur bacteria. What characteristics do these processes share and how are they different?

IV • Respiratory Processes Defined by Electron Acceptor

Anaerobic respiration occurs when an alternative electron acceptor is substituted for oxygen in respiration. Many forms of anaerobic respiration are possible using oxidized nitrogen and sulfur compounds, and some metals and organic compounds support the process as well.

We examined the process of aerobic respiration in Chapter 3 and reviewed the bioenergetics of respiration in Section 14.1. Here we consider the details of **anaerobic respiration** in its many variations found in the microbial world. Diverse compounds function as electron acceptors in anaerobic respirations (Figure 14.1), and each acceptor has its own redox properties and is typically linked to a specific group or groups of microbes. We begin with a common form of anaerobic respiration in which nitrate functions as electron acceptor.

14.11 Nitrate Reduction and Denitrification

Inorganic nitrogen compounds are some of the most common electron acceptors in anaerobic respiration. Table 14.3 summarizes the relevant forms of inorganic nitrogen with their oxidation states. One of the most common alternative electron acceptors for dissimilative purposes is nitrate (NO_3^-), which can be reduced with two electrons to nitrite (NO_2^-), or reduced further to nitric oxide (NO), nitrous oxide (N_2O), or dinitrogen (N_2). Because NO, N_2O, and N_2 are all gases, they can be lost from the environment, and their biological production is called **denitrification** (Figure 14.27).

Some nitrate reducers, for example *Escherichia coli*, are not true denitrifiers, but only carry out the first step (NO_2^- to NO_3^-) in the process (Figure 14.27and ◀ Figure 3.23). Moreover, some organisms can reduce NO_2^- to ammonia (NH_3) in a dissimilative process. But it is the production of gaseous products—*denitrification*—that is of greatest global significance because it consumes fixed nitrogen and produces some polluting gases.

Denitrifying Microorganisms and Their Ecological Activities

Many denitrifying *Bacteria* are phylogenetically *Proteobacteria* and physiologically facultative aerobes. *Pseudomonas* species, for example, are typically strong denitrifiers. Aerobic respiration occurs when O_2 is present, even if NO_3^- is also present in the medium. Many denitrifying bacteria also reduce other electron acceptors anaerobically, such

TABLE 14.3 Oxidation states of key nitrogen compounds

Compound	Oxidation state of N atom
Organic N (—NH_2)	−3
Ammonia (NH_3)	−3
Nitrogen gas (N_2)	0
Nitrous oxide (N_2O)	+1 (average per N)
Nitric oxide (NO)	+2
Nitrite (NO_2^-)	+3
Nitrogen dioxide (NO_2)	+4
Nitrate (NO_3^-)	+5

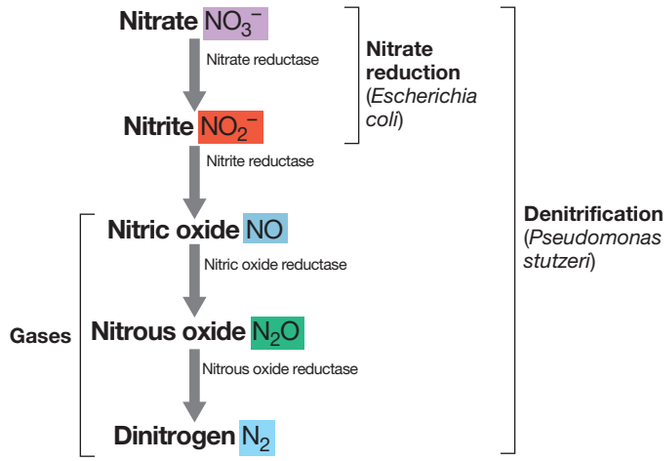


Figure 14.27 Steps in the dissimilative reduction of nitrate. Some organisms can carry out only the first step. All enzymes involved are derepressed by anoxic conditions. Also, some bacteria are known that can reduce NO_3^- to NH_4^+ in dissimilative metabolism. Note that colors used here match those used in Figure 14.28.

as Fe^{3+} and certain organic electron acceptors (Figure 14.1), and some denitrifiers can even ferment. Thus, denitrifying bacteria are metabolically diverse in terms of alternative energy-generating mechanisms. Some species of *Archaea* can grow anaerobically by nitrate reduction to nitrite, and several others can denitrify. Interestingly, at least one eukaryote has also been shown to be a denitrifier. The protist *Globobulimina pseudospinescens*, a shelled amoeba (a foraminiferan, ▶ Section 18.6), can denitrify and likely employs this form of metabolism in its habitat, anoxic marine sediments.

Denitrification is a process with significant ecological ramifications. For agricultural purposes, denitrification is a detrimental process, as it removes nitrate—often intentionally added by grain and other crop farmers as potassium nitrate fertilizer—from the soil. Gaseous products of denitrification other than N_2 (N_2O and NO) are also of significant environmental concern. N_2O is a strong greenhouse gas (contributing to climate change) and can also be converted to NO by sunlight; NO reacts with and consumes ozone (O_3) in the upper atmosphere to form NO_2^-. When it rains, NO_2^- returns to Earth as nitrous acid (HNO_2) in *acid rain*. In contrast to these environmentally harmful processes, for a desirable process such as sewage treatment, denitrification (as well as anammox, see earlier) is beneficial. This is because denitrification removes fixed nitrogen, a major trigger of algal growth if a nitrate-rich sewage effluent is released into rivers or lakes (▶ Sections 22.6 and 22.7).

Biochemistry of Dissimilative Nitrate Reduction

The electron transport pathway of denitrification is shown in **Figure 14.28**. The enzyme that catalyzes the first step of dissimilative nitrate reduction is *nitrate reductase*, a molybdenum-containing, membrane-integrated enzyme whose synthesis is repressed by O_2. All subsequent enzymes of the pathway are coordinately regulated and thus also repressed by O_2. In addition to anoxic conditions, nitrate must also be present before these enzymes are fully expressed.

The biochemistry of dissimilative nitrate reduction has been studied in detail in *E. coli*, in which NO_3^- is reduced only to NO_2^- (◀ Section 3.10) and *Paracoccus denitrificans* and *Pseudomonas stutzeri*, in which denitrification occurs. In *P. denitrificans* and *P. stutzeri*, nitrogen oxides are formed enzymatically from NO_2^- by nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Figure 14.28). NO and N_2O are gaseous intermediates that are free to escape from the cell, and N_2O in particular is a major product of denitrification, though these intermediates are often reduced all the way to N_2. During these electron transport reactions, a proton motive force is established (Figure 14.28), and ATPase couples this to the synthesis of ATP.

We move on from coverage of nitrogen compounds as electron acceptors in anaerobic respiration to consider sulfur compounds as electron acceptors in the next section. A diverse group of organisms will be in play here that generate a potentially toxic product but one that is central to the sulfur cycle. Following that, we round out this part of the chapter by considering a few key metals and organic compounds as electron acceptors.

Check Your Understanding

- Under what condition would you expect the genes for denitrification to be repressed?
- If the final product of denitrification is N_2 then why is N_2O released into the environment?
- Where is dissimilative nitrate reductase found in the cell? What unusual metal does it contain?

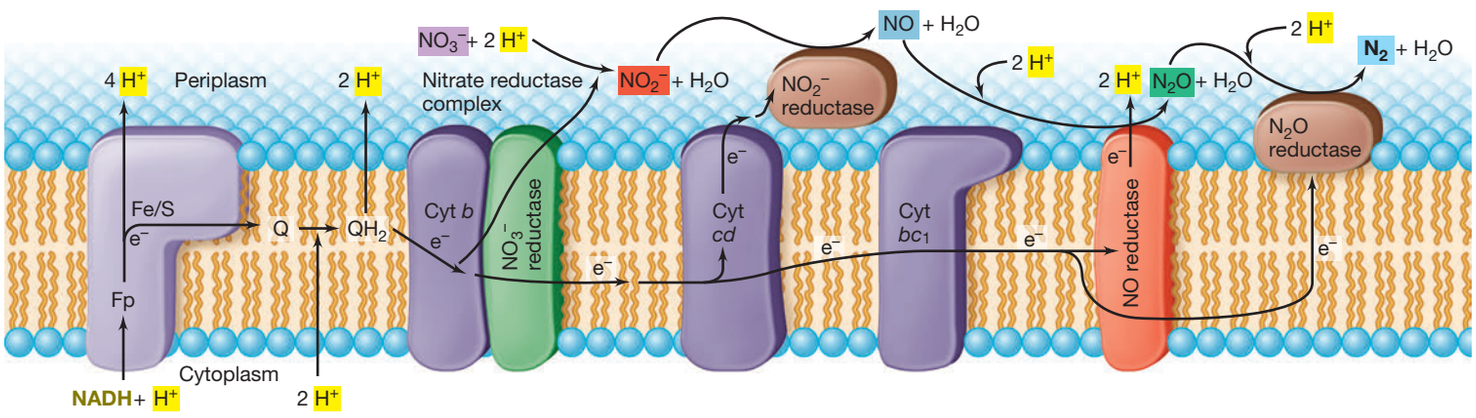


Figure 14.28 Denitrification. Scheme for electron transport in membranes of *Pseudomonas stutzeri* during denitrification. Nitrate and nitric oxide reductases are integral membrane proteins, whereas nitrite and nitrous oxide reductases are periplasmic enzymes. Fp, flavoprotein; Q, ubiquinone; Cyt, cytochrome.

14.12 Sulfate and Sulfur Reduction

Several inorganic sulfur compounds are important electron acceptors in anaerobic respiration, and **Table 14.4** lists the oxidation states of the key compounds. Sulfate (SO_4^{2-}), the most oxidized form of sulfur, is reduced by the *sulfate-reducing bacteria*, a highly diverse group of obligately anaerobic bacteria widely distributed in nature. The product of sulfate reduction is hydrogen sulfide, H_2S (which occurs mostly as HS^- at pH 7), an important and potentially toxic natural product that participates in many biogeochemical processes (▶ Sections 21.4, 22.6, and 22.7). Sulfate-reducing species in the genus *Desulfovibrio*, in particular *D. desulfuricans*, have been widely studied, and the general properties of sulfate-reducing bacteria are discussed in Section 15.11.

As was the case with nitrate metabolism (Section 14.11), it is necessary to distinguish between assimilative and dissimilative sulfate metabolism. Most microbes can incorporate sulfate for biosynthetic purposes to make cysteine, methionine, and many other organosulfur compounds; this is *assimilative* sulfate metabolism. By contrast, the ability to use sulfate as an electron acceptor for energy conservation requires its large-scale reduction and is restricted to the sulfate-reducing bacteria. H_2S is produced on a very large scale by these organisms and is excreted from the cell, free to be oxidized by air, used by other organisms, or combined with metals to form metal sulfides. Although a major electron donor to many microbes, an environmental concern of H_2S production is the fact that sulfide is highly toxic to higher organisms such as fish and wildlife. When anoxic conditions develop in sulfate-containing waters, the production of H_2S can cause die-offs of fish and other aquatic organisms and reduce water quality for recreational use or consumption.

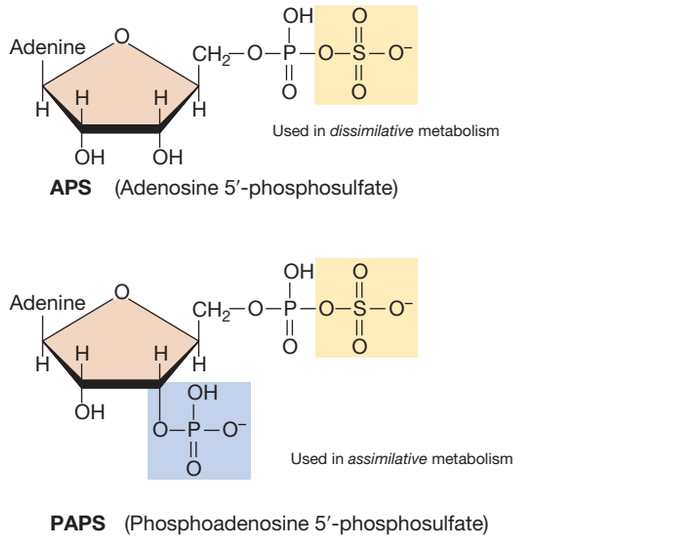
Biochemistry and Energetics of Sulfate Reduction

As shown in Figure 14.1, SO_4^{2-} is an energetically much less favorable electron acceptor than is O_2 or NO_3^-. Hydrogen (H_2) is used as an electron donor by virtually all species of sulfate reducers, whereas the use of organic electron donors is more restricted. For example, lactate and pyruvate are widely used by sulfate-reducing bacteria found in freshwater anoxic environments while acetate and longer-chain fatty acids are widely used by marine sulfate-reducing bacteria. Many morphological and physiological types of sulfate-reducing bacteria are known, and with the exception of *Archaeoglobus* (▶ Section 17.4), a genus of *Archaea*, all known sulfate reducers are *Bacteria* (▶ Section 15.11).

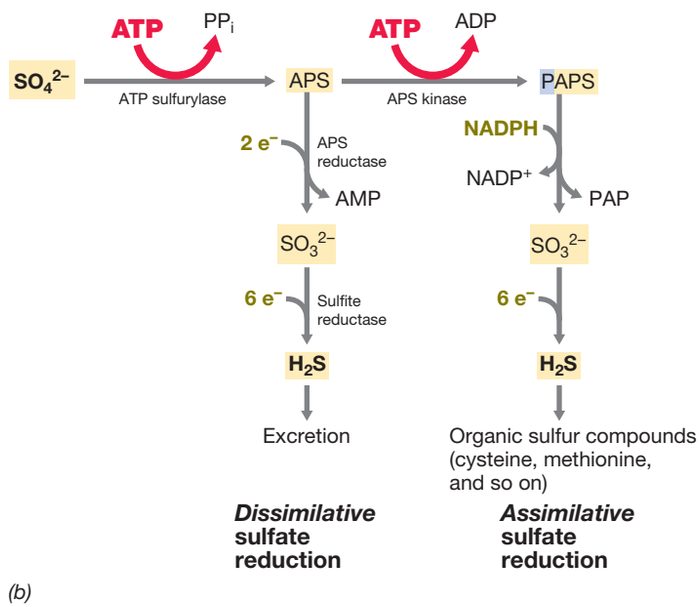
TABLE 14.4 Sulfur compounds for sulfate reduction

Compound	Oxidation state of S atom
Organic S (R—SH)	−2
Sulfide (H_2S)	−2
Elemental sulfur (S^0)	0
Thiosulfate (—S–SO_3^{2-})	−2/ + 6
Sulfur dioxide (SO_2)	+4
Sulfite (SO_3^{2-})	+4
Sulfate (SO_4^{2-})	+6

The reduction of SO_4^{2-} to H_2S requires eight electrons and proceeds through a number of intermediate stages. The reduction of SO_4^{2-} requires that it first be *activated* in a reaction requiring ATP. The enzyme ATP sulfurylase catalyzes the attachment of SO_4^{2-} to a phosphate of ATP, forming *adenosine phosphosulfate* (*APS*) as shown in **Figure 14.29*a***. Activation raises the extremely electronegative E_0' of the SO_4^{2-}/SO_3^{2-} couple (−0.52 V) to near 0 V, making reduction of the sulfate moiety possible with electron donors such as NADH (−0.32 V). In *dissimilative* sulfate reduction, the SO_4^{2-} in APS is reduced directly to sulfite (SO_3^{2-}) by the enzyme APS reductase with the release of AMP. In *assimilative* reduction, by contrast, a



(a)



(b)

Figure 14.29 Biochemistry of sulfate reduction: Activated sulfate. *(a)* Two forms of active sulfate can be made, adenosine 5′-phosphosulfate (APS) and phosphoadenosine 5′-phosphosulfate (PAPS). Both are derivatives of adenosine diphosphate (ADP), with the second phosphate of ADP being replaced by SO_4^{2-}. *(b)* Schemes of assimilative and dissimilative sulfate reduction.

second phosphate is added to APS to form *phosphoadenosine phosphosulfate* (*PAPS*) (Figure 14.29*a*), and only then is the SO_4^{2-} reduced. However, in both cases the product of sulfate reduction is sulfite (SO_3^{2-}), and once SO_3^{2-} is formed, it is reduced to H_2S by a sulfite reductase enzyme (Figure 14.29*b*).

Sulfate reduction offers only a very low free-energy yield and so organisms have developed a variety of mechanisms to optimize electron flow and energy conservation. During dissimilative sulfate reduction, electron transport reactions generate a proton motive force and this drives ATP synthesis by ATPase. *Cytochrome* c_3, a periplasmic low-potential cytochrome (**Figure 14.30**), is a major intermediate in these reactions. Cytochrome c_3 can accept electrons from several different periplasmic hydrogenase enzymes and can transfer these electrons to a number of different membrane-associated protein complexes. **Hydrogenases** are enzymes that perform redox reactions that produce or consume H_2. Sulfate reducers that use organic substrates typically do so by oxidizing these substrates to produce H_2 that then feeds into the electron transport chain by way of these periplasmic hydrogenases and cytochrome c_3 (Figure 14.30).

Dissimilative sulfate reduction requires several enzymes that perform *flavin-based electron bifurcation* (Section 14.1). For example, the reduction of APS to SO_3^{2-} requires the activity of the APS reductase complex (Figure 14.30). The APS reductase complex includes membrane-bound proteins that funnel electrons from a reduced quinone to a cytoplasmic enzyme that performs an electron confurcation reaction (Section 14.1) combining 2 e^- from reduced ferredoxin (Fd_{red}) and 2 e^- from the quinone to reduce 2 APS to 2 SO_3^{2-}. In addition, the dissimilative reduction of SO_3^{2-} to HS^- occurs in two steps. The first step is a two-electron transfer mediated by dissimilatory sulfite reductase (DsrAB); this reaction forms a heterodisulfide bond between S (from SO_3^{2-}) and the protein DsrC (Figure 14.30). The heterodisulfide is reduced in a four-electron transfer mediated by an electron-bifurcating heterodisulfide reductase (Hdr, reaction 4 in Figure 14.30). This Hdr reaction is similar to a key reaction in methanogenesis (see

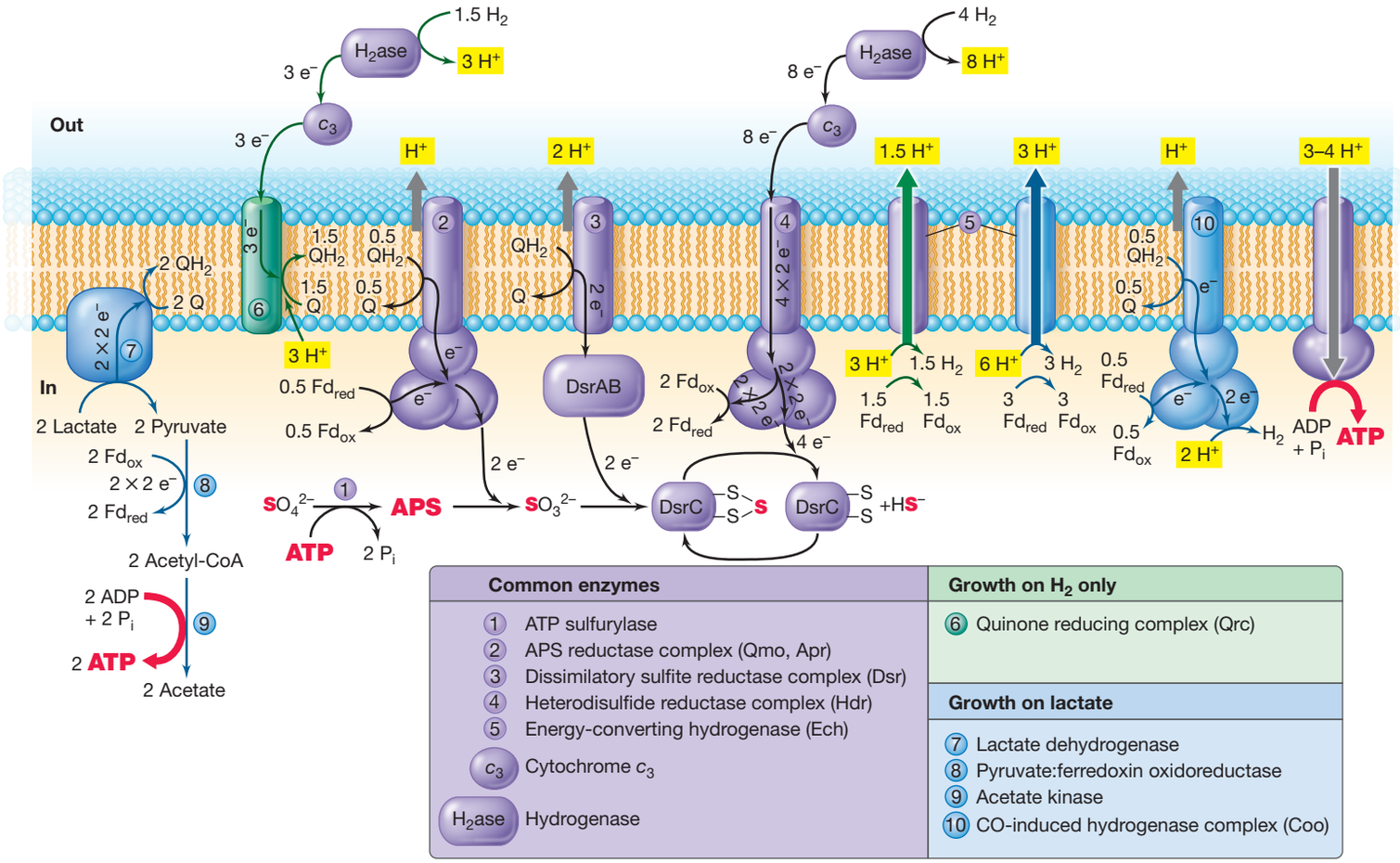


Figure 14.30 Electron transport and energy conservation in sulfate-reducing bacteria. H_2 serves as primary electron donor for sulfate reduction, and growth on organic compounds such as lactate result in the production of H_2 as an intermediate. The periplasmic cytochrome c_3 transfers electrons from a system of periplasmic hydrogenases (H_2ase) to membrane-bound proteins that mediate electron transfer reactions. Core components of electron transfer are shown in purple, and also shown are components required for growth exclusively on H_2 (in green) and on lactate (in blue). The scheme is proposed to include three bifurcating enzymes. Electron bifurcation at the APS reductase complex results in the donation of electrons to APS. Electron bifurcation at the heterodisulfide reductase complex (Hdr) results in the donation of electrons to Dsr-C and the release of HS^-. An electron confurcation at the CO-induced hydrogenase complex (Coo) contributes to redox balance during growth on lactate. Energy-converting hydrogenases (see Section 14.17) contribute to energy conservation in addition to quinone cycling.

Section 14.15 and Figure 14.38). These cells also balance electron flow using an **energy-converting hydrogenase**, a membrane-bound hydrogenase enzyme that uses the exergonic reduction of protons by Fd_{red} to generate a pmf (Figure 14.30). This reaction has a standard free-energy change ($\Delta G^{0\prime}$) close to zero, but under natural conditions, the ΔG is typically exergonic. A net of about two ATP is produced for each SO_4^{2-} reduced to HS^- by H_2, and the reaction is

$$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O$$
$$\Delta G^{0\prime} = -152\ kJ$$

Oxidation of organic compounds, such as lactate, is used to produce H_2 through energy-converting hydrogenases or by electron confurcation (Figure 14.30). This H_2 crosses the cytoplasmic membrane and is oxidized by the periplasmic hydrogenase to electrons—which are fed back into the system—and protons, which establish the proton motive force (Figure 14.30). When lactate or pyruvate is the electron donor, ATP is produced not only from pmf but also by substrate-level phosphorylation during the oxidation of pyruvate to acetate plus CO_2 (reaction 9 in Figure 14.30). Many strains are ultimately unable to further oxidize acetate and so while some acetate is assimilated during biosynthetic reactions, the remainder is excreted from the cell.

Marine but not freshwater species of sulfate-reducing bacteria can couple sulfate reduction and the oxidation of acetate (and longer-chain fatty acids) to CO_2:

$$CH_3COO^- + SO_4^{2-} + 3\,H^+ \rightarrow 2\,CO_2 + H_2S + 2\,H_2O$$
$$\Delta G^{0\prime} = -57.5\ kJ$$

The mechanism for acetate oxidation in most of these species is the *reductive acetyl-CoA pathway*, a series of reversible reactions used by many anaerobes for acetate synthesis or acetate oxidation (Section 14.14). A few sulfate-reducing bacteria can also grow autotrophically with H_2. Under these conditions, the organisms use the reductive acetyl-CoA pathway for making acetate as a carbon source. Such species can be cultured in a completely organic-free medium containing only mineral salts, sulfate, CO_2, and H_2.

Special Metabolisms of Sulfate-Reducing Bacteria

Certain species of sulfate-reducing bacteria can catalyze reactions not characteristic of all species. These include *sulfur reduction*, *disproportionation*, and even *phosphite oxidation*. Most sulfate-reducing bacteria can also conserve energy from the reduction of elemental sulfur to sulfide ($S^0 + 2\,H \rightarrow H_2S$). The electrons for sulfur reduction come from H_2 or any of a number of organic compounds. For example, *Desulfuromonas acetoxidans* can oxidize acetate or ethanol to CO_2 coupled to the reduction of S^0 to H_2S.

Many microbes can reduce elemental sulfur but not sulfate in anaerobic respiration. These are the *sulfur reducers*, a diverse group of *Bacteria* and *Archaea* that often coexist with sulfate-reducing bacteria in anoxic, sulfur-rich habitats. Sulfur reducers lack the capacity to activate sulfate to APS (Figure 14.29), and presumably this is what excludes them from using SO_4^{2-} as an electron acceptor.

Disproportionation is a process in which one molecule of a substance is oxidized while a second molecule is reduced, ultimately forming two different products. For example, *Desulfovibrio sulfodismutans* can disproportionate thiosulfate ($S{-}SO_3^{2-}$) as follows:

$$S{-}SO_3^{2-} + H_2O \rightarrow H_2S + SO_4^{2-} \quad \Delta G^{0\prime} = -21.9\ kJ/reaction$$

Note that in this reaction, the right-hand sulfur atom of $S{-}SO_3^{2-}$ is oxidized (forming SO_4^{2-}), while the left-hand atom is reduced (forming H_2S). The free energy available from the oxidation of thiosulfate by *D. sulfodismutans* is insufficient to couple to substrate-level phosphorylation and so instead is coupled to a proton "pump" that uses the minimal energy available in the reaction to establish a proton motive force. Other partially reduced sulfur compounds such as sulfite (SO_3^{2-}) and sulfur (S^0) can also be disproportionated.

At least one sulfate-reducing bacterium can couple phosphite (HPO_3^-) oxidation to SO_4^{2-} reduction. This bacterium, *Desulfotignum phosphitoxidans*, is an autotroph and also a strict anaerobe. The chemolithotrophic reaction carried out by *D. phosphitoxidans* yields phosphate and sulfide:

$$4\,HPO_3^- + SO_4^{2-} + H^+ \rightarrow 4\,HPO_4^{2-} + HS^- \quad \Delta G^{0\prime} = -364\ kJ$$

The natural sources of phosphite are likely to be organophosphorous compounds called *phosphonates* that are generated from the anoxic degradation of nucleic acids, phospholipids, and other cellular sources. Along with sulfur respiration, sulfur disproportionation (also a chemolithotrophic process), and H_2 utilization, phosphite oxidation underscores the diversity of metabolic reactions carried out by sulfate-reducing bacteria.

Check Your Understanding

- What mechanisms do sulfate reducers use to balance electron flow and conserve energy when growing on organic substrates such as lactate?
- Why is cytochrome c_3 so important for energy conservation in sulfate-reducing bacteria?
- What difference in metabolism allows some sulfate reducers to oxidize organic carbon substrates all the way to CO_2 while others stop at acetate?

14.13 Other Electron Acceptors

Bacteria can perform a wide diversity of anaerobic respirations (Figure 14.1) including the respiration of several metals, metalloids, and associated minerals. In addition, certain organic and halogenated compounds can also serve as important electron acceptors in anaerobic respiration. We consider these forms of anaerobic respiration here.

Metal and Metalloid Reduction

A variety of metals, metalloids (elements such as arsenic, As, whose properties are intermediate between those of a metal and a nonmetal), and associated minerals can be reduced through anaerobic respiration. Ferric iron (Fe^{3+}) and manganic ion (Mn^{4+}) are the most common such electron acceptors, but other possible electron acceptors include As^{5+}, Co^{3+}, Cr^{6+}, Se^{6+}, Tc^{7+}, Te^{6+}, U^{6+}. The reduction potential of the Fe^{3+}/Fe^{2+} couple is +0.2 V (at pH 7), and that of the Mn^{4+}/Mn^{2+} couple is +0.8 V; thus, several electron donors can couple to Fe^{3+} and Mn^{4+} reduction. A major challenge for metal-reducing organisms is that oxidized minerals of Fe and Mn are often insoluble, making transport into the cell difficult if not impossible. Hence, cells performing metal respiration need a mechanism for exporting electrons outside of the cell to allow the reduction to occur on the outer cell surface.

A number of different systems have evolved in diverse bacteria for reducing extracellular insoluble substrates. One well-studied example is *Geobacter sulfurreducens*, a gram-negative bacterium that can couple the oxidation of H_2, acetate, or other organic electron donors to the reduction of a number of different metals and minerals (▶ Section 15.13). *G. sulfurreducens* can oxidize acetate using the citric acid cycle (◀ Section 3.6), and electrons make their way to electron transport by way of NADH. The electrons pass through quinones and are used to generate a pmf before reducing *c*-type cytochromes in the periplasm. These periplasmic cytochromes transfer electrons to a transmembrane porin–cytochrome complex embedded in the outer membrane. Finally, the electrons are transferred to specialized type IV pili (◀ Section 2.6) that are electrically conductive (**Figure 14.31**). These electrically conductive type IV pili are called **nanowires** because they function like electrical wires. The pili are enriched in aromatic amino acids that function to conduct electrons. Attached to the pili are multiheme *c*-type cytochromes that function as an adaptor to allow electrons to flow out of the pili and into an insoluble terminal electron acceptor, such as ferrihydrite (Fe_2O_3).

Other inorganic substances can function as electron acceptors for anaerobic respiration, including the metalloids selenium, tellurium, and arsenic, and various oxidized chlorine compounds (some of these are shown in Figure 14.1). For example, the sulfate-reducing bacterium *Desulfotomaculum auripigmentum* can reduce AsO_4^{3-} to AsO_3^{3-}, and sulfate to sulfide, precipitating the yellow mineral orpiment (As_2S_3) in the process (**Figure 14.32**). This process is an example of *biomineralization*, the formation of a mineral by bacterial activity. The presence of soluble forms of arsenic in groundwater is a major problem in many parts of the world and so microbes that convert arsenic from a soluble to an insoluble form provide a means of detoxifying contaminated habitats. Metal- and metalloid-reducing bacteria are of great importance in groundwater chemistry, and their metabolic activities are often used to remediate contaminated sites (▶ Section 22.3).

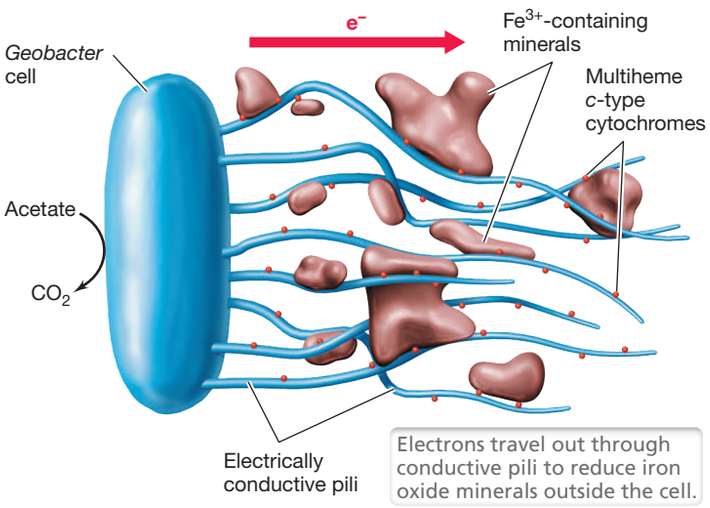


Figure 14.31 Iron-reducing bacteria reduce extracellular electron acceptors. Iron oxides are insoluble and cannot be transported into the cell. The bacterium *Geobacter* makes electrically conductive type IV pili (nanowires) that transfer electrons out of the cell where they reduce oxidized iron minerals.

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Figure 14.32 Biomineralization during arsenate reduction by the sulfate-reducing bacterium *Desulfotomaculum auripigmentum*. Left, appearance of culture bottle after inoculation. Right, following growth for 2 weeks and biomineralization of arsenic trisulfide, As_2S_3, also called orpiment. Center, synthetic sample of As_2S_3.

Organic and Halogenated Electron Acceptors

Certain organic compounds can be electron acceptors in anaerobic respirations. Of those listed in Figure 14.1, the compound that has been most extensively studied is *fumarate*, a citric acid cycle intermediate that is reduced to succinate; the reaction in this anaerobic respiration is the opposite of the reaction that takes place in the citric acid cycle aerobically (◀ Figure 3.12). The role of fumarate as an electron acceptor for anaerobic respiration derives from the fact that the fumarate/succinate couple has a reduction potential near 0 V, which allows coupling of fumarate reduction to the oxidation of NADH, FADH, or H_2. Many facultatively aerobic bacteria can grow anaerobically on fumarate as electron acceptor, including *Escherichia coli*.

Trimethylamine oxide (TMAO) and dimethyl sulfoxide (DMSO) (Figure 14.1) are also important organic electron acceptors. TMAO is a product of marine fish, and several bacteria can reduce it to trimethylamine (TMA), which has a strong odor and flavor (the odor of spoiled seafood is due primarily to TMA produced by bacterial action). Dimethyl sulfoxide (DMSO), which is reduced to dimethyl sulfide (DMS), is a common natural product and is found in both marine and freshwater environments. The reduction potentials of the TMAO/TMA and DMSO/DMS couples are about the same (near +0.15 V, Figure 14.1) and so the electron transport chains that terminate with TMAO or DMSO reductases typically contain cytochromes of the *b* type.

Several halogenated organic compounds function as electron acceptors in **reductive dechlorination** (also called *dehalorespiration*). For example, the sulfate-reducing bacterium *Desulfomonile* grows anaerobically with H_2 or organic electron donors and chlorobenzoate as an electron acceptor that is reduced to benzoate and hydrochloric acid (HCl):

$$C_7H_4O_2Cl^- + 2\,H \rightarrow C_7H_5O_2^- + HCl$$

Several other anaerobic bacteria can reductively dechlorinate, and some specialize in the use of chlorinated compounds as electron acceptors for anaerobic respiration. For example, *Dehalobacter* and *Dehalococcoides* oxidize H_2 and reduce tetrachloroethylene (TCE) to dichloroethylene and ethene, respectively. *Dehalococcoides* can also

reduce polychlorinated biphenyls (PCBs). TCE and PCBs are widespread organic pollutants that seep into groundwater and freshwater environments from underground storage tanks, leaking transformers, and other human inputs. However, removal of the chlorine groups from these molecules greatly reduces their toxicity and hence reductive dechlorination is not only a form of anaerobic respiration but also an environmentally significant process of bioremediation (▶ Sections 22.3–22.5).

We now explore the fascinating metabolisms of species of *Bacteria* and *Archaea* that specialize in the transformations of compounds that contain only one carbon: the C_1 metabolizers.

Check Your Understanding

- With H_2 as electron donor, why is reduction of Fe^{3+} a more favorable reaction than reduction of fumarate?
- Explain two different processes of anaerobic respiration that can be used to remediate contaminated environments.
- How can bacteria transfer electrons to an insoluble extracellular electron acceptor?

V • One-Carbon (C_1) Metabolism

Acetogenesis and methanogenesis are energy-conserving metabolisms that exploit exergonic reductions of CO_2 to form acetate and methane, respectively. Methanotrophs oxidize CH_4 while methylotrophs oxidize C_1 compounds other than CH_4.

Carbon dioxide (CO_2) and methane (CH_4) are abundant in many anoxic habitats, and a wide diversity of microbes have evolved metabolic pathways that conserve energy from either the reduction of CO_2 or the oxidation of CH_4. A number of the enzymatic reactions in one-carbon metabolisms are unique. In this part of the chapter, we consider the metabolism of organisms that either consume or produce C_1 compounds, highlighting the major similarities and differences.

14.14 Acetogenesis

Two major groups of strictly anaerobic microbes use CO_2 as an electron acceptor for energy conservation. One of these is the *acetogens*, and we discuss them here. The other group, the *methanogens*, are considered in the next section. Hydrogen (H_2) is a major electron donor for both groups of organisms, and an overview of their energy metabolism, **acetogenesis** and **methanogenesis**, is shown in Figure 14.33. Both processes are linked to ion pumps, of either protons (H^+) or sodium ions (Na^+), as the mechanism of energy conservation, and these pumps fuel ATP synthases in the membrane. The pathway of acetogenesis also conserves energy in a substrate-level phosphorylation reaction.

Organisms and Pathway

Acetogens carry out the reaction

$$4\,H_2 + H^+ + 2\,HCO_3^- \rightarrow CH_3COO^- + 4\,H_2O \quad \Delta G^{0\prime} = -105\ \text{kJ}$$

In addition to H_2, electron donors for acetogenesis include various C_1 compounds such as methanol, several methoxylated aromatic

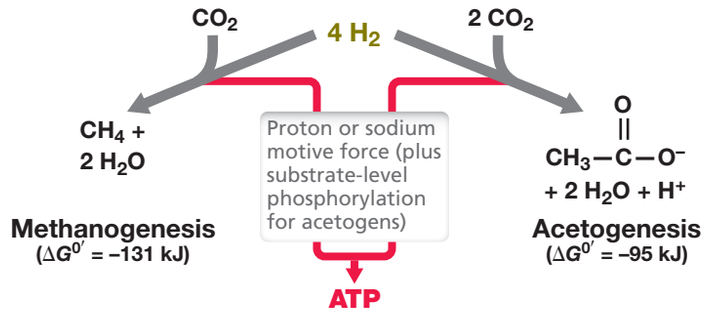


Figure 14.33 The contrasting processes of methanogenesis and acetogenesis. Note the difference in free energy released in the reactions.

compounds, sugars, organic and amino acids, alcohols, and certain nitrogen bases, depending on the organism. Many acetogens can also grow by fermentation or by the anaerobic respiration of nitrate (NO_3^-) or thiosulfate ($S_2O_3^{2-}$). However, CO_2 reduction is a major feature of the acetogenic lifestyle, with CO_2 serving both as electron acceptor and as an intermediate through which carbon is assimilated into cellular material.

A major unifying thread among acetogens is the pathway of CO_2 reduction. Acetogens reduce CO_2 to acetate by the reductive acetyl-CoA pathway (also called the *Wood–Ljungdahl pathway*), the major pathway in obligate anaerobes for CO_2 fixation and the production of acetate (Section 14.2). The reactions of the reductive acetyl-CoA pathway are reversible, and some microbes reverse this pathway to oxidize acetate.

Acetogens such as *Acetobacterium woodii* and *Clostridium aceticum* can grow either chemoorganotrophically by fermentation of sugars (reaction A) or chemolithotrophically and autotrophically through the reduction of CO_2 to acetate with H_2 as electron donor (reaction B). In either case, the sole organic product is *acetate*:

$$\text{(A)} \qquad C_6H_{12}O_6 \rightarrow 3\,CH_3COO^- + 3\,H^+$$

$$\text{(B)} \qquad 2\,HCO_3^- + 4\,H_2 + H^+ \rightarrow CH_3COO^- + 4\,H_2O$$

When growing on glucose, acetogens use glycolysis (◀ Figure 3.11) to oxidize the glucose into two molecules of pyruvate and two molecules of NADH. The pyruvate is then further oxidized to produce two molecules of acetate:

$$\text{(C)} \qquad 2\ \text{Pyruvate}^- \rightarrow 2\ \text{acetate}^- + 2\,CO_2 + 2\,\text{NADH}$$

The CO_2 generated in reaction (C) is then used as a terminal electron acceptor in the reductive acetyl-CoA pathway. The NADH generated during glycolysis and pyruvate oxidation is used as an electron donor in CO_2 reduction. Starting from pyruvate, then, the overall production of acetate can be written as

$$2\ \text{Pyruvate}^- + 4\,H \rightarrow 3\ \text{acetate}^- + H^+$$

Most acetogenic bacteria that produce acetate in energy metabolism are gram-positive *Bacteria*, and many are species of the genera *Clostridium* or *Acetobacterium*. A few other gram-positive and many different gram-negative *Bacteria* and *Archaea* use the reductive acetyl-CoA pathway for autotrophic purposes, reducing CO_2 to acetate as a source of cell carbon; these include autotrophic sulfate-reducing bacteria (Section 14.12), anammox bacteria (Section 14.10), and

methanogens (Section 14.15). And finally, some microbes run the acetyl-CoA pathway in the *reverse direction* as a means of oxidizing acetate to CO_2; these include acetate-utilizing methanogens and sulfate-reducing bacteria. The acetyl-CoA pathway is thus a metabolically highly versatile series of reactions.

The Reductive Acetyl-CoA Pathway and Energy Conservation in Acetogenesis

Unlike other autotrophic pathways (Section 14.2), the reductive acetyl-CoA pathway of CO_2 fixation is not a cycle. Instead, it catalyzes the reduction of CO_2 along two linear pathways, with one molecule of CO_2 being reduced to the methyl group of acetate (the methyl branch of the pathway) and the other to the carbonyl group of acetate (the carbonyl branch of the pathway). These two C_1 units are then combined to form acetyl-CoA (**Figure 14.34**).

A key enzyme of the acetyl-CoA pathway is *carbon monoxide* (*CO*) *dehydrogenase*. CO dehydrogenase contains Ni, Zn, and Fe as cofactors and catalyzes the reaction

$$CO_2 + H_2 \rightarrow CO + H_2O$$

The CO produced by CO dehydrogenase ends up as the *carbonyl* carbon of acetate (reaction 1 in Figure 14.34). The methyl group of acetate originates from the reduction of CO_2 by a series of reactions in which the coenzyme *tetrahydrofolate* (THF) plays a major role (Figure 14.34). The methyl group is then transferred from tetrahydrofolate to a cobalt- and iron-containing *corrinoid iron–sulfur protein* (CoFeSP) coenzyme. In the final step of the pathway, the methyl group is combined with CO by the activity of both CO dehydrogenase and acetyl-CoA synthase to form acetyl-CoA. Conversion of acetyl-CoA to acetate by the combined activities of the enzymes phosphotransacetylase and acetate kinase is the last step in the pathway, generating one ATP by substrate-level phosphorylation (Figure 14.34; see Table 14.5). However, this ATP is consumed in the first step of the acetyl-CoA pathway. Considering this, how then do acetogens get their ATP?

Acetogens conserve energy by the generation of an ion motive force. In *Acetobacterium woodii*, the best-studied acetogen, the ion motive force is generated by activity of the *Rnf complex*; this enzyme uses reduced ferredoxin (Fd_{red}) as electron donor and NAD^+ as electron acceptor. The Rnf complex pumps one Na^+ across the membrane for each electron exchanged, thereby generating a Na^+ motive force, which can be used to make ATP using a Na^+-dependent ATP synthase (Figure 14.34). Alternatively, other acetogens such as *Clostridium ljungdahlii* use a proton motive force instead of a sodium

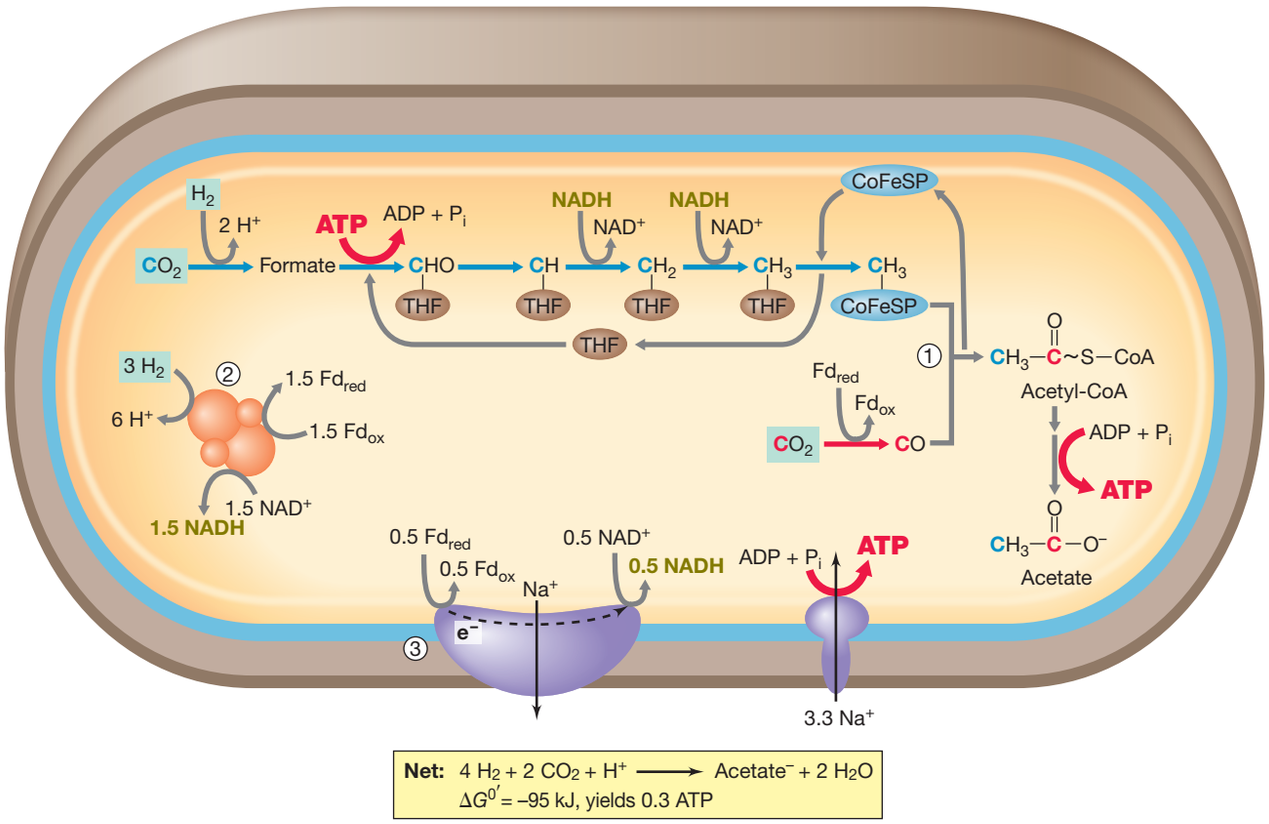


Figure 14.34 Reactions of acetogenesis from H_2 and CO_2 in *Acetobacterium woodii*. The reductive acetyl-CoA pathway is used to reduce CO_2 to acetate. The pathway has a methyl branch (blue arrows) and a carbonyl branch (red arrows). Carbon monoxide from the carbonyl branch and a methyl group from the methyl branch are combined into acetyl-CoA by carbon monoxide dehydrogenase ①, a key enzyme for the pathway. Note that the reductive acetyl-CoA pathway itself does not conserve energy. An electron-bifurcating hydrogenase ② (see also Figure 14.2) is used to reduce ferredoxin (Fd_{red}), and energy is conserved at the Rnf complex ③, which generates a Na^+ motive force. THF, tetrahydrofolate; CoFeSP, Co/Fe-containing corrinoid iron–sulfur protein.

motive force. The Fd_{red} required by the Rnf cluster is provided by a flavin-based electron bifurcation reaction (Figure 14.2) in which the oxidation of 2 H_2 is coupled to the reduction of one Fd_{ox} and one NAD^+ (Figure 14.34). This bifurcation reaction is critical for producing the reduced ferredoxin required to conserve energy during acetogenic growth on H_2. Ultimately, only 0.3 ATP are produced for every 4 H_2 and 2 CO_2 that are consumed by the acetogens, making these organisms minimalists in terms of the energy that can be conserved from their metabolism.

As we have seen, acetogens start with a C_1 or C_6 compound and make a C_2 product in acetate. We now examine methanogens, whose metabolism starts with a C_1 or C_2 (or rarely a C_3) compound and makes a C_1 product, natural gas (CH_4).

Check Your Understanding

- What is the purpose of CO dehydrogenase?
- If acetogens conserve energy using the Rnf complex, then what is the purpose of the reductive CoA pathway?
- What is electron bifurcation and what role does it play in acetogens?

14.15 Methanogenesis

UNIT 4

The biological production of methane—*methanogenesis*—is catalyzed by a diverse assemblage of strictly anaerobic *Archaea* called the **methanogens**. These organisms are common in freshwater sediments and wetlands (**Figure 14.35**), the guts of many animals including humans, sewage sludge digesters (▶ Section 22.8), and other bioreactors. The reduction of CO_2 by H_2 to form CH_4 is a major pathway of methanogenesis and is a form of anaerobic respiration. We consider the basic properties, phylogeny, and taxonomy of the methanogens in Section 17.2. Here we focus on their bioenergetics and unique biochemistry.

Mastering Microbiology
Art Activity: Figure 14.47 Methanogenesis from CO_2 plus H_2

John A. Breznak

Figure 14.35 Methanogenesis. Methane is collected in a funnel from swamp sediments where it was produced by methanogens and then ignited in a demonstration experiment at Woods Hole, Massachusetts (USA).

C_1 Carriers in Methanogenesis

Methanogenesis from CO_2 requires eight electrons, and these electrons are added two at a time. This leads to intermediary oxidation states of the carbon atom from +4 (CO_2) to −4 (CH_4). Several novel coenzymes participate in methanogenesis and can be divided into two classes: (1) those that carry the C_1 unit along its path of enzymatic reduction (*C_1 carriers*) and (2) those that donate electrons (*redox coenzymes*) (**Figure 14.36**). We consider the C_1 carriers first.

The coenzyme *methanofuran* (MF) is required for the first step of methanogenesis. Methanofuran contains the five-membered furan ring and an amino nitrogen atom that binds CO_2 (Figure 14.36*a*). *Methanopterin* (MP, Figure 14.36*b*, also called *tetrahydromethanopterin*) is a methanogenic coenzyme that resembles the vitamin folic acid and plays a role analogous to that of tetrahydrofolate (see Figure 14.34) by carrying the C_1 unit in the intermediate steps of CO_2 reduction to CH_4. *Coenzyme M* (CoM) (Figure 14.36*c*) is required for the terminal step of methanogenesis, the reduction of the methyl group ($-CH_3$) to CH_4. Although not a C_1 carrier, the nickel (Ni^+)-containing tetrapyrrole *coenzyme F_{430}* (Figure 14.36*d*) also participates in the terminal step of methanogenesis as part of the methyl reductase enzyme complex (discussed later).

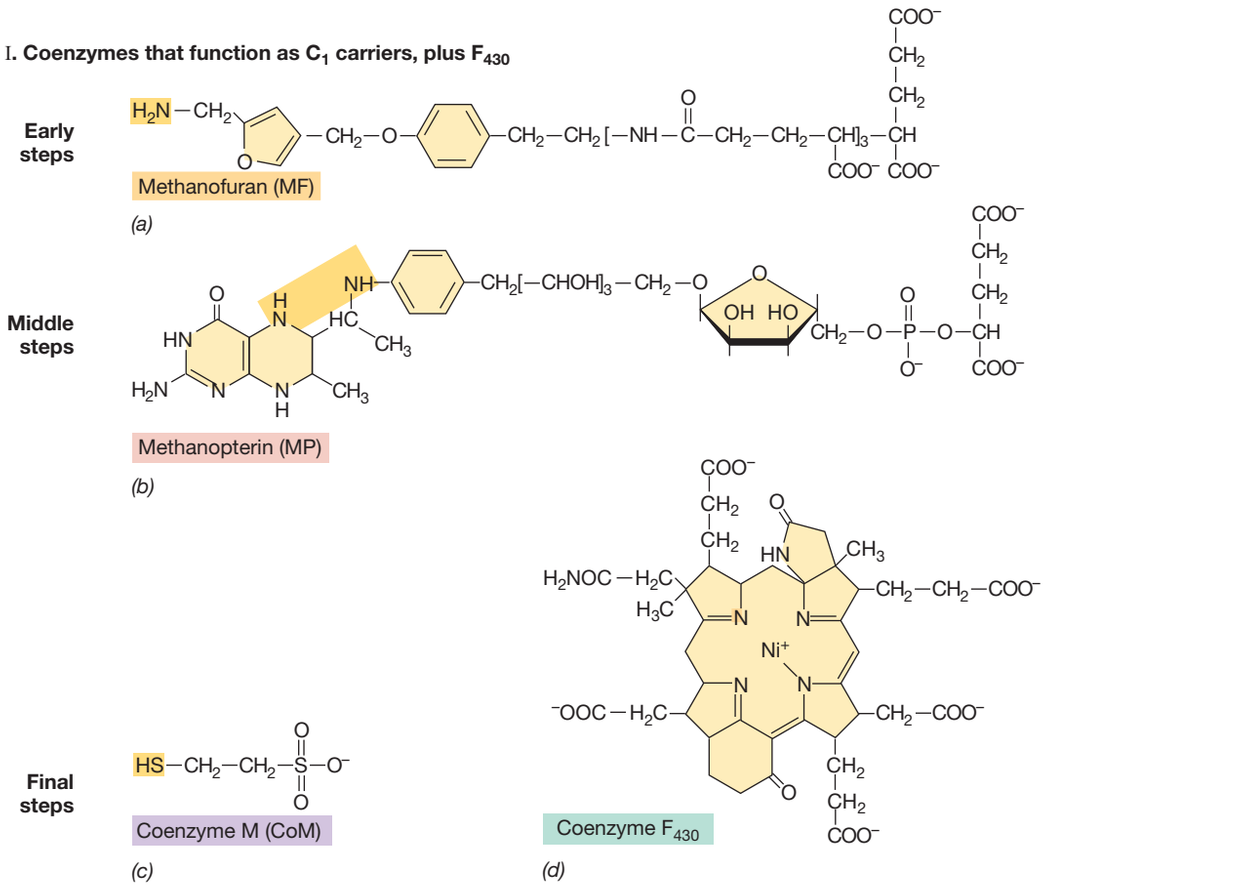
Redox Coenzymes

The coenzymes F_{420} and *7-mercaptoheptanoylthreonine phosphate* (also called coenzyme B, CoB) are electron donors in methanogenesis. Coenzyme F_{420} (Figure 14.36*e*) is a flavin derivative, structurally resembling the flavin coenzyme FMN (◀ Figure 3.15). F_{420} participates in methanogenesis as the electron donor in several steps of CO_2 reduction (see Figure 14.38). Coenzyme F_{420} takes its name from the fact that its oxidized form absorbs light at 420 nm and fluoresces blue-green. Such fluorescence is useful for the microscopic identification of a methanogen (**Figure 14.37**). CoB is required for the terminal step of methanogenesis catalyzed by the *methyl reductase enzyme complex*. As shown in Figure 14.36*f*, the structure of CoB resembles the vitamin pantothenic acid, which is part of acetyl-CoA (◀ Figure 3.8).

Methanogenesis from CO_2 + H_2

Nearly all methanogens can use H_2 as the source of electrons for the reduction of CO_2 to CH_4. Some can also use a few additional substrates including acetate, formate, methanol, and methylamines. Methanogens use the reductive acetyl-CoA pathway (Sections 14.2 and 14.14) to assimilate CO_2 into cellular material. With respect to energy conservation, two major groups of methanogens are differentiated by the presence or absence of cytochromes. We will first consider methanogenesis from H_2 and CO_2 in methanogens that lack cytochromes (**Figure 14.38**):

1. CO_2 is activated by a methanofuran-containing enzyme and reduced to the formyl (CHO) level. The immediate electron donor is ferredoxin (Fd_{red}), a strong reductant with a reduction potential (E_0') of about −0.45 V.



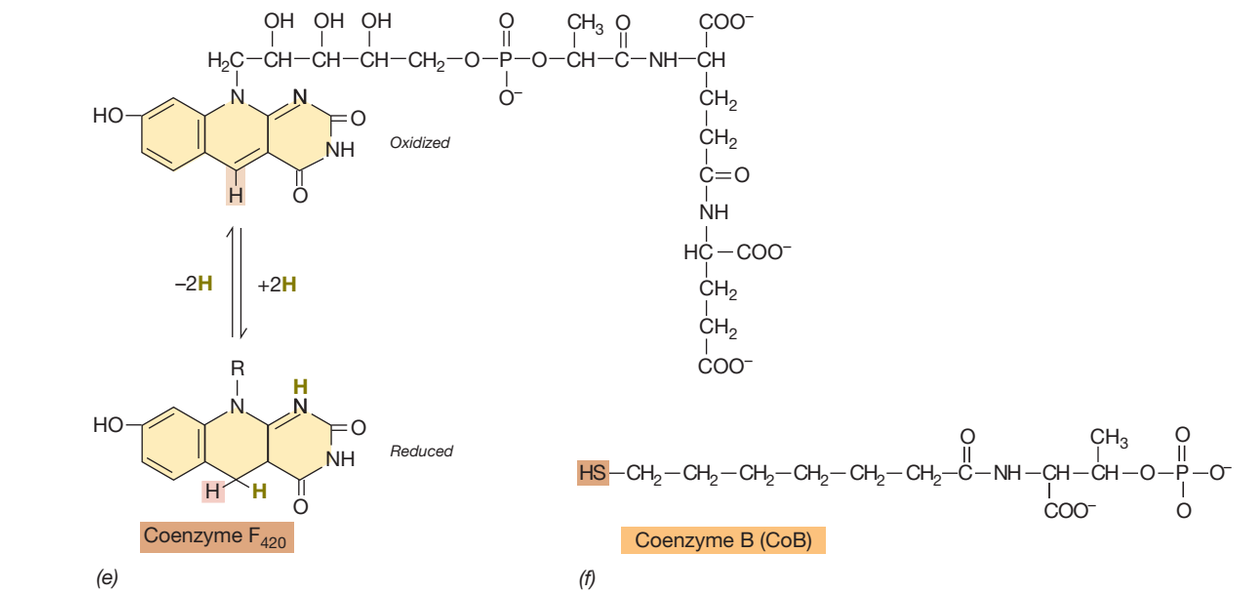


Figure 14.36 **Coenzymes of methanogenesis.** The atoms shaded in brown or yellow are the sites of oxidation–reduction reactions (brown in F_{420} and CoB) or the position to which the C_1 moiety is attached during the reduction of CO_2 to CH_4 (dark yellow in methanofuran, methanopterin, and coenzyme M). The same colors used to highlight a particular coenzyme name (CoB is orange, for example) are also used in Figures 14.38 and 14.39 to follow the reactions in each figure. Coenzyme F_{430} participates in the terminal step of methanogenesis catalyzed by the enzyme methyl reductase, with the methyl group binding to Ni^+ in F_{430} prior to its reduction to CH_4.

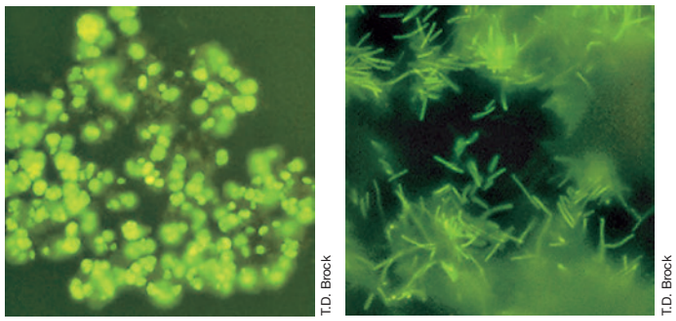


Figure 14.37 Fluorescence due to the methanogenic coenzyme F_{420}. *(a)* Autofluorescence in cells of the methanogen *Methanosarcina barkeri* due to the presence of the unique electron carrier F_{420}. A single cell is about 1.7 μm in diameter. The organisms were made visible by excitation with blue light in a fluorescence microscope. *(b)* F_{420} fluorescence in cells of the methanogen *Methanobacterium formicicum*. A single cell is about 0.6 μm in diameter.

2. The formyl group is transferred from methanofuran (MF) to an enzyme containing methanopterin (MP). It is subsequently dehydrated and reduced in two separate steps (total of 4 H) to the methylene and methyl levels. The 2 H required for each of these reductions are derived from H_2 and transferred by F_{420}.
3. The methyl group is transferred from MP to an enzyme containing CoM by the enzyme methanopterin:coenzyme M methyltransferase. This highly exergonic reaction results in energy conservation because it drives 2 Na^+ across the membrane, resulting in the formation of a sodium motive force.
4. Methyl-S-CoM is reduced to CH_4 by methyl-coenzyme M reductase. In this reaction, F_{430} and CoB are required. Coenzyme F_{430} removes the CH_3 group from CH_3-S-CoM, forming a Ni^+-CH_3 complex. This complex is reduced by CoB, generating CH_4 and a heterodisulfide bond between CoM and CoB (CoM-S—S-CoB).

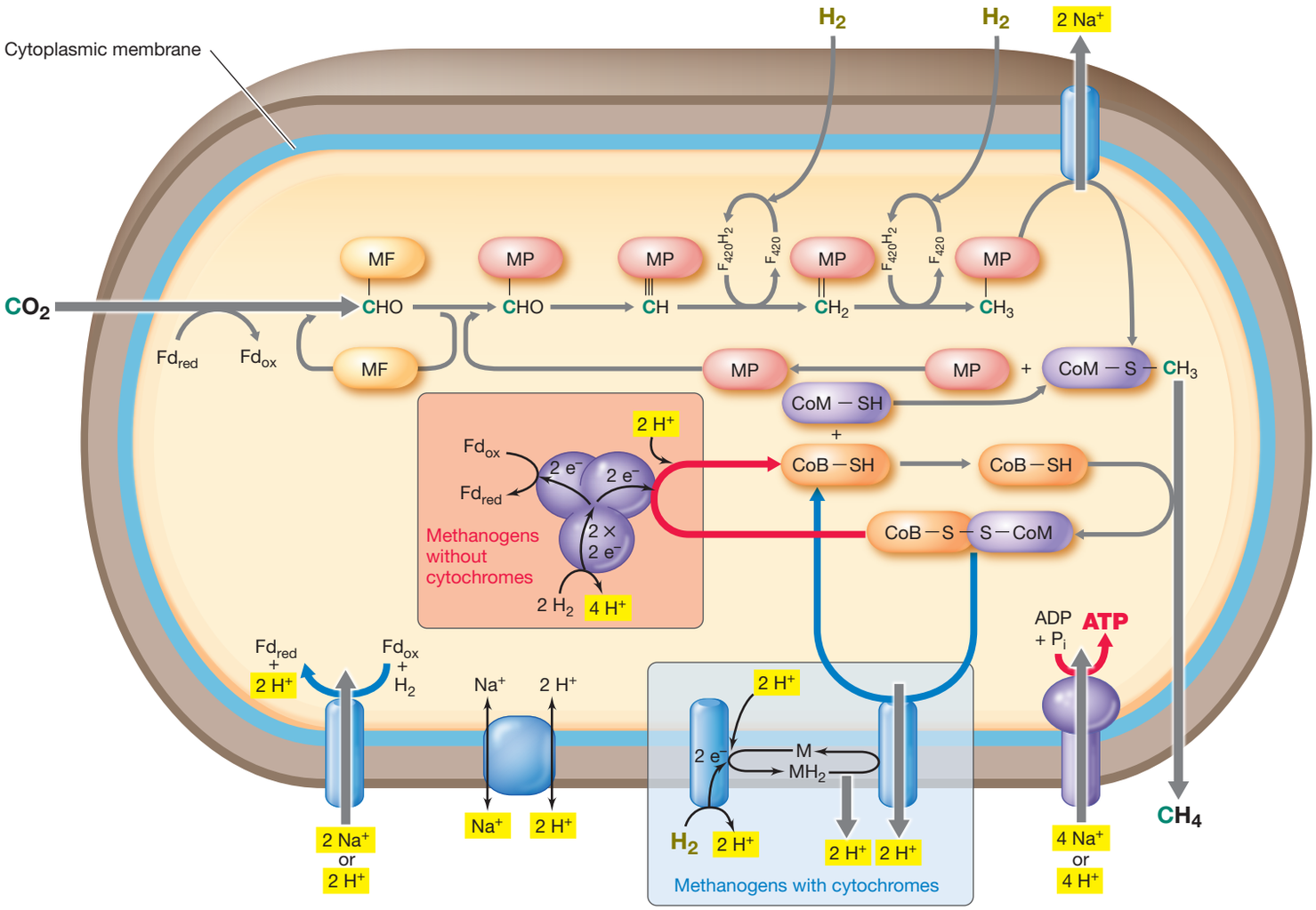


Figure 14.38 Methanogenesis from CO_2 plus H_2. The carbon atom reduced is highlighted in green, and the source of electrons is highlighted in brown. See Figure 14.36 for the structures of the coenzymes. Methanogens without cytochromes reduce the heterodisulfide bond in CoM-S-S-CoB using an electron bifurcation reaction (red box and reaction arrow) that also generates reduced ferredoxin (Fd_{red}). Methanogens with cytochromes conserve more energy by using CoM-S-S-CoB as the electron acceptor in electron transport (blue box and arrow). MF, methanofuran; MP, methanopterin; CoM, coenzyme M; $F_{420}H_2$, reduced coenzyme F_{420}; F_{430}, coenzyme F_{430}; Fd, ferredoxin; CoB, coenzyme B; M, methanophenazine.

5. Free CoM and CoB are regenerated by the reduction of CoM-S—S-CoB ($E_0' = -0.14$) catalyzed by heterodisulfide reductase through a flavin-based electron-bifurcation reaction (see red-shaded region in Figure 14.38; see also Figure 14.2) with H_2 (−0.42 V) as the electron donor. The high reduction potential of CoM-S—S-CoB is used to drive electrons in the bifurcation reaction to reduce ferredoxin. The Fd_{red} so generated is then used for CO_2 reduction in the first step of the pathway.

Under standard conditions, the free energy of methanogenesis from $H_2 + CO_2$ is −131 kJ/mol; however, the free energy available under natural conditions is likely in the range of −17 to −40 kJ/mol. In methanogens that lack cytochromes, a sodium motive force drives ATP formation by activity of a Na^+-linked ATPase. The heterodisulfide reductase reaction produces a net of two H^+ in the cytoplasm and these are exchanged for one Na^+ by an H^+:Na^+ antiporter in the membrane (Figure 14.38). Hence, the production of one CH_4 molecule from $H_2 + CO_2$ yields a net of one Na^+ ion pumped across the membrane, sufficient to form less than 0.5 ATP.

In methanogens containing cytochromes, H_2 is oxidized at a membrane-bound hydrogenase containing *b*-type cytochromes. This enzyme passes electrons to a membrane-associated electron carrier called *methanophenazine* (M, highlighted in blue in Figure 14.38). Methanophenazine in turn passes electrons to a membrane-bound heterodisulfide reductase complex that reduces CoM-S—S-CoB (Figure 14.38). The net result of these electron transport reactions is the generation of a pmf. Methanogens with cytochromes have H^+-linked ATPases in place of the Na^+-fueled ATPases found in many other methanogens. Since Fd_{red} is still required for CO_2 reduction, some of the pmf drives Fd_{red} formation using electrons from H_2 (Figure 14.38). The net result is that methanogens with cytochromes conserve more energy than those without, forming >1ATP per molecule of CH_4 formed from $H_2 + CO_2$.

In methanogens we thus see at least two mechanisms for energy conservation that have different energy yields: (1) a sodium motive force (which can be converted to a proton motive force) linked to the methyl transferase reaction, and (2) a proton motive force linked to cytochrome-dependent electron transport reactions. In addition to these energetic differences, cytochrome-containing methanogens are more metabolically versatile than those that lack cytochromes, with many species able to metabolize acetate, methanol, and other methylated compounds, as we see next.

Methanogenesis from Methyl Compounds and Acetate

Cytochrome-containing methanogens, such as *Methanosarcina*, can form CH_4 from acetate and certain methylated compounds, as well as from $H_2 + CO_2$. Methyl compounds such as methanol are catabolized by donating methyl groups to an enzyme containing a corrinoid coenzyme to form CH_3–corrinoid. Corrinoids are the parent structures of compounds such as vitamin B_{12} and contain a porphyrin-like ring with a central cobalt atom. The CH_3–corrinoid complex then transfers the methyl group to CoM, yielding CH_3-S-CoM (**Figure 14.39*a***). Energy is then conserved during electron transport reactions in the same manner as for growth on $H_2 + CO_2$ (Figure 14.38). However, in the absence of H_2, the reducing power for driving electron transport reactions comes from running the methanogenic pathway in reverse from CH_3-S-CoM to produce CO_2, one molecule of Fd_{red}, and two molecules of $F_{420}H_2$. These reduced $F_{420}H_2$ molecules can donate electrons to the electron transport chain in place of H_2 (Figure 14.39*a*). In addition, the Fd_{red} can be oxidized by an energy-converting hydrogenase (see Section 14.12) to produce H_2 (which can donate electrons to the electron transport chain along with $F_{420}H_2$) and contribute further to pmf formation. The net result for growth on methanol is that one molecule of methanol must be oxidized to CO_2 for every three reduced to CH_4.

When acetate is the substrate for methanogenesis by acetate-degrading methanogens (called *acetoclastic methanogens*), it is first activated to acetyl-CoA, which interacts with CO dehydrogenase from the acetyl-CoA pathway (Section 14.14 and Figure 14.34). The methyl group of acetate is then transferred to the corrinoid enzyme, ultimately forming CH_3-MP (Figure 14.39*b*). Formation of CH_4 from CH_3-MP proceeds as in the $H_2 + CO_2$ pathway (Figure 14.38), resulting in the formation of both a Na^+ and a H^+ motive force. Simultaneously, the CO group is oxidized to yield CO_2 and Fd_{red} (Figure 14.39*b*). The reduced ferredoxin can be oxidized by an energy-converting hydrogenase (see Figure 14.43) to produce H_2 and contribute further to pmf formation.

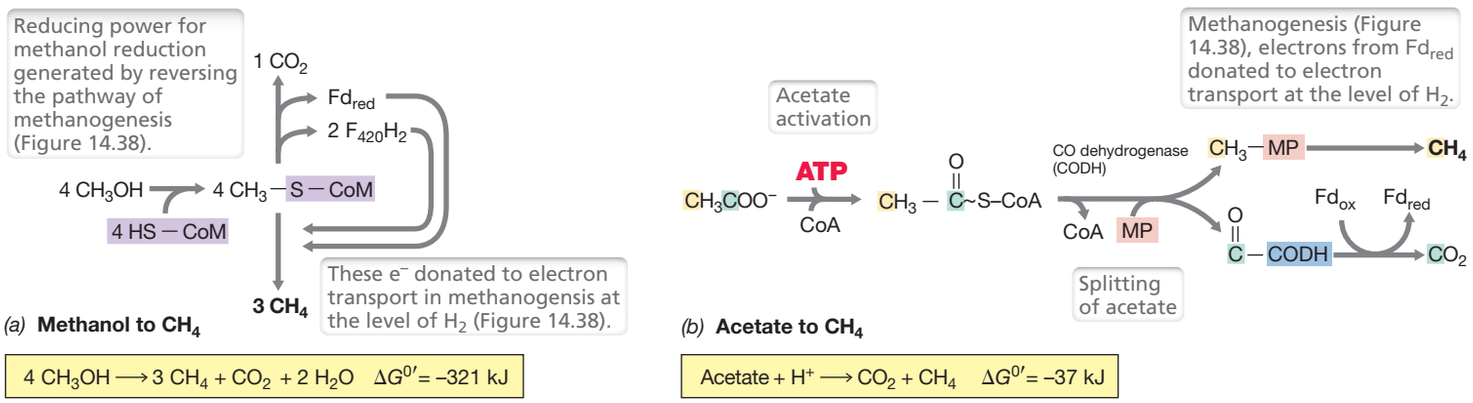


Figure 14.39 Methanogenesis from methanol and acetate. Both reactions funnel carbon into the methanogenic pathway (Figure 14.38) but at different points. *(a)* For growth on methanol (CH_3OH), redox balance is achieved because one CH_3OH is oxidized to CO_2 for every 3 reduced to CH_4. *(b)* Acetate is split into CH_4 and CO_2. Abbreviations and color-coding are as in Figures 14.36 and 14.38; CODH, carbon monoxide dehydrogenase.

Autotrophy

Autotrophy in methanogens is supported by the reductive acetyl-CoA pathway (Sections 14.2 and 14.14). As we have just seen, parts of this pathway are already integrated into the catabolism of methanol and acetate by methanogens that contain cytochromes (Figure 14.39). However, methanogens lack the tetrahydrofolate-driven series of reactions of the acetyl-CoA pathway that lead to the production of a methyl group (Figure 14.34). But this is not a problem because methanogens produce methyl groups during methanogenesis from H_2 and CO_2. In addition, methylotrophic methanogens derive the required methyl groups directly from their electron donors. Finally, acetoclastic methanogens have no need for CO_2 fixation because acetyl-CoA, which is formed during acetate oxidation (Figure 14.39*b*), can be funneled directly into biosynthesis.

Check Your Understanding

- Why is it that methanogens lacking cytochromes are unable to form CH_4 from methyl compounds?
- Explain why methanogens that contain cytochromes can conserve more energy than those that lack cytochromes.
- Explain how a reaction that produces a free energy yield of less than −31.8 kJ/mol, and produces a net translocation of just a single Na^+ ion, is enough to drive the formation of ATP.

14.16 Methanotrophy

Methane (CH_4) and many other C_1 organic compounds can be catabolized both aerobically and anaerobically. In this section, we consider the oxidation of these compounds by **methylotrophs**, organisms that use organic compounds that lack C—C bonds as electron donors and carbon sources. The oxidations of CH_4 and methanol (CH_3OH) have been the best-studied reactions, and we focus here on the oxidation of CH_4 as an example of a methylotrophic lifestyle.

Aerobic Methane Oxidation

Not all methylotrophs can use methane. **Methanotrophs** are those methylotrophs that can use CH_4, and methanotrophy has been well studied in aerobic methanotrophs such as the gram-negative bacterium *Methylococcus capsulatus*. The steps in CH_4 oxidation to CO_2 can be summarized as

$$CH_4 \rightarrow CH_3OH \rightarrow CH_2O \rightarrow HCOO^- \rightarrow CO_2$$

The initial step in the *aerobic* oxidation of CH_4 is catalyzed by the enzyme *methane monooxygenase* (MMO). Monooxygenases incorporate one oxygen atom from O_2 into a carbon compound (see Section 14.23 and Figure 14.54*a*). *M. capsulatus* contains two MMOs, one cytoplasmic (soluble MMO, sMMO) and the other membrane-integrated (particulate MMO, pMMO). In the MMO reaction, an atom of oxygen is introduced into CH_4, forming CH_3OH, and the second atom of O is reduced to form H_2O. CH_3OH is oxidized to formaldehyde (CH_2O) and then to CO_2, yielding NADH that can be used to conserve energy in electron transport to O_2. In addition, formaldehyde can be siphoned off to provide carbon for biosynthesis through one of two different pathways.

C_1 Assimilation by Aerobic Methanotrophs

At least two distinct pathways exist for the incorporation of C_1 units into cell material in methanotrophs. The **serine pathway** is outlined in **Figure 14.40*a***. In this pathway, acetyl-CoA is synthesized from one molecule of CH_2O and one molecule of CO_2. The serine pathway requires reducing power and energy in the form of two molecules each of NADH and ATP, respectively, for each acetyl-CoA synthesized. The serine pathway employs a number of enzymes of the citric acid cycle and one enzyme, *serine transhydroxymethylase*, unique to the pathway (Figure 14.40*a*).

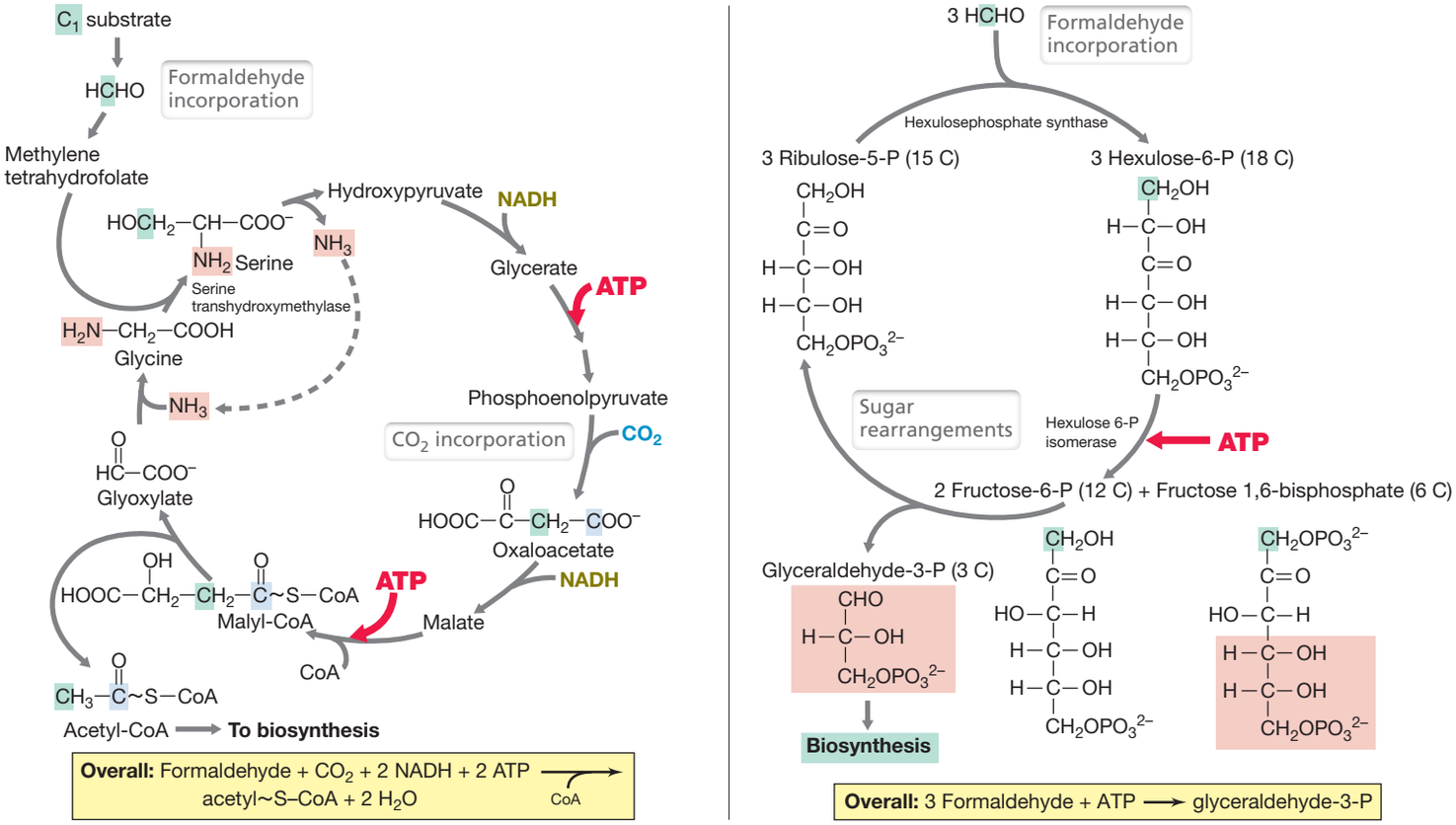
An alternative pathway for C_1 incorporation is the **ribulose monophosphate pathway** (Figure 14.40*b*). This pathway is more energy efficient than the serine pathway because *all* of the carbon for cell material is derived from CH_2O. Because CH_2O is at the same oxidation level as cell material, no reducing power is needed for its incorporation. Hence, all of the NADH from the oxidation of methane can be oxidized in the electron transport chain.

The ribulose monophosphate pathway consumes one molecule of ATP for each molecule of glyceraldehyde 3-phosphate (G-3-P) synthesized (Figure 14.40*b*); two G-3-Ps can then be converted into glucose by reversal of the glycolytic pathway (◀ Figure 3.11). Enzymes unique to the ribulose monophosphate pathway are *hexulosephosphate synthase*, which condenses one molecule of formaldehyde with one molecule of ribulose 5-phosphate, and *hexulose 6-P isomerase*, which forms fructose derivatives that can be converted into glyceraldehyde 3-P (Figure 14.40*b*). The remaining enzymes of this pathway are enzymes of intermediary metabolism widely distributed in bacteria.

Anaerobic Oxidation of Methane (AOM)

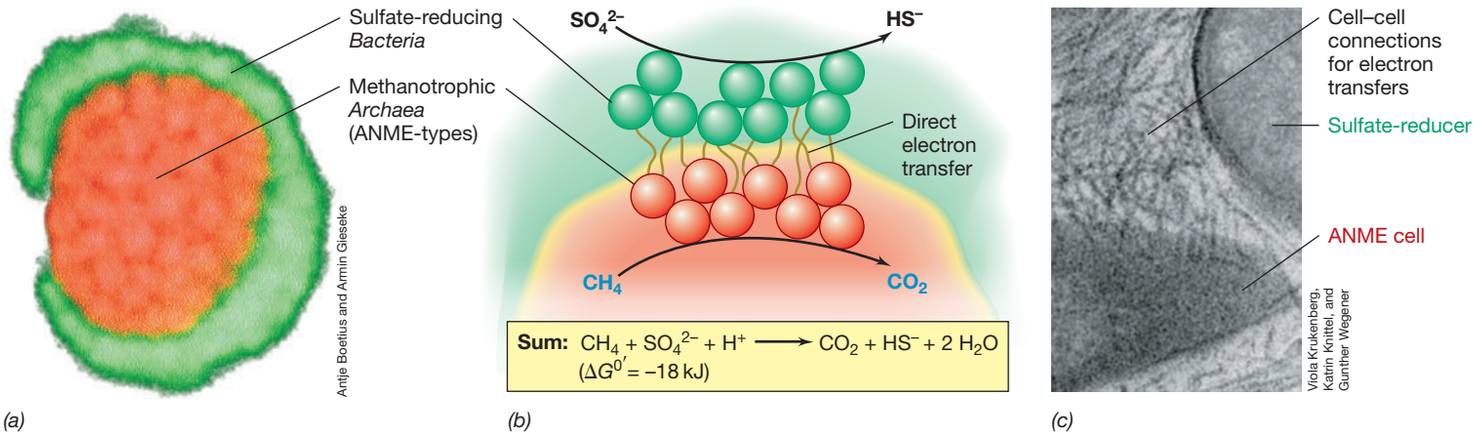
The *anaerobic* oxidation of methane uses a variety of enzymes and cofactors common to other forms of C_1 metabolism; however, anaerobic methanotrophy shows some novel features, as well. Methane can be oxidized anaerobically by a microbial association (called a *consortium*) of two organisms, a sulfate-reducing bacterium (SRB) plus a species of *Archaea* phylogenetically related to methanogens. These consortia thrive in anoxic marine sediments and are responsible for oxidizing more than 90% of the methane produced there. The components of the consortium coexist in spatially structured aggregates (**Figure 14.41**). The archaeal component, called ANME (*an*aerobic *me*thanotroph), of which there are several different types, oxidizes CH_4 as an electron donor. Electrons from methane oxidation are then transferred to the sulfate reducer, which uses them to reduce SO_4^{2-} to H_2S (Figure 14.41*b*).

ANME *Archaea* oxidize CH_4 to CO_2 by reversing the steps of methanogenesis (Figure 14.38). This process is endergonic but is made possible by the SRB partner organism, which consumes electrons from ANME, thereby making the oxidation CH_4 to CO_2 energetically favorable. Remarkably, electrons are transferred between the ANME and SRB partners by *direct electron transfer* (Figure 14.41*b*, *c*). Cells of ANME make electrically conductive multiheme cytochromes that span their outer cell layer and transfer electrons from the cytoplasmic membrane to the outside of the cell. The SRB have similar large electrically conductive cytochromes as well as pili that serve as electrically conductive "nanowires" (Section 14.13). These pili can



(a) **The serine pathway** (b) **The ribulose monophosphate pathway**

Figure 14.40 The serine and ribulose monophosphate pathways for the assimilation of C_1 units into cell material by methylotrophic bacteria. *(a)* Serine pathway. The product, acetyl-CoA, is used as the starting point for making new cell material. The key enzyme of the pathway is serine transhydroxymethylase. *(b)* Ribulose monophosphate pathway. Three molecules of CH_2O are required, with the product being glyceraldehyde 3-phosphate. The key enzyme of this pathway is hexulosephosphate synthase. The sugar rearrangements require enzymes of the pentose phosphate pathway (◀ Figure 3.31).



(a) (b) (c)

Figure 14.41 Anaerobic oxidation of methane. *(a)* Methane-oxidizing cell aggregates from marine sediments. The aggregates contain methane-oxidizing (methanotrophic) *Archaea* (red) surrounded by sulfate-reducing bacteria (green). Each cell type has been stained by a different FISH probe (▶ Section 19.5). The aggregate is about 30 μm in diameter. *(b)* Mechanism for the cooperative degradation of CH_4. Electrons are transferred from the archaeal methanotrophic (ANME) cell to the sulfate reducer by direct electron transfer through the "nanowire" connections. *(c)* Transmission electron micrograph of a section through a methane-oxidizing aggregate showing an ANME cell transferring electrons to a sulfate reducer.

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be more than 1 μm in length and electrically connect the cells within aggregates to facilitate the two microbes' metabolisms (Figure 14.41*b*, *c*; ▶ Section 23.3). Such direct electron transfer likely explains why some ANME can also use insoluble metals such as iron (Fe^{3+}) and manganese oxides (Mn^{4+}) as terminal electron acceptors (Section 14.13) in CH_4 oxidation. Since these insoluble materials cannot enter the cell, the cell has to "reach out" to them in order to transfer electrons.

AOM is not limited to consortia of ANME and SRB. ANME *Archaea* include *Methanoperedens nitroreducens*, which uses nitrate (NO_3^-) as a terminal electron acceptor for the anaerobic oxidation of CH_4. This organism couples reverse methanogenesis to the reduction of NO_3^- to NO_2^-. *M. nitroreducens* can be found in consortia with denitrifying bacteria that then use NO_2^- as an electron acceptor. These consortia are active in anoxic environments where CH_4 and NO_3^- coexist, such as certain freshwater sediments.

Intra-Aerobic Methanotrophy

The methanotrophic denitrifying bacterium *Methylomirabilis oxyfera* is an obligate anaerobe that catalyzes AOM linked to NO_2^- as an electron acceptor. *M. oxyfera* can grow on CH_4 in pure culture and has a highly unusual polygonal morphology (**Figure 14.42**). Analysis of the *M. oxyfera* genome reveals all of the genes required for the *aerobic* oxidation of CH_4 to CO_2. *M. oxyfera* is also able to reduce NO_2^- to N_2, and it has most of the genes required for denitrification although it lacks nitric oxide reductase and nitrous oxide reductase (Section 14.11 and Figure 14.28). This leads to the interesting question of why an *anaerobic* bacterium would use *aerobic* pathways for CH_4 oxidation.

The answer to this conundrum is that *M. oxyfera* reduces nitrite in a novel way. The organism reduces NO_2^- to nitric oxide (NO) as a normal denitrifying bacterium would, but then *M. oxyfera* does something remarkable: The organism generates O_2 through the reaction $2\,NO \rightarrow N_2 + O_2$ and proceeds to use this O_2 as the electron acceptor for CH_4 oxidation. Because *M. oxyfera* produces its own O_2, its methanotrophic metabolism has been termed *intra-aerobic methanotrophy*. As it turns out, O_2 is toxic for the anaerobic *M. oxyfera*. However, if the O_2 is consumed (by its reduction to H_2O using electrons from the oxidation of CH_4) as soon as it is produced, O_2 never accumulates, and the organism's environment remains anoxic.

We transition to Part VI now and consider metabolisms in which ATP is not a product of electron transport reactions. These are the fermentations—metabolisms in which there is no lack of physiological diversity.

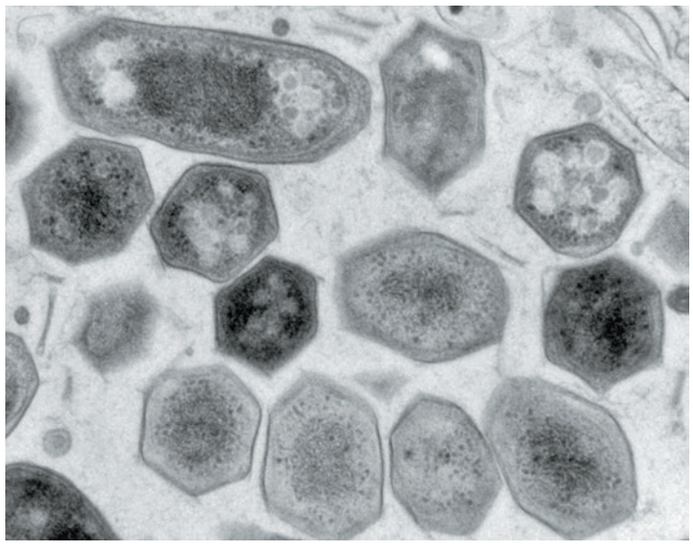

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Figure 14.42 The cell morphology of *Methylomirabilis oxyfera*. The denitrifying methanotroph *M. oxyfera* has a unique polygonal morphology as revealed in this transmission electron micrograph of cells from a microbial community grown in a bioreactor. A cell is about 0.5 μm in diameter.

Mastering Microbiology
Art Activity: Figure 14.54 The essentials of fermentation

Check Your Understanding

- When using CH_4 as electron donor, why is *Methylococcus capsulatus* an obligate aerobe?
- In which two ways does the ribulose monophosphate pathway save energy over reactions of the serine pathway?
- What is unique about methanotrophy in *Methylomirabilis oxyfera*?

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VI • Fermentation

Fermentations are anaerobic metabolisms that do not require external electron acceptors. Fermenting microbes achieve redox balance and conserve energy by forming various fermentation products that are excreted from the cell.

Thus far we have considered phototrophy and many types of aerobic and anaerobic respiration; these processes are unified by the fact that they all conserve energy primarily by using an ion gradient (H^+ or Na^+) that fuels photophosphorylation or oxidative phosphorylation. Here we turn our focus to *fermentation*, in which energy conservation depends primarily upon substrate-level phosphorylation reactions.

14.17 Energetic and Redox Considerations

Fermentation is a form of chemotrophic metabolism defined by the lack of an external electron acceptor. In fermentation, cells conserve energy primarily by substrate-level phosphorylation and they achieve redox balance by donating electrons to metabolic intermediates excreted as fermentation products. Fermentative organisms face two major challenges. First, their reliance on substrate-level phosphorylation means that they typically conserve much less energy than respiratory organisms. Second, their lack of external electron acceptors makes it difficult to achieve redox balance.

A tremendous diversity of fermentation reactions are possible. Many fermentative organisms, such as *Escherichia coli*, perform diverse metabolic reactions and only ferment when external electron acceptors are absent and conditions are anoxic. However, there are many more organisms, such as most lactic acid bacteria and the clostridia, which have exclusively fermentative metabolisms. Over the next few sections, we will see that fermentative organisms have evolved diverse strategies for facing the challenges of energy scarcity and redox balance.

Energy-Rich Compounds and Substrate-Level Phosphorylation

Energy can be conserved by substrate-level phosphorylation from many different compounds. However, as we learned in Chapter 3, central to an understanding of substrate-level phosphorylation is the concept of *energy-rich compounds* (◀ Figure 3.8). These are organic compounds that contain an energy-rich phosphate bond or a molecule of coenzyme A. The bond is "energy-rich" because its hydrolysis is highly exergonic. **Table 14.5** lists some energy-rich compounds formed during metabolism; the hydrolysis of most of these yields sufficient free energy to be coupled to ATP synthesis ($\Delta G^{0\prime} = -31.8$ kJ/mol). If an organism can form one of these compounds during fermentative metabolism, it can make ATP by transferring the phosphate bond from the energy-rich compound to ADP to form ATP— in other words, substrate-level phosphorylation.

Many anaerobic bacteria produce acetate or other fatty acids as a major or minor fermentation product. The production of fatty acids offers the organism the opportunity to conserve energy by substrate-level phosphorylation. The key to this process is the production of a coenzyme-A derivative of the fatty acid, since these are energy-rich compounds (Table 14.5). For example, acetyl-CoA can be converted to acetyl phosphate and the phosphate group subsequently transferred to ADP, yielding ATP (see Figures 14.46 and 14.47). Fatty acid production is common in fermentations, and if the fatty acid is metabolized through a CoA intermediate, the potential for ATP synthesis by substrate-level phosphorylation is a possibility.

Achieving Redox Balance

In any fermentation there must be atomic and redox balance. That is, the total number of each type of atom and electrons in the reaction products must balance those in the reactants (the substrates). Redox balance is achieved by the excretion of fermentation products, reduced substances such as acids or alcohols that are made as products of the fermentation. However, many reactions are difficult to balance, and so fermentative organisms have evolved alternative mechanisms for achieving redox balance.

TABLE 14.5 Energy-rich compounds that can couple to substrate-level phosphorylation[a]

Compound	Free energy of hydrolysis, $\Delta G^{0\prime}$ (kJ/mol)[b]
Acetyl-CoA	−35.7
Propionyl-CoA, butyryl-CoA, or caproyl-CoA	−35.6
Succinyl-CoA	−35.1
Acetyl phosphate, butyryl phosphate	−44.8
1,3-Bisphosphoglycerate	−51.9
Carbamyl phosphate	−39.3
Phosphoenolpyruvate	−51.6
1,3-Bisphosphoglycerate	−51.9
Adenosine phosphosulfate (APS)	−88
N^{10}Formyltetrahydrofolate	−23.4
Energy of hydrolysis of ATP ($ATP \rightarrow ADP + P_i$)	−31.8

[a]Data from Thauer, R.K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev. 41*: 100.
[b]The $\Delta G^{0\prime}$ values shown here are for "standard conditions," which are not necessarily those of cells. Including heat loss, the energy costs of making an ATP are more like −60 kJ/mol than −31.8 kJ/mol, and the energy of hydrolysis of the energy-rich compounds shown here is thus likely higher. But for simplicity and comparative purposes, the values in this table will be taken as the actual energy released per reaction.

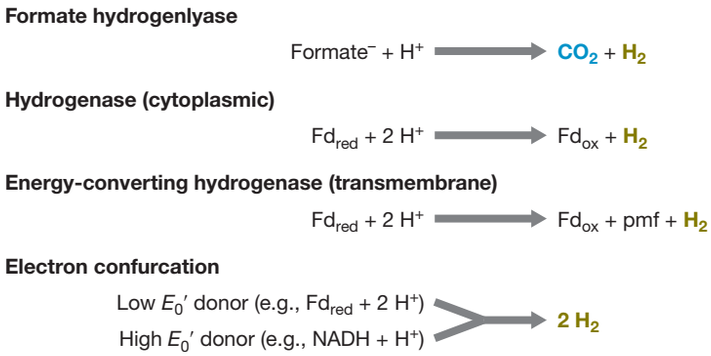


Figure 14.43 Production of H_2 to achieve redox balance during fermentation. Several mechanisms exist for reducing protons to H_2, thereby allowing the cell to achieve redox balance by exporting electrons. Energy-converting hydrogenases allow for coupling of H_2 production with energy conservation by pumping an H^+ across the membrane to generate a proton-motive force.

In many fermentations, redox balance is facilitated by the production of molecular hydrogen (H_2). Fermentative organisms can produce H_2 in several ways (**Figure 14.43**). First, H_2 can be produced from the C_1 fatty acid formate using the enzyme *formate hydrogenlyase*. This mechanism is the source of fermentative H_2 production used by many facultative aerobes including most enteric bacteria such as *E. coli*. Second, H_2 can be produced from proton reduction using the iron–sulfur protein *ferredoxin* (Fd_{red}), in a reaction catalyzed by a *hydrogenase* enzyme. Many organisms perform this reaction using a membrane-bound *energy-converting hydrogenase* because this enzyme couples H_2 production to proton pumping, thereby contributing to pmf formation and energy conservation (Figure 14.43).

Finally, some organisms produce H_2 using electron confurcation (Figure 14.43, see also Section 14.1). The advantage of electron confurcation is that it provides the ability to regenerate NAD^+ by producing H_2 from NADH at low energetic cost. Consider that $\Delta G^{0\prime} = +17$ kJ/mol for the confurcation reaction producing two H_2, while $\Delta G^{0\prime} = +20$ kJ/mol for the direct coupling of NADH to H_2 production; hence, confurcation allows the cell to produce two H_2 for less energy than it takes to produce one H_2 from the oxidation of NADH by reverse electron transport.

Fermentative organisms excrete H_2 to achieve redox balance by reoxidizing electron carriers such as NAD(P)H and Fd_{red}. The four mechanisms for H_2 production (Figure 14.43) all have $\Delta G^{0\prime}$ very close to zero under standard conditions. However, fermentation can produce copious amounts of NADH and Fd_{red} that would accumulate in the cell if these electron carriers were not reoxidized. Under actual physiological conditions, then, these H_2 production reactions are usually slightly exergonic, and in the case of energy-converting hydrogenases (Figure 14.43) can even be used to conserve energy. Another factor to consider is that H_2, excreted as a waste product by

fermentative organisms, is a very powerful electron donor for respiratory organisms, and this reality typically keeps H_2 levels in anoxic environments extremely low.

With H_2 we thus see that the waste product of one organism becomes a resource for another. This metabolic coupling, in which one organism's waste is another organism's resource, is a theme that runs throughout the microbial world. With these bioenergetic and redox principles in mind, we explore the metabolic diversity of fermentations beginning with species that produce acidic fermentation products, common and widespread bacteria in most anoxic environments.

Check Your Understanding

- Why is H_2 produced during many types of fermentation?
- Why is acetate formation in fermentation energetically beneficial to the cell?
- What is an energy-converting hydrogenase, and what role does it play in achieving redox balance?

14.18 Lactic and Mixed-Acid Fermentations

Fermentations are categorized by either the substrate fermented or the products formed. Table 14.6 lists some major fermentations classified on the basis of the products formed, such as alcohol, lactic acid, or other reduced organic compounds. Other fermentations are classified by the substrate fermented rather than the fermentation product; for instance, the amino acid, purine/pyrimidine, or succinate fermentations. We begin with two very common fermentations of sugars in which lactic acid is the sole or major product.

Lactic Acid Fermentation

Lactic acid bacteria are gram-positive nonsporulating bacteria that produce lactic acid as a major or sole fermentation product from the fermentation of sugars (▶ Section 16.6). Two fermentative patterns are observed. One, called **homofermentative**, yields a single fermentation product, lactic acid. The other, called **heterofermentative**, yields products in addition to lactate, mainly ethanol plus CO_2.

Figure 14.44 summarizes pathways for the fermentation of glucose by homofermentative and heterofermentative lactic acid bacteria. The differences observed can be traced to the presence or absence of the enzyme *aldolase*, a key enzyme of glycolysis (◀ Figure 3.11). Homofermentative lactic acid bacteria contain aldolase and produce *two* molecules of lactate from glucose by the glycolytic pathway (Figure 14.44*a*). Heterofermenters lack aldolase and thus cannot break down fructose bisphosphate to triose phosphate. Instead, they oxidize glucose 6-phosphate to 6-phosphogluconate and then decarboxylate this to ribulose 5-phosphate and on to xylulose 5-phosphate. The latter is then converted to triose phosphate and acetyl phosphate by the key enzyme *phosphoketolase* (Figure 14.44*b*). The early steps in catabolism by heterofermentative lactic acid bacteria are those of the pentose phosphate pathway (◀ Figure 3.31).

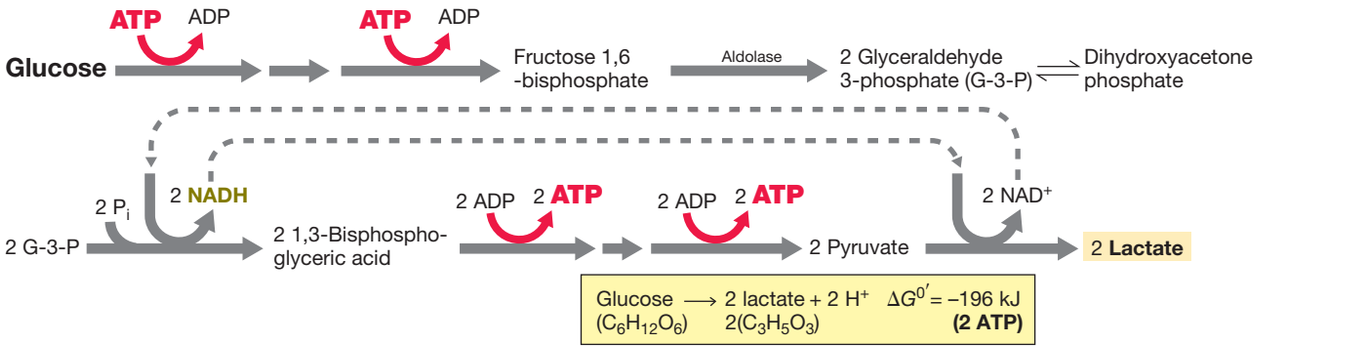
In heterofermenters, triose phosphate is converted to lactic acid with the production of ATP (Figure 14.44*b*). However, to achieve redox balance the acetyl phosphate produced is used as an electron acceptor and is reduced by NADH (generated during the production of pentose phosphate) to ethanol. This occurs without ATP synthesis because the energy-rich CoA bond is lost during ethanol formation. Because of this, heterofermenters produce only *one* ATP per glucose instead of the *two* ATP per glucose produced by homofermenters. In addition, because heterofermenters decarboxylate 6-phosphogluconate, they produce CO_2 as a fermentation product; homofermenters do not produce CO_2. Thus, an easy way to differentiate a homofermenter from a heterofermenter is to observe for the production of CO_2 in laboratory cultures.

Entner–Doudoroff Pathway

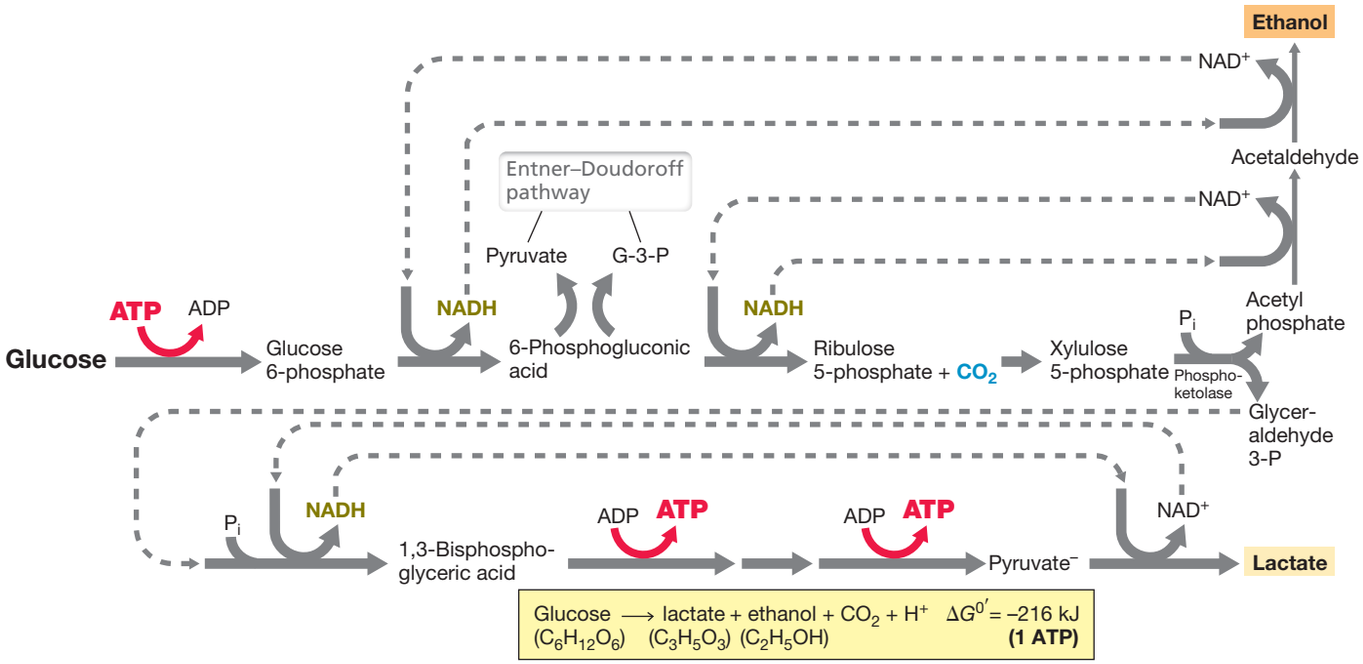
The initial stages of glucose fermentation often rely on glycolysis (◀ Figure 3.11) or a variant of the glycolytic pathway called the *Entner–Doudoroff pathway* (this variation is shown in Figure 14.44*b*). In the Entner–Doudoroff pathway, glucose 6-phosphate is oxidized to 6-phosphogluconic acid and NADPH; the 6-phosphogluconic

TABLE 14.6 Common fermentations and their energetics and example organisms

Type	Reaction	Energy yield ($\Delta G^{0\prime}$, kJ/mol)	Organisms
Alcoholic	Hexose → 2 ethanol + 2 CO_2	−239	Yeast, *Zymomonas*
Homolactic	Hexose → 2 lactate$^-$ + 2 H^+	−196	*Streptococcus*, some *Lactobacillus*
Heterolactic	Hexose → lactate$^-$ + ethanol + CO_2 + H^+	−216	*Leuconostoc*, some *Lactobacillus*
Propionic acid	3 Lactate$^-$ → 2 propionate$^-$ + acetate$^-$ + CO_2 + H_2O	−170	*Propionibacterium, Clostridium propionicum*
Mixed acid	Hexose → lactate$^-$ + acetate$^-$ + succinate^{2-} + formate$^-$ + ethanol + H_2 + CO_2	Depends on product ratio	Enteric bacteria including *Escherichia*, *Salmonella*, *Shigella*
Butanediol	Hexose → 2,3-butanediol + ethanol + lactate$^-$ + acetate$^-$ + succinate^{2-} + formate$^-$ + H_2 + CO_2	Depends on product ratio	*Klebsiella*, *Enterobacter*
Butyric acid	Hexose → butyrate$^-$ + 2 H_2 + 2 CO_2 + H^+	−264	*Clostridium butyricum*
Butanol	2 Hexose → butanol + acetone + 5 CO_2 + 4 H_2	−468	*Clostridium acetobutylicum*
Caproate/Butyrate	6 Ethanol + 3 acetate$^-$ → 3 butyrate$^-$ + caproate$^-$ + 2 H_2 + 4 H_2O + H^+	−183	*Clostridium kluyveri*



(a) **Homofermentative**



(b) **Heterofermentative**

Figure 14.44 The fermentation of glucose in *(a)* homofermentative and *(b)* heterofermentative lactic acid bacteria. Note that no ATP is made in reactions leading to ethanol formation in heterofermentative organisms.

acid is dehydrated and split into pyruvate and glyceraldehyde 3-phosphate (G-3-P), a key intermediate of the glycolytic pathway. G-3-P is then catabolized as in glycolysis, generating NADH and two ATP, and used as an electron acceptor to balance redox reactions as occurs in both lactic acid fermentations (Figure 14.44).

Because pyruvate is formed directly in the Entner–Doudoroff pathway and cannot yield ATP as can G-3-P (Figure 14.44), the Entner–Doudoroff pathway yields only half the ATP of the glycolytic pathway. Organisms using the Entner–Doudoroff pathway therefore share this physiological characteristic with heterofermentative lactic acid bacteria (Figure 14.44*b*). *Zymomonas*, an obligately fermentative gram-negative bacterium, and *Pseudomonas*, a strictly respiratory bacterium (▶ Section 16.4), are major genera that employ the Entner–Doudoroff pathway for glucose catabolism, although a modified version of this pathway is present in some species of hyperthermophilic and extremely halophilic *Archaea*.

Mixed-Acid Fermentations

In *mixed-acid fermentations* (Table 14.6), characteristic of enteric bacteria such as *Escherichia coli* and relatives (▶ Section 16.3), three different acids—*acetic*, *lactic*, and *succinic*—are formed from the fermentation of glucose or other sugars that can be converted into glucose. Ethanol, CO_2, and H_2 are also typically formed as fermentation products. Glycolysis (◀ Figure 3.11) is the pathway used to degrade glucose by mixed-acid fermenters.

Some enteric bacteria produce acidic products in lower amounts than *E. coli* and balance redox in their fermentations by producing larger amounts of neutral products. One key neutral product is the four-carbon alcohol *butanediol*. In this variation of the mixed-acid fermentation, the main products observed are butanediol, ethanol, CO_2, and H_2 (**Figure 14.45**). In the mixed-acid fermentation of *E. coli*, equal amounts of CO_2 and H_2 are produced, whereas in a

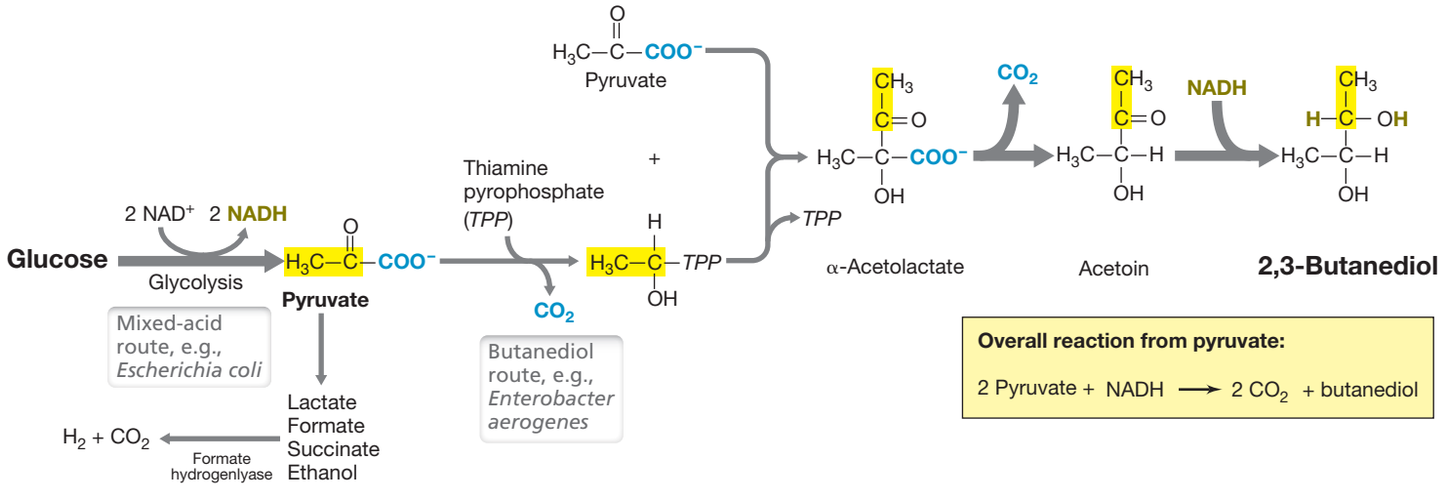


Figure 14.45 Butanediol production and mixed-acid fermentations. Note how only one NADH but two molecules of pyruvate are used to make one butanediol. This leads to redox imbalance and the production of more ethanol by butanediol producers than by mixed-acid fermenters. A yellow highlight is used to show the path that two of the four pyruvate carbons take to end up in α-acetolactate.

butanediol fermentation, considerably more CO_2 than H_2 is produced. This is because mixed-acid fermenters produce CO_2 only from formic acid by means of the enzyme *formate hydrogenlyase* (Figure 14.45, see also Figure 14.43):

$$HCOOH \rightarrow H_2 + CO_2$$

By contrast, butanediol producers, such as *Enterobacter aerogenes*, produce CO_2 and H_2 from formic acid but also produce two additional molecules of CO_2 during the formation of each molecule of butanediol. However, because butanediol production consumes only one-half of the NADH generated in glycolysis, more ethanol is produced by these organisms than by non–butanediol fermenters in order to achieve redox balance (Figure 14.45).

Lactic acid and enteric bacteria are facultative, meaning that they can grow either aerobically or anaerobically. By contrast, some fermenters are obligate anaerobes and we explore their fermentation patterns now.

Check Your Understanding

- How can homo- and heterofermentative metabolism be differentiated in pure cultures of lactic acid bacteria?
- Why does butanediol production lead to greater ethanol production than the mixed-acid fermentation of *Escherichia coli*?
- How and why does *E. coli* produce H_2 during fermentation?

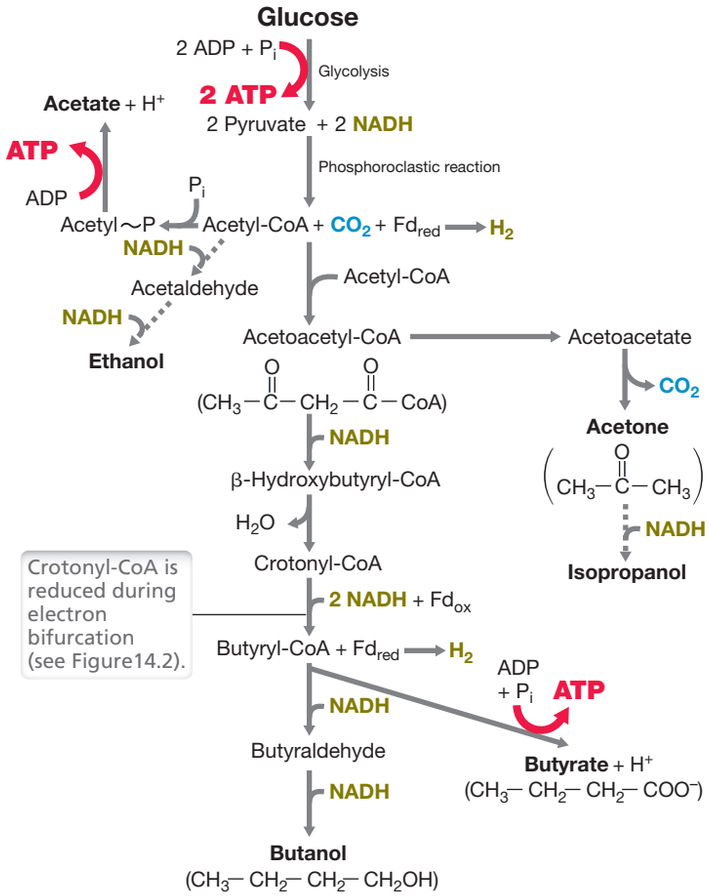
14.19 Fermentations of Obligate Anaerobes

Many fermentative organisms are obligate anaerobes. These organisms grow under highly reducing conditions, often use enzymes that cannot tolerate O_2, and often produce H_2 as a fermentation product. Species of *Clostridium* (▶ Section 16.8) and their relatives are the most well known of this group. Different species of clostridia ferment different groups of substrates, including sugars, amino acids, purines and pyrimidines, and a few other compounds. In all cases, ATP synthesis is linked primarily to substrate-level phosphorylation. However, we will see that many obligately anaerobic fermentative organisms can also conserve some energy through the generation of pmf. We begin with *saccharolytic* (sugar-fermenting) clostridia.

Sugar Fermentation by *Clostridium* Species

A number of clostridia ferment sugars, producing *butyric acid* and H_2 as major fermentation products. A generalized scheme for the biochemical steps in the formation of butyric acid and neutral products from sugars is shown in **Figure 14.46**. In saccharolytic clostridia, such as the well-studied *Clostridium pasteurianum*, 1.5 glucose is converted to 3 pyruvate and 3 NADH via the glycolytic pathway, and each pyruvate is split to yield acetyl-CoA, CO_2, and Fd_{red} (the "phosphoroclastic reaction" in Figure 14.46). One acetyl-CoA gives rise to acetate and an ATP and the other two are combined and reduced to butyrate to form another ATP. One NADH is reoxidized during the formation of β-hydroxybutyryl-CoA and the other 2 NADH are oxidized in an electron bifurcation reaction (Figure 14.2) that couples the exergonic reduction of crotonyl-CoA to the endergonic reduction of Fd_{ox}. Redox balance is achieved ultimately by a cytoplasmic hydrogenase that uses Fd_{red} to reduce protons to H_2 (Figure 14.43).

The actual fermentation products observed differ between species, and the ratio of these products is influenced by environmental conditions. *Clostridium acetobutylicum* provides a classic example of this pattern. During the early stages of the butyric fermentation, *C. acetobutylicum* produces mostly butyrate and a small amount of acetate, similar to that of *C. pasteurianum*. But the production of butyrate and acetate leads to acid production (Figure 14.46), and acidification of the environment eventually inhibits growth of *C. acetobutylicum*. Hence, as the pH of the medium drops, the organism shifts its fermentation products toward acetone and butanol, "neutral products" that do not lower the pH of the environment. However, if the pH of the medium is kept neutral by buffering, butyric acid production continues, and very little acetone and butanol will be formed because butyrate formation provides a higher yield of ATP (Figure 14.46).



1.5 Glucose + H_2O ⟶ acetate + butyrate + 3 CO_2 + 4 H_2 + H^+
$\Delta G^{0'}$ = –250 kJ **(3–4 ATP/glucose)**

2 Glucose ⟶ acetone + butanol + 5 CO_2 + 4 H_2
$\Delta G^{0'}$ = –468 kJ **(2 ATP/glucose)**

Figure 14.46 The butyric acid and butanol/acetone fermentation. All fermentation products from glucose are shown in bold (dashed lines indicate minor products). Note how the production of acetate and butyrate lead to additional ATP by substrate-level phosphorylation. By contrast, formation of butanol and acetone reduces the ATP yield because the butyryl-CoA to butyrate step is bypassed. The production of butyryl-CoA is an electron bifurcation reaction (Figure 14.2) in which 2 NADH donate electrons to crotonyl-CoA and Fd_{ox}. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin.

Interestingly, the production of butanol is a consequence of the production of acetone. For each acetone produced, two NADH cannot be reoxidized in the reduction of crotonyl-CoA. To achieve redox balance, the cell then uses butyrate as an electron acceptor, producing butanol as a fermentation product (Figure 14.46). Although neutral product formation helps *C. acetobutylicum* keep its environment from becoming too acidic, there is an energetic price to pay.

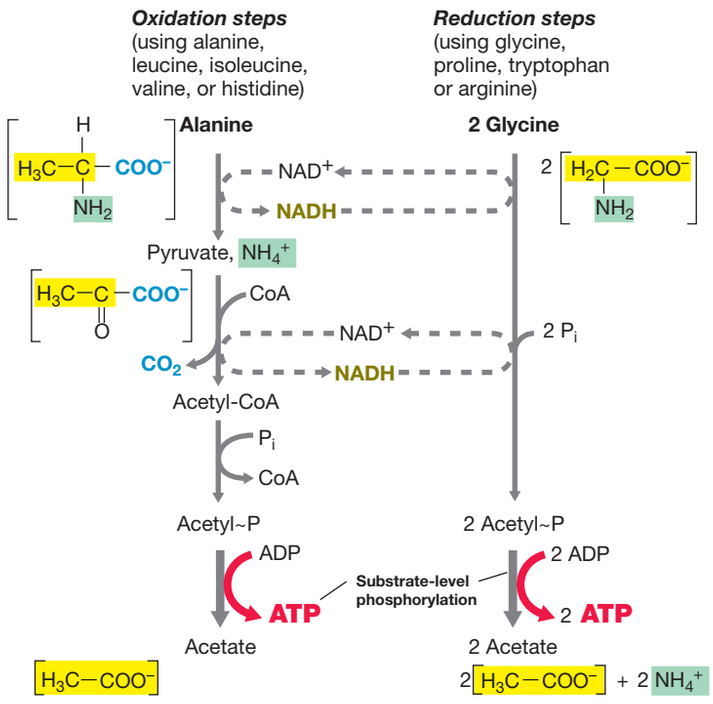
Amino Acid Fermentation by Clostridia and the Stickland Reaction

Some clostridia ferment amino acids. These are the *proteolytic* clostridia, organisms that degrade proteins released from dead organisms. Some of these, such as the animal pathogen *Clostridium tetani* (tetanus), are strictly proteolytic, while other species are both saccharolytic and proteolytic.

Depending on the species, some proteolytic clostridia ferment individual amino acids, typically glutamate, glycine, alanine, cysteine, histidine, serine, or threonine. The biochemistry behind these fermentations is quite complex, but the metabolic strategy is simple. In virtually all cases, the amino acids are catabolized in such a way as to eventually yield a fatty acid–CoA derivative, typically acetyl (C_2), butyryl (C_4), or caproyl (C_6). From these, ATP is produced by substrate-level phosphorylation (Table 14.5). Other typical products of amino acid fermentation include ammonia (NH_3) and CO_2.

Some clostridia ferment only an amino acid *pair*. In this situation one amino acid functions as the electron donor and is oxidized, whereas the other amino acid is the electron acceptor and is reduced. This *coupled* amino acid fermentation is called a **Stickland reaction**, named for the scientist who discovered it. For example, *Clostridium sporogenes* ferments glycine and alanine, and in this reaction, alanine is the electron donor and glycine is the electron acceptor (**Figure 14.47**). The products of the Stickland reaction are invariably NH_3, CO_2, and a carboxylic acid with one fewer carbons than the amino acid that was oxidized (Figure 14.47).

Many of the products of amino acid fermentation by clostridia are foul-smelling substances, and the odor that results from putrefaction is mainly a result of clostridial activity. In addition to fatty



Overall: Alanine + 2 glycine + 2 H_2O ⟶ 3 $acetate^-$ + CO_2 + 3 NH_4^+
$\Delta G^{0'}$ = –186 kJ **(3 ATP)**

Figure 14.47 The Stickland reaction. This example shows the co-catabolism of the amino acids alanine and glycine. The structures of key substrates, intermediates, and products are shown in brackets to allow the chemistry of the reaction to be followed. Note that in the reaction shown, alanine is the electron donor and glycine is the electron acceptor.

acids, other odoriferous compounds produced in proteolytic fermentations include hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH, derived from sulfur-containing amino acids), cadaverine (from lysine), putrescine, and NH_3. Purines and pyrimidines (◀ Figure 3.34), released from the degradation of nucleic acids, lead to many of the same fermentation products and also yield ATP by substrate-level phosphorylation from the hydrolysis of fatty acid–CoA derivatives (Table 14.5) produced in their respective fermentative pathways.

Energy-Converting Hydrogenases

Pyrococcus furiosus is a hyperthermophilic species of *Archaea* that grows optimally at 100°C (▶ Section 17.4) by fermenting sugars and small peptides. It uses the early reactions of glycolysis (◀ Figure 3.11) to convert glucose into two glyceraldehyde 3-phosphate, but then produces two molecules of 3-phosphoglycerate instead of two 1,3-bisphosphoglycerate, as normally occurs in glycolysis (**Figure 14.48**). In so doing, *P. furiosus* forgoes the ability to make ATP from the energy-rich phosphate bonds in 1,3-bisphosphoglycerate and instead forms Fd_{red}. Additional ATP and Fd_{red} are formed in the subsequent conversion of pyruvate to acetate (Figure 14.48). *P. furiosus* is then able to use an energy-converting hydrogenase (Figure 14.43) to reoxidize Fd_{red} and achieve redox balance, but this hydrogenase also has the ability to translocate one Na^+ across the membrane for each proton reduced. A Na^+-linked ATPase then uses the sodium motive force to synthesize ATP (Figure 14.48).

Ultimately, *P. furiosus* obtains two-thirds (4 ATP) of its energy from substrate-level phosphorylation and one-third (2 ATP) from oxidative phosphorylation driven by proton respiration linked to a Na^+ pump. By using this strategy, *P. furiosus* yields a net of 4 ATP per glucose (Figure 14.48), whereas lactic acid bacteria only obtain 2 ATP per glucose (Figure 14.44). Like *P. furiosus*, many obligately anaerobic fermentative organisms use energy-converting hydrogenases to achieve redox balance and thereby conserve some energy in the form of a proton or sodium motive force. Is this respiration or is it fermentation? Some metabolic reactions are difficult to classify, but the majority of energy is conserved in substrate-level phosphorylation, a trait that is a defining feature of fermentative metabolisms.

Is fermentation the "end of the line" in terms of the energy extracted from the initial substrate? Not quite, as we see in the final three sections of this part of the chapter.

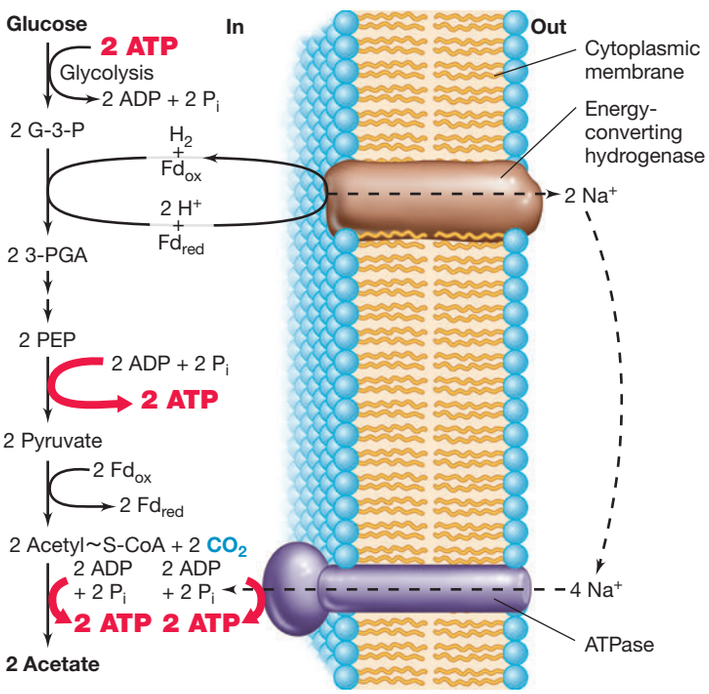


Figure 14.48 Modified glycolysis and proton reduction in anaerobic respiration in the hyperthermophile *Pyrococcus furiosus*. Hydrogen (H_2) production is linked to H^+ pumping by an energy-converting hydrogenase (see Figure 14.43) that receives electrons from reduced ferredoxin (Fd_{red}).Compare this figure with classical glycolysis in Figure 3.11. G-3-P, glyceraldehyde 3-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate.

Check Your Understanding

- Why does *Clostridium acetobutylicum* need to produce butanol as a fermentation product when it is producing acetone?
- What type of substrates are fermented by saccharolytic clostridia? By proteolytic clostridia?
- What aspects of metabolism in *Pyrococcus furiosus* are unusual for a fermentative organism?

14.20 Secondary Fermentations

In addition to classifying fermentations by the substrate fermented or product(s) produced (Section 14.18 and Table 14.6), fermentations can be classified as *primary* or *secondary*. Primary fermentations are carried out by organisms that break down and ferment various polymers and monomers of carbohydrate, protein, and fat to reduced products including acids and alcohols as well as H_2 and CO_2. **Secondary fermentations** use these fermentation products as substrates for additional fermentation reactions whose products are mainly volatile fatty acids (such as butyrate, propionate, and acetate), H_2, and CO_2.

Propionic Acid Fermentation

The gram-positive bacterium *Propionibacterium* and some related bacteria produce *propionic acid* as a major fermentation product from either glucose or lactate. Lactate, a fermentation product of the lactic acid bacteria (Section 14.18), is probably the major substrate for propionic acid bacteria in nature, where these two groups live in close association. *Propionibacterium* is an important agent in the ripening of Emmental (Swiss) cheese, which gets its unique bitter and nutty taste from the propionic and acetic acids produced; the CO_2 produced during the fermentation forms bubbles that leave the characteristic holes (eyes) in the cheese.

Figure 14.49 shows the reactions leading from lactate to propionate. When glucose is the starting substrate, it is first catabolized to pyruvate by the glycolytic pathway. Then pyruvate, produced either from glucose or from the oxidation of lactate, is converted to acetate plus CO_2 or carboxylated to form methylmalonyl-CoA; the latter is converted into oxaloacetate and, eventually, propionyl-CoA

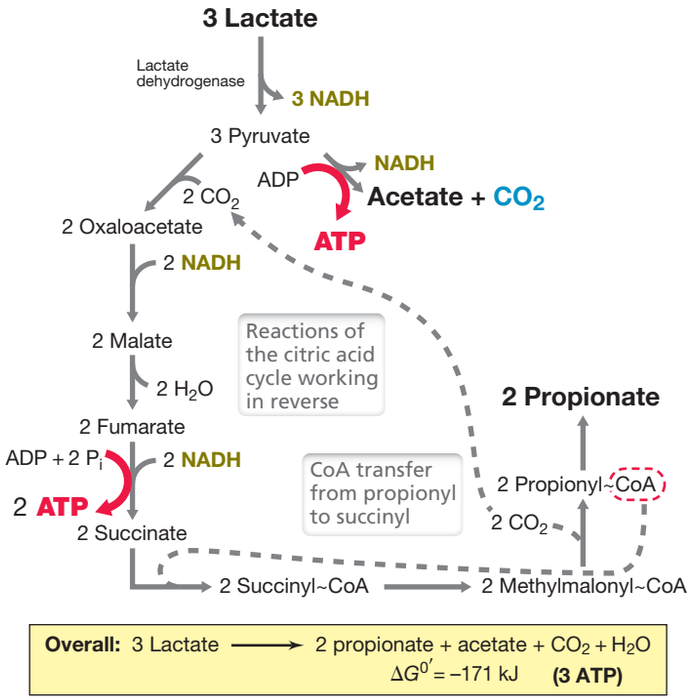


Figure 14.49 **The propionic acid fermentation of *Propionibacterium*.** Products are shown in bold. The four NADH made from the oxidation of three lactate are reoxidized in the reduction of oxaloacetate and fumarate, and the CoA group from propionyl-CoA is exchanged with succinate during the formation of propionate.

(Figure 14.49). Propionyl-CoA reacts with succinate in a step catalyzed by the enzyme CoA transferase, producing succinyl-CoA and propionate. This results in a lost opportunity for ATP production from propionyl-CoA (Table 14.5) but avoids the energetic costs of having to activate succinate with ATP to form succinyl-CoA. The succinyl-CoA is then isomerized to methylmalonyl-CoA and the cycle is complete when propionate is formed and CO_2 regenerated (Figure 14.49).

NADH is oxidized in the steps between oxaloacetate and succinate. The reduction of fumarate to succinate (Figure 14.49) is linked to electron transport reactions (Section 14.13) and the formation of a proton motive force; this yields one ATP by oxidative phosphorylation. The propionate pathway also converts some pyruvate to acetate plus CO_2, which allows for additional ATP to be made by substrate-level phosphorylation because this reaction proceeds through the energy-rich intermediate acetyl-CoA (Table 14.5 and Figure 14.49). Thus, in the propionate fermentation, both substrate-level *and* oxidative phosphorylation occur.

Propionate is also formed in the fermentation of succinate by the bacterium *Propionigenium*, but by a completely different mechanism than that described here for *Propionibacterium*. *Propionigenium*, to be considered in Section 14.21, is phylogenetically and ecologically unrelated to *Propionibacterium*, but aspects of its energy metabolism are of considerable interest from the standpoint of metabolic diversity and the energetic limits to life.

Clostridium kluyveri Fermentation

Clostridium kluyveri ferments two substrates: *ethanol plus acetate*. This is an unusual secondary fermentation in that ethanol is the electron *donor* and acetate is the electron *acceptor*, and butyrate, caproate, and H_2 are the products. The overall reaction in the caproate/butyrate fermentation is shown in Table 14.6 and begins with 6 ethanol and 3 acetate as substrates. The oxidation of 6 ethanol to 6 acetyl-CoA results in the production of 12 NADH. Then, using the same reactions we saw in butyric acid fermentation (Figure 14.46), one acetyl-CoA is used to produce acetate and ATP by substrate-level phosphorylation. Next, the 3 acetate molecules are activated by adding CoA, giving a total of 8 acetyl-CoA. These molecules are used in the butyric acid pathway to accept electrons from the 12 NADH generated earlier, thereby producing 4 butyryl-CoA (as in Figure 14.46).

As in the butyric acid fermentation, the production of 4 butyryl-CoA is mediated by an electron bifurcation reaction (Figure 14.2) producing 4 Fd_{red}, two of which give rise to H_2. However, *C. kluyveri*, unlike *C. pasteurianum*, has a membrane-bound NAD^+–ferredoxin oxidoreductase (RnfA–G) that uses the exergonic reduction of NAD^+ by Fd_{red} to pump protons across the membrane, thereby generating a pmf (Rnf is also found in acetogens, see Figure 14.34). The two remaining Fd_{red} are used by the Rnf enzyme complex to produce 2 NADH and to pump 4 H^+ across the membrane, enough to make one ATP by oxidative phosphorylation. Finally, we are left with 2 NADH, 1 acetyl-CoA, and 4 butyryl-CoA. The CoA are transferred from 3 of the butyryl-CoA to activate the 3 acetate at the beginning of the pathway, producing 3 butyrate. Lastly, the 2 remaining NADH are used to make caproate from the final butyryl-CoA and the acetate produced earlier in the pathway, yielding the final fermentation products: 3 butyrate, 1 caproate, and 2 H_2.

We can see several themes at work in the fermentation of *C. kluyveri*. First, we see the modularity of metabolism in the way that *C. kluyveri* has adapted the butyric acid pathway to perform a butyrate-based fermentation that is distinct from that of *C. pasteurianum*. Second, we can see how metabolic intermediates are often recycled and reused within fermentative pathways to achieve redox balance. Third, we see that organisms can enhance their metabolic efficiency by using mechanisms such as electron bifurcation and using reduced ferredoxin as electron donor to generate a proton motive force with protons or NAD^+ as electron acceptor.

In the final two sections of this part of the chapter, we will see more examples of microbes that employ innovative strategies for living at the thermodynamic edge of life.

Check Your Understanding

- What aspects of energy conservation are shared by both *Propionibacterium* and *Clostridium kluyveri*?
- What is meant by the terms primary and secondary fermentation?
- How is the Rnf enzyme complex of *Clostridium kluyveri* similar to the energy-converting hydrogenase of *Pyrococcus furiosus*? How are they different?

14.21 Fermentations That Lack Substrate-Level Phosphorylation

Certain fermentations yield insufficient energy to synthesize ATP by substrate-level phosphorylation (that is, less than −31.8 kJ, Table 14.5), yet still support anaerobic growth without external

electron acceptors. In these cases, catabolism of the compound is linked to ion pumps that establish an ion gradient across the cytoplasmic membrane. Examples of these include the fermentation of succinate by *Propionigenium modestum* and the fermentation of oxalate by *Oxalobacter formigenes*.

Propionigenium modestum

Propionigenium modestum was first isolated in anoxic enrichment cultures lacking electron acceptors and fed succinate as an electron donor. *Propionigenium* inhabits marine and freshwater sediments and can also be isolated from the human oral cavity. The organism is a gram-negative short rod and, phylogenetically, is a species of *Fusobacteria*. During studies of the physiology of *P. modestum*, it was shown to require sodium chloride (NaCl) for growth and to catabolize succinate under strictly anoxic conditions:

$$\text{Succinate}^{2-} + H_2O \rightarrow \text{propionate}^- + HCO_3^- \quad \Delta G^{0\prime} = -20.5\ \text{kJ}$$

This is a disproportionation reaction (Section 14.12) in which part of the succinate molecule is reduced to propionate and the other part is oxidized to CO_2 (**Figure 14.50*a***). Energy is conserved by an unusual enzyme that decarboxylates (S)-methylmalonyl-CoA and adds a proton to it to produce propionyl-CoA. The enzyme uses the energy released from this decarboxylation reaction to translocate two Na^+ across the membrane. Energy is then conserved using a Na^+-linked ATPase as we have seen for acetogens (Section 14.14), methanogens (Section 14.15), and *Pyrococcus* (Section 14.19).

In a related decarboxylation reaction, the bacterium *Malonomonas rubra* decarboxylates the C_3 dicarboxylic acid malonate, forming acetate plus CO_2. As for *Propionigenium*, energy metabolism in *Malonomonas* is linked to Na^+ and a sodium-driven ATPase. But the free energy available from malonate fermentation by *Malonomonas* (−17.4 kJ) is even less than that of succinate fermentation by *P. modestum*. *Sporomusa*, an endospore-forming bacterium and also an acetogen (Section 14.14), is also capable of fermenting malonate, as are a few other anaerobic *Bacteria*.

Oxalobacter formigenes

Oxalobacter formigenes is a bacterium present in the intestinal tract of animals, including humans. It performs a disproportionation reaction using the C_2 dicarboxylic acid oxalate, reducing one part of the molecule to formate and oxidizing the other part to CO_2 (Figure 14.50*b*). Oxalate degradation by *O. formigenes* is thought to be important in the human colon for preventing the accumulation of oxalate, a substance that can form calcium oxalate kidney stones. *O. formigenes* is a gram-negative strict anaerobe that carries out the following reaction:

$$\text{Oxalate}^{2-} + H_2O \rightarrow \text{formate}^- + HCO_3^- \quad \Delta G^{0\prime} = -26.7\ \text{kJ}$$

As in the catabolism of succinate by *P. modestum*, insufficient energy is available from this reaction to drive ATP synthesis by substrate-level phosphorylation (Table 14.5). In addition, remarkably, the organism does not have a mechanism for pumping ions out of the cell. Energy is conserved because protons are consumed in the cytoplasm during the oxidation of oxalate and production of formate. Looked at another way, a divalent molecule (oxalate) enters the cell while a univalent molecule (formate) is excreted, and this

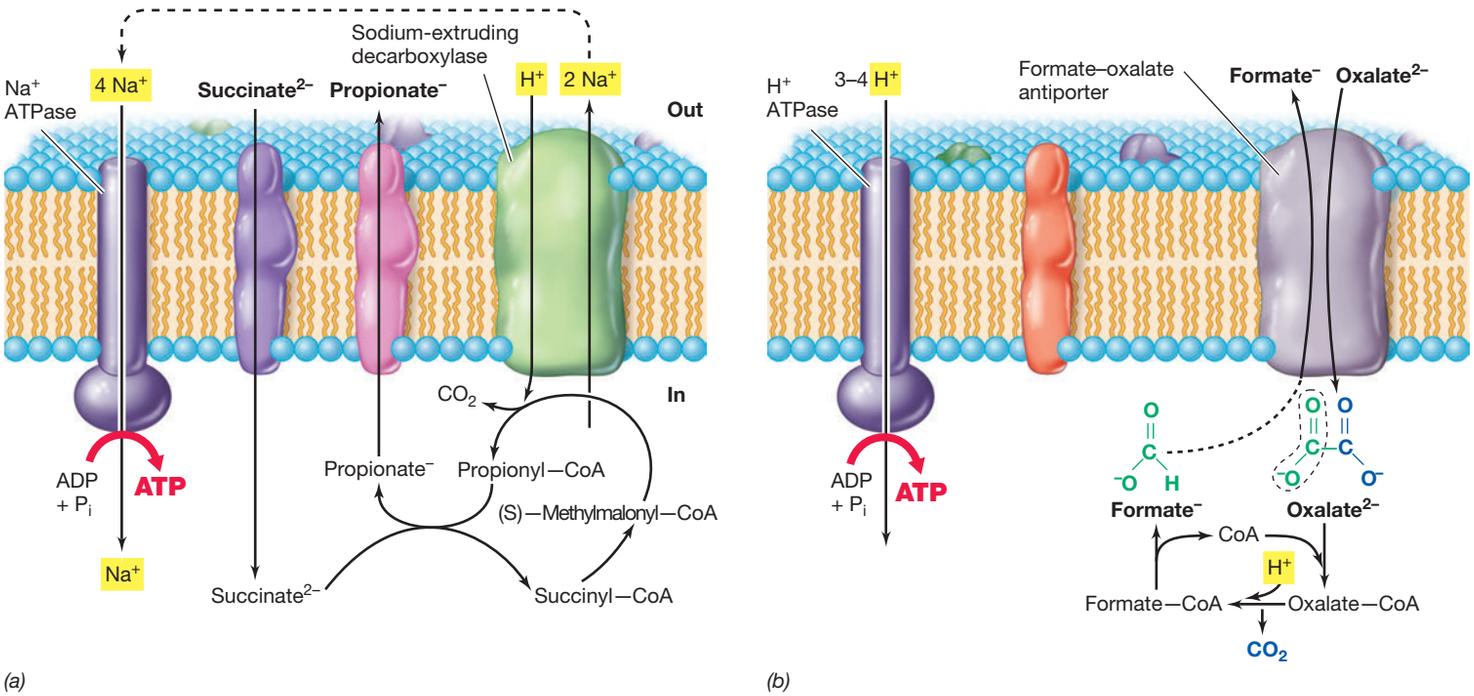


Figure 14.50 The unique fermentations of succinate and oxalate. *(a)* Succinate fermentation by *Propionigenium modestum*. Sodium export is linked to the energy released by succinate decarboxylation, and a sodium-translocating ATPase produces ATP. *(b)* Oxalate fermentation by *Oxalobacter formigenes*. Oxalate import and formate export by a formate–oxalate antiporter (◀ Figure 2.6) consume cytoplasmic protons. ATP synthesis is linked to a proton-driven ATPase. All substrates and products of the fermentation are shown in black bold.

contributes to the energy of the electrochemical gradient. Together, these forces allow for the formation of a proton motive force sufficient to drive ATP synthesis (Figure 14.50*b*).

What Can Be Learned from the Decarboxylations of Succinate and Oxalate?

The unique aspect of decarboxylation-type fermentations is that ATP is made without substrate-level phosphorylation *or* electron transport reactions. In both cases, ATP synthesis is driven by a disproportionation reaction in which a redox reaction is used to generate an ion gradient directly, either by consuming protons, as in *Oxalobacter*, or by fueling an ion pump, as in *Propionigenium*. We also see this trend reflected in energy-converting hydrogenases (as in some sulfate reducers, acetogens, methanogens, and fermenters) and in the Rnf enzyme complex (as in some acetogens and fermenters), which both couple a redox reaction directly to an ion-pumping mechanism. All of these mechanisms offer an important lesson in microbial bioenergetics: ATP synthesis from reactions that yield less than −31.8 kJ/mol is still possible if the reaction can be coupled to an ion pump.

At a minimum, then, an energy-conserving reaction must yield sufficient free energy to pump at least one ion. This energy requirement is estimated to be near −12 kJ. Reactions that release less free energy than this should not be able to drive ion pumps and should therefore not be potential energy-conserving reactions. However, as we will see in the next section, bacteria are known that push this theoretical limit even lower, and their energetics, consequently, are still incompletely understood. These are the syntrophs, bacteria living on the energetic margin of existence.

Check Your Understanding

- Why does *Propionigenium modestum* require sodium for growth?
- Of what benefit is the organism *Oxalobacter* to human health?
- How can a fermentation that yields insufficient free energy to make an ATP still support growth?

14.22 Syntrophy

There are many examples in microbiology of **syntrophy**, a situation in which two different microbes cooperate to perform a metabolic reaction that neither can accomplish alone. Most syntrophic reactions are secondary fermentations in which organisms ferment the fermentation products of other anaerobes. We will see in Chapter 21 how syntrophy is often a key step in anaerobic food webs and in the anaerobic metabolisms that lead to the production of methane in nature. Here we consider the microbiology and energetic aspects of syntrophy.

Interspecies Electron Transfer

Many organic compounds can be degraded syntrophically, including even aromatic and aliphatic hydrocarbons. But the major compounds of interest in syntrophic environments are fatty acids and alcohols. The heart of syntrophic reactions is *interspecies electron transfer*, defined as a relationship between two organisms in which one species serves as the electron acceptor for another species that is the electron donor. This relationship can be direct (*direct interspecies electron transfer*, DIET), mediated by either direct contact between the cells or by direct electrical connections established by using nanowires (Section 14.13). Alternatively, the relationship can be mediated (*mediated interspecies electron transfer*, MIET) by metabolic products that are exchanged by diffusion between cells. H_2 mediates many syntrophic partnerships since many fermentative organisms readily produce it and many respiratory organisms readily consume it. We will encounter examples of DIET in Chapter 23. Here we consider only how the exchange of metabolic products allows both syntrophic partners to thrive.

As an example of syntrophy, consider the fermentation of ethanol to acetate plus H_2 by the syntroph *Pelotomaculum* coupled to the production of methane (**Figure 14.51**). The syntroph carries out a reaction whose standard free-energy change ($\Delta G^{0\prime}$) is positive. Hence, in pure culture, the organism will not grow. However, the H_2 produced by *Pelotomaculum* can be used as an electron donor by a methanogen to produce methane, an exergonic reaction. When the two reactions are summed, the overall reaction is exergonic (Figure 14.51*a*), and when *Pelotomaculum* and a methanogen are cultured together (cocultured), both organisms grow.

A second example of syntrophy is the oxidation of a fatty acid such as butyrate to acetate plus H_2 by the fatty acid–oxidizing bacterium *Syntrophomonas* (**Figure 14.52*a***). *Syntrophomonas* oxidizes butyrate by reversing most steps in the butyric acid fermentation pathway (see Figure 14.46). However, the electron-bifurcating enzyme of butyric acid fermentation is replaced by an enzyme that oxidizes butyryl-CoA to produce NADH (Figure 14.52*a*). The two NADH produced in butyrate oxidation are oxidized in an electron

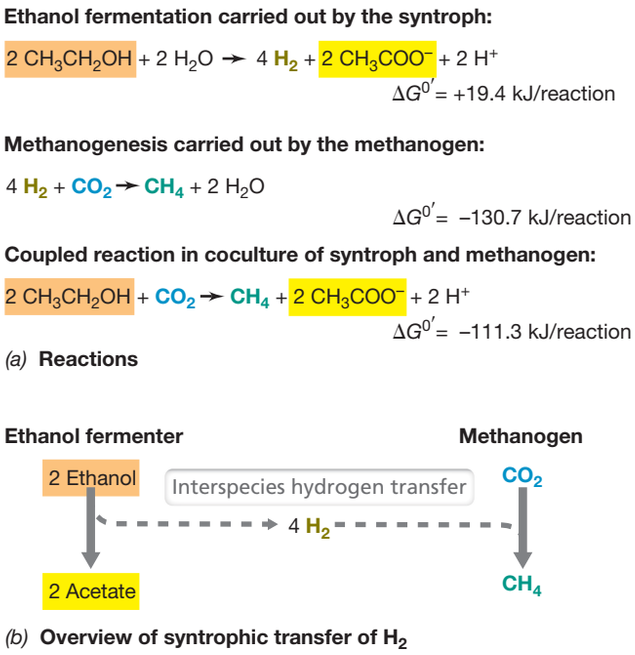
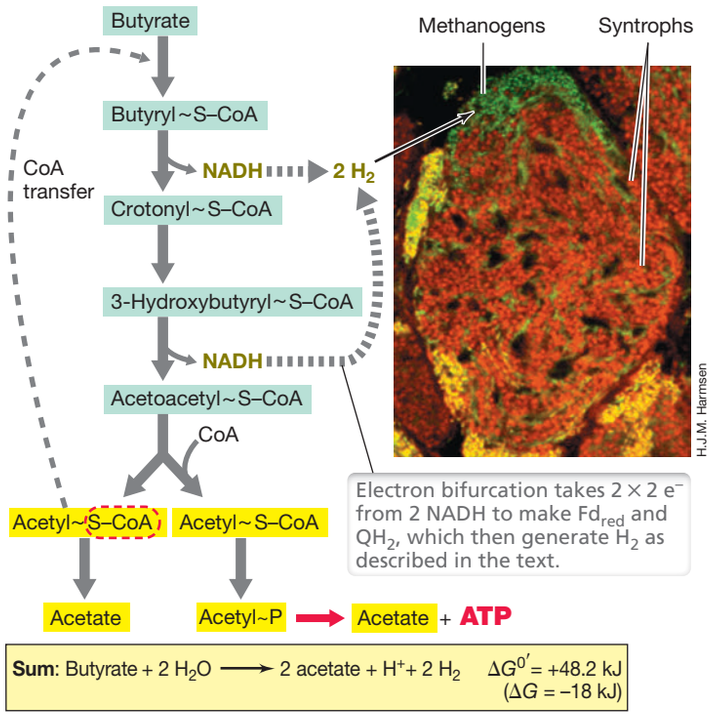
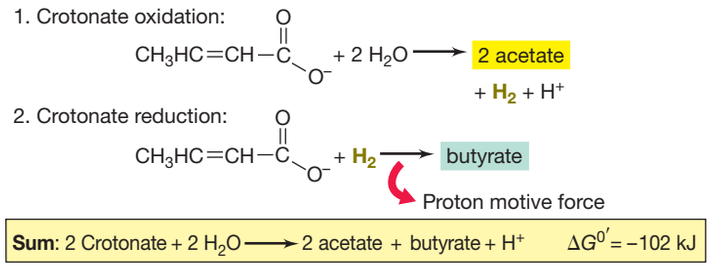


Figure 14.51 Syntrophy: Interspecies H_2 transfer. Shown is the fermentation of ethanol to methane and acetate by syntrophic association of an ethanol-oxidizing syntroph and a H_2-consuming partner (in this case, a methanogen). *(a)* Reactions involved. The two organisms share the energy released in the coupled reaction. *(b)* Overview of the syntrophic transfer of H_2.



(a) **Syntrophic culture**



(b) **Pure culture**

Figure 14.52 Energetics of growth of *Syntrophomonas* in syntrophic culture and in pure culture. *(a)* In syntrophic culture, growth requires a H_2-consuming organism, such as a methanogen. H_2 production is driven by reverse electron transport because the E_0' values of the NADH couple are more electropositive than that of $2\ H^+/H_2$. *(b)* In pure culture, energy conservation is linked to anaerobic respiration with crotonate reduction to butyrate. Inset: photomicrograph of cells of a fatty acid–degrading syntrophic bacterium (red) in association with a methanogen (green-yellow).

bifurcation reaction to reduce Fd_{ox} and menaquinone (Q). The Fd_{red} is then used by a cytoplasmic hydrogenase to produce one H_2, and the QH_2 is used in a membrane-associated endergonic reaction to reduce $2\ H^+$ to H_2. The free-energy change of this overall reaction is even more unfavorable than that of ethanol oxidation and in pure culture *Syntrophomonas* will obviously not grow on butyrate (Figure 14.52*a*). However, as with ethanol fermentation by *Pelotomaculum*, if the H_2 produced by *Syntrophomonas* is consumed by a partner organism, *Syntrophomonas* will grow on butyrate in coculture with the H_2-consuming partner. How does this occur?

Energetics of H_2 Transfer

In a syntrophic relationship, the removal of H_2 by a partner organism shifts the equilibrium of the entire reaction and pulls it in the direction of product formation; this can greatly affect the energetics of the reaction. Recall from our consideration of the principles of free energy (◀ Section 3.3) that the concentration of reactants and products in a reaction can have a major effect on the reaction's energetics. Said another way, the $\Delta G^{0\prime}$ of a reaction calculated under *standard* conditions may well differ from the ΔG of the reaction calculated under *actual* conditions (◀ Figure 3.7). At very low H_2 concentration, the energetics of oxidizing butyrate (or ethanol, or other fatty acids) to acetate plus H_2 become exergonic. For example, if a partner organism keeps the concentration of H_2 extremely low, the oxidation of butyrate by *Syntrophomonas* yields −18 kJ/mol (Figure 14.52*a*).

While H_2 transfer is characteristic of many syntrophic associations, particularly those associated with fermentation, it is also possible to have syntrophic associations in which *only electrons* are transferred. In direct electron transfer, such as the anoxic consumption of CH_4 by the ANME–sulfate reducer consortium (Section 14.16 and Figure 14.41), we learned that some respiratory syntrophs can use electrically conductive proteins (large multiheme cytochromes and "nanowires") to transfer electrons directly between cells separated by significant distances. In such syntrophic reactions, direct electron transfer does not depend on diffusion rates or H_2 concentrations because the electron transfer results from a direct connection between two cells.

Ecology of Syntrophs

Ecologically, syntrophic bacteria are key links in the anoxic steps of the carbon cycle (▶ Section 21.2). Syntrophs consume highly reduced fermentation products and release a key product, H_2, for anaerobic respirations. Without syntrophs, a bottleneck would develop in anoxic environments in which electron acceptors (other than CO_2) were limiting. By contrast, when conditions are oxic or alternative electron acceptors are abundant, syntrophic relationships are unnecessary. For example, if O_2 or NO_3^- is available as an electron acceptor, the energetics of the respiration of a fatty acid or an alcohol is so favorable that syntrophic relationships are unnecessary. Thus, syntrophy is characteristic of anoxic catabolisms in which primarily methanogenesis or acetogenesis are the terminal processes in the ecosystem. Methanogenesis is a major process in anoxic wastewater biodegradation, and microbiological studies of sludge granules that form in such systems have shown the close physical relationship that develops between H_2 producer and H_2 consumer in such habitats (Figure 14.52*a* inset).

The final part of this chapter, Part VII, considers the catabolism of a specific class of substrates: hydrocarbons. These electron donors, common in nature, can be degraded both aerobically and anaerobically, and once again, metabolic diversity is on display.

Check Your Understanding

- Give an example of interspecies H_2 transfer. How is it that both organisms benefit from this process?
- Could *Syntrophomonas* grow in a syntrophic association with a butyric acid fermenter like *Clostridium pasteurianum*?
- How does *Syntrophomonas* produce H_2, and what makes this possible?

VII • Hydrocarbon Metabolism

Hydrocarbons are organic compounds that are devoid of oxygen and can be catabolized both aerobically and anaerobically. Hydrocarbon catabolism requires oxygen, and anaerobically, a series of unique reactions solves this biochemical conundrum.

Hydrocarbons, molecules that contain only carbon and hydrogen atoms, are widely used by microbes as electron donors, and we wrap up our coverage of metabolic diversity with a consideration of this process. Unlike methane, hydrocarbons containing two or more carbons typically have to be oxygenated before they can be catabolized. We first consider the aerobic catabolism of aliphatic and aromatic hydrocarbons, where this oxygenation involves O_2. We then proceed with a consideration of anoxic hydrocarbon metabolism, a situation where oxygenation of the hydrocarbon is still necessary, but where O_2 obviously plays no role.

14.23 Aerobic Hydrocarbon Metabolism

We previously discussed the role of molecular oxygen (O_2) as an *electron acceptor* in energy-generating reactions. By contrast, O_2 also plays an important role as a *reactant* in the catabolism of hydrocarbons, and oxygenase enzymes are key players in the process.

Oxygenases and Aliphatic Hydrocarbon Oxidation

Oxygenases are enzymes that catalyze the incorporation of O_2 into organic compounds and in some cases, inorganic compounds (Section 14.9). There are two classes of oxygenases: *dioxygenases*, which catalyze the incorporation of *both atoms* of O_2 into the molecule, and *monooxygenases*, which catalyze the incorporation of *only one* of the two oxygen atoms of O_2 into an organic compound with the second atom of O_2 being reduced to H_2O. For most monooxygenases, the required electron donor is NADH or NADPH.

In the initial oxidation step of a saturated aliphatic (straight-chain) hydrocarbon, one of the atoms of O_2 is incorporated, typically at a terminal carbon atom. This reaction is catalyzed by a monooxygenase, and a typical reaction sequence is shown in **Figure 14.53*a***. The end product of the reaction sequence is a fatty acid of the same length as the original hydrocarbon. The fatty acid is then oxidized by *beta-oxidation*, a series of reactions in which two carbons of the fatty acid are split off at a time (Figure 14.53*b*). During beta-oxidation, NADH is formed and is oxidized in the electron transport chain for energy conservation purposes (◀ Figure 3.19). A single round of beta-oxidation releases acetyl-CoA plus a new fatty acid that is two carbon atoms shorter than the original fatty acid. The process of beta-oxidation is then repeated, and another acetyl-CoA molecule is released. The acetyl-CoA formed by beta-oxidation is either oxidized through the citric acid cycle (◀ Figure 3.12) or used

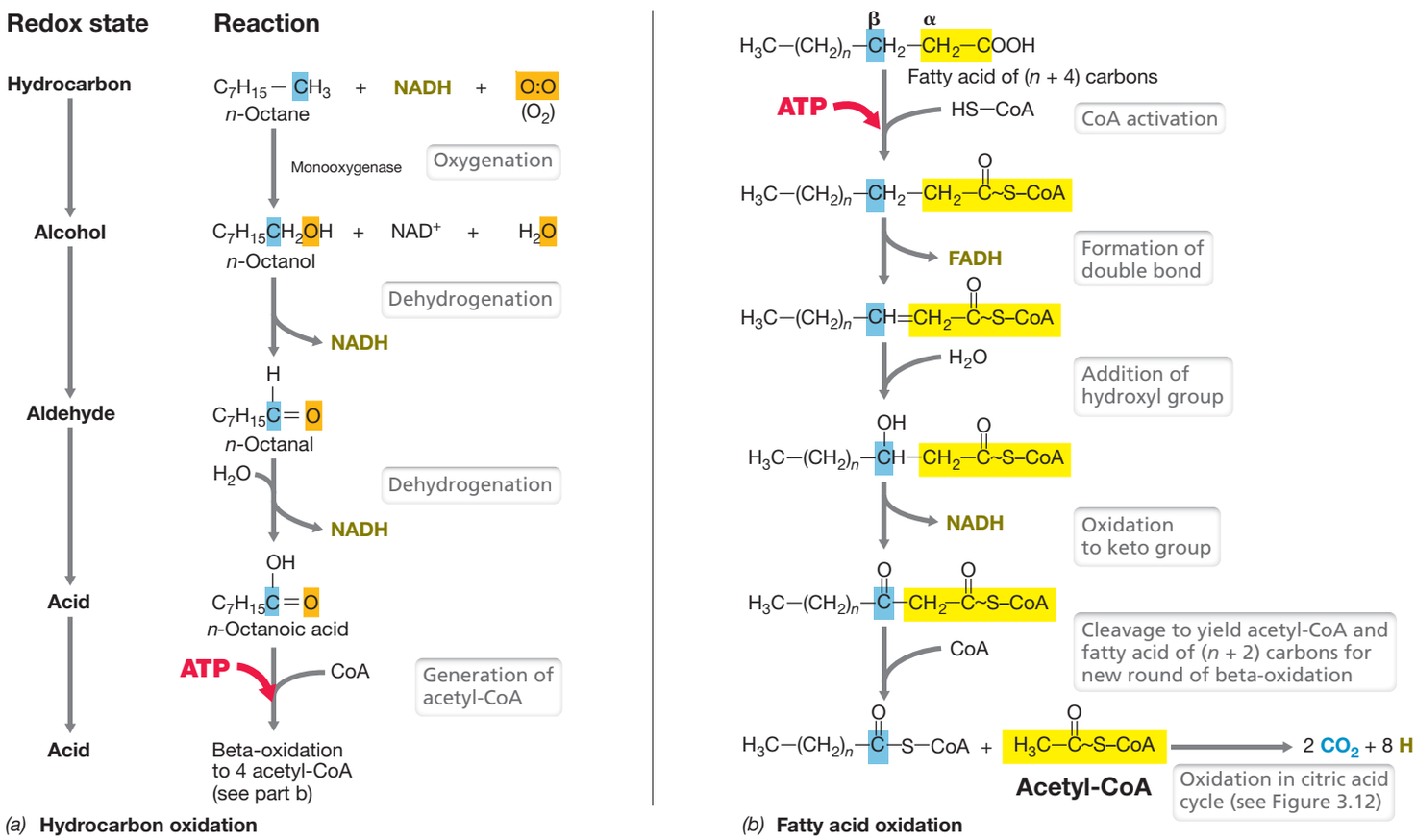


Figure 14.53 Monooxygenase activity and beta-oxidation. *(a)* Steps in the oxidation of an aliphatic hydrocarbon, the first of which is catalyzed by a monooxygenase. *(b)* Fatty acid oxidation by beta-oxidation leads to the successive formation of acetyl-CoA.

to make new cell material. With the exception of how the hydrocarbon is oxygenated, much of the biochemistry of anoxic hydrocarbon catabolism is the same as that shown for aerobic catabolism (Figure 14.53), with beta-oxidation reactions being of prime importance in both cases.

Aromatic Hydrocarbon Oxidation

Many aromatic (ringed) hydrocarbons can also be used as electron donors aerobically by microorganisms. The metabolism of these compounds, some of which contain multiple rings, such as naphthalene or biphenyls, typically has as its initial stage the formation of catechol or a structurally related compound via catalysis by oxygenase enzymes, as shown in **Figure 14.54**. Once catechol is formed it can be cleaved and further degraded into compounds that can enter the citric acid cycle, such as succinate, acetyl-CoA, and pyruvate.

Several steps in the aerobic catabolism of aromatic hydrocarbons require oxygenases. Figure 14.54*a–c* shows four different oxygenase-catalyzed reactions, one using a monooxygenase, two using a ring-cleaving dioxygenase, and one (toluene dioxygenase) using a ring-hydroxylating mechanism. As in aerobic aliphatic hydrocarbon catabolism (Figure 14.53), aromatic compounds, whether single- or multi-ringed, are typically oxidized completely to CO_2, with electrons entering an electron transport chain or used to make new cell material.

Check Your Understanding

- How do monooxygenases differ in function from dioxygenases?
- What is the final product of catabolism of a hydrocarbon?
- What is meant by the term beta-oxidation?

14.24 Anaerobic Hydrocarbon Metabolism

Although aerobic hydrocarbon oxidation is a major process in nature, anaerobic hydrocarbon oxidation linked to the reduction of nitrate, sulfate, or ferric iron as electron acceptors in anaerobic respirations (Sections 14.11–14.13) is also possible. And, as for the aerobic process, both aliphatic and aromatic hydrocarbons can be degraded anaerobically.

Aliphatic Hydrocarbons

Aliphatic hydrocarbons are substrates for denitrifying and sulfate-reducing bacteria. Saturated aliphatic hydrocarbons as long as C_{20} support growth, although shorter-chain hydrocarbons are more soluble and therefore more readily catabolized. The mechanism of anoxic hydrocarbon degradation has been well studied for hexane (C_6H_{14}) metabolism in denitrifying bacteria, organisms that use NO_3^- as an electron acceptor (▶ Section 15.10). However, the mechanism appears to be the same for the anoxic catabolism of longer-chain hydrocarbons and for anoxic hydrocarbon oxidation linked to other electron acceptors, and so we focus on the hexane/nitrate system here.

In anoxic hexane metabolism, hexane is modified on carbon atom 2 by attachment of a molecule of *fumarate*, a C_4 intermediate of the citric acid cycle (◀ Figure 3.12), forming the compound *1-methylpentylsuccinate* (**Figure 14.55*a***). The enzymatic addition of fumarate to hexane oxygenates the hydrocarbon without the need for O_2 and allows the molecule to be further catabolized anaerobically. Following the addition of coenzyme A, a series of reactions occurs that includes beta-oxidation (Figure 14.53*b*) and regeneration of fumarate (Figure 14.55*a*). The electrons released during beta-oxidation generate a proton motive force and are consumed in nitrate or sulfate reduction (Sections 14.11 and 14.12, respectively).

Aromatic Hydrocarbons

Aromatic hydrocarbons can also be degraded anaerobically through anaerobic respirations. For anoxic catabolism of the aromatic hydrocarbon toluene, oxygen needs to be added and this also occurs by the addition of fumarate (Figure 14.55*b*). The reaction series eventually yields benzoyl-CoA, which is further degraded by ring reduction (see Figure 14.56). Benzene

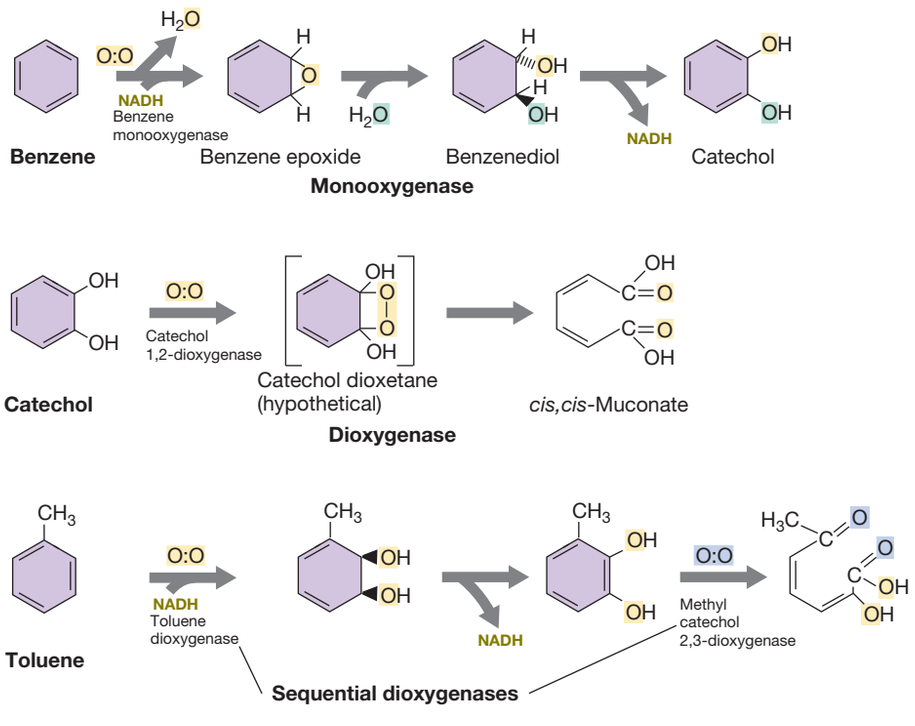


Figure 14.54 Roles of oxygenases in catabolism of aromatic compounds. Monooxygenases introduce one atom of oxygen from O_2 into a substrate, whereas diooxygenases introduce both atoms of oxygen. *(a)* Hydroxylation of benzene to catechol by a monooxygenase in which NADH is an electron donor. *(b)* Cleavage of catechol to *cis, cis*-muconate by an intradiol ring-cleavage dioxygenase. *(c)* The activities of a ring-hydroxylating dioxygenase and an extradiol ring-cleavage dioxygenase in the degradation of toluene. The oxygen atoms that each enzyme introduces are distinguished by different colors. Compare aerobic toluene catabolism to anoxic toluene catabolism shown in Figure 14.55*b*.

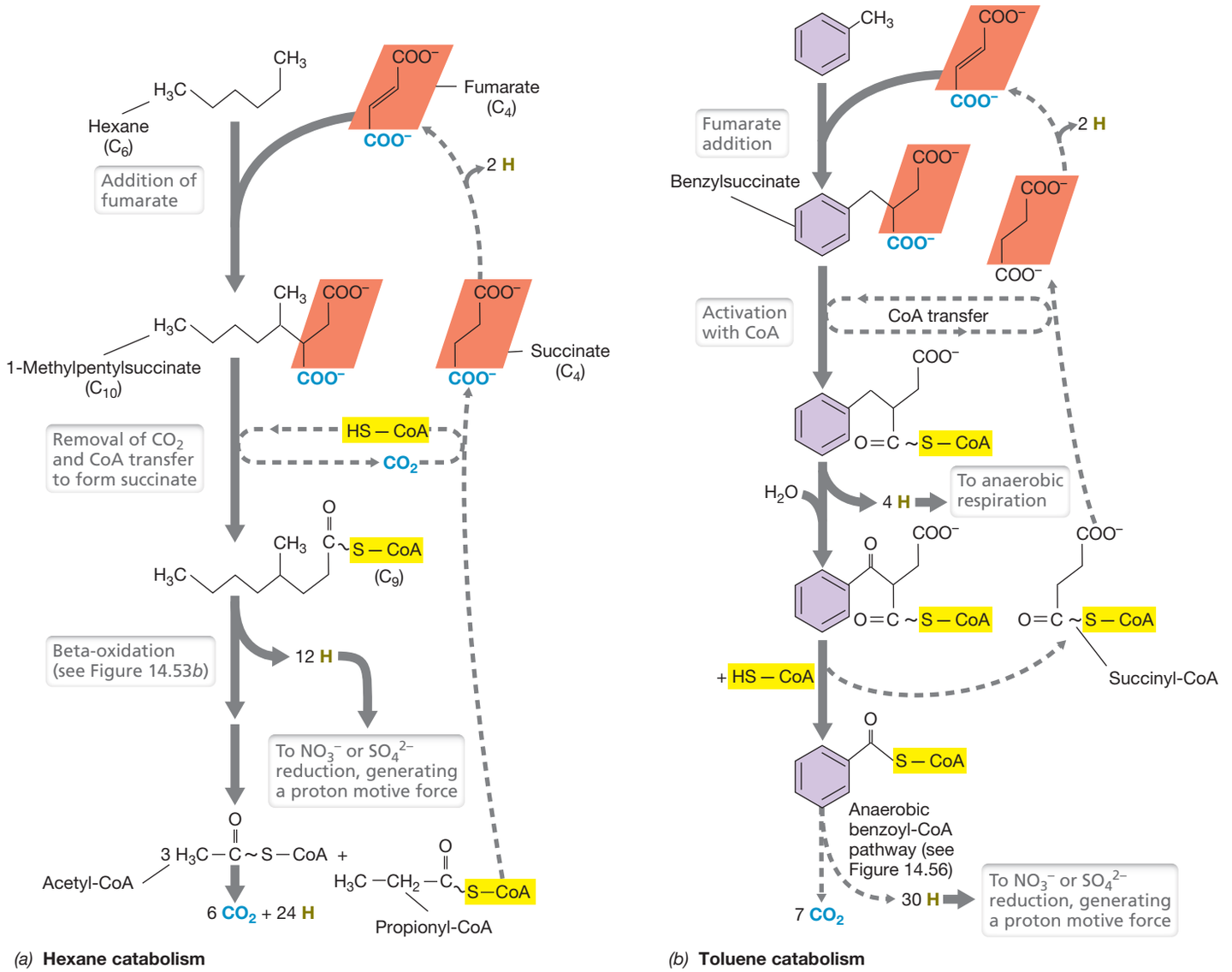


Figure 14.55 **Anoxic catabolism of two hydrocarbons.** *(a)* In anoxic catabolism of the aliphatic hydrocarbon hexane, the addition of fumarate provides the oxygen atoms necessary to form a fatty acid derivative that can be catabolized by beta-oxidation (see Figure 14.53*b*) to yield acetyl-CoA. Electrons (H) generated from hexane catabolism are used to reduce sulfate or nitrate in anaerobic respirations. *(b)* Fumarate addition during the anoxic catabolism of the aromatic hydrocarbon toluene forms benzylsuccinate.

(C_6H_6) can also be catabolized anaerobically, likely by a similar mechanism. Multi-ringed aromatic hydrocarbons such as naphthalene ($C_{10}H_8$) can be degraded by certain sulfate-reducing and denitrifying bacteria. But in contrast to other hydrocarbons, the oxygenation of multi-ringed hydrocarbons occurs by the addition of CO_2 to the ring to form a carboxylic acid derivative rather than by fumarate addition. However, this carboxylation reaction serves the same biochemical purpose as oxygenase reactions (Figures 14.53*a* and 14.54) or the addition of fumarate (Figure 14.55); an O atom becomes part of the hydrocarbon and facilitates its catabolism.

Many bacteria can catabolize certain aromatic hydrocarbons anaerobically, including even fermentative and phototrophic purple bacteria. However, except for toluene, only aromatic compounds that already contain an O atom are degraded, typically by a common mechanism. In contrast to aerobic catabolism that occurs by way of ring *oxidation* (Figure 14.54), anaerobic catabolism proceeds by ring *reduction*. The anaerobic degradation of aromatic hydrocarbons is often facilitated by their conversion into benzoate followed by aromatic ring cleavage and benzoate catabolism performed by the *benzoyl-CoA pathway* (**Figure 14.56**). Benzoate catabolism in this pathway begins by forming the coenzyme A derivative followed by ring cleavage to yield fatty or dicarboxylic acids (Figure 14.56) that can be further catabolized to intermediates of the citric acid cycle (◀ Figure 3.12).

As we reach the end of our survey of metabolic diversity, we need not keep in mind all of the metabolic details, for they are numerous and formidable, but instead, we need to see the "metabolic big picture" formed by the major themes of this chapter: the modularity of

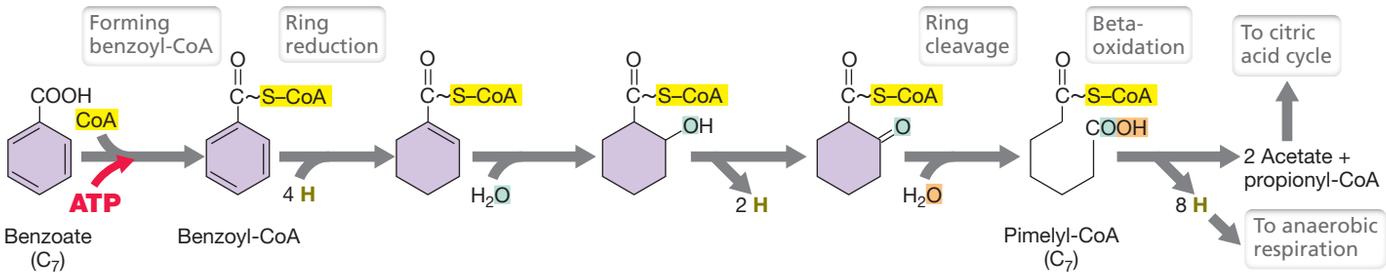


Figure 14.56 Anoxic degradation of benzoate by the benzoyl-CoA pathway. This pathway operates in the purple phototrophic bacterium *Rhodopseudomonas palustris* and many other facultative bacteria, both phototrophic and chemotrophic. Note that all intermediates of the pathway are bound to coenzyme A. The acetate produced is further catabolized in the citric acid cycle.

metabolism, and the key processes of phototrophy, chemolithotrophy, and autotrophy, respirations by electron donor or electron acceptor, C_1 metabolisms, fermentations, and hydrocarbon metabolism. These overarching principles will guide us through the next four chapters where our focus will be the diversity of the microbes themselves rather than their metabolisms. Chapters 15–17 in particular will cover many of the *Bacteria* and *Archaea* whose metabolisms we have described here, and we will witness there the many cases in which metabolic diversity and prokaryotic diversity are inextricably linked.

Check Your Understanding

- What is the benzoyl-CoA pathway, and how might it participate in the anaerobic degradation of toluene?
- How is hexane oxygenated during anoxic catabolism?

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CHAPTER REVIEW

I • Introduction to Metabolic Diversity

14.1 All cells must conserve energy, have a source of reducing power, and achieve redox balance in their metabolism. Energy is conserved through substrate-level phosphorylation, oxidative phosphorylation, or photophosphorylation. Reducing power is supplied by electron donors or must be generated in endergonic reactions. Redox balance is achieved by using terminal electron acceptors (in respiration) or fermentation products (including H_2) to export electrons from the cell.

Q What are reverse electron transport and electron bifurcation, and what role do they play in generating reducing power and achieving redox balance?

14.2 Autotrophy is supported in most phototrophic and chemolithotrophic bacteria by the Calvin cycle, in which the enzyme RuBisCO plays a key role. The reverse citric acid and hydroxypropionate cycles are autotrophic pathways in green sulfur and green nonsulfur bacteria, respectively.

Q What is a carboxysome, and what is its role in CO_2 fixation? Plants do not have carboxysomes; how might this affect the efficiency with which they fix CO_2?

II • Phototrophy

14.3 In photosynthesis, ATP is generated from light and then consumed in the reduction of CO_2. Two forms of photosynthesis are known: oxygenic, where O_2 is produced (for example, in cyanobacteria), and anoxygenic, where it is not (for example, in purple and green bacteria). Photosynthetic reaction centers and photosynthetic pigments reside in membranes where the light reactions of photosynthesis are carried out.

Q What are the functions of light-harvesting and reaction center chlorophylls?

14.4 Accessory pigments including carotenoids and phycobilins absorb light and transfer the energy to reaction center chlorophyll, thus broadening the wavelengths of light usable in photosynthesis. Carotenoids also play an important photoprotective role in preventing photooxidative damage to cells.

Q What accessory pigments are present in phototrophs, and what are their functions?

14.5 In anoxygenic phototrophs, electron transport reactions in photosynthetic membranes are driven by the photosynthetic reaction center, forming a proton motive force and ATP.

Q Why is reverse electron transport necessary in purple sulfur bacteria but not in green sulfur bacteria?

14.6 In oxygenic photosynthesis, H_2O donates electrons to drive CO_2 fixation, and O_2 is a by-product. There are two separate but interconnected photosystems in oxygenic phototrophs, PSI and PSII, whereas anoxygenic phototrophs contain a single photosystem.

Q How does the reduction potential (E_0') of chlorophyll *a* in PSI and PSII differ? Why must the reduction potential of PSII chlorophyll *a* be so highly electropositive?

III • Respiratory Processes Defined by Electron Donor

14.7 Reduced sulfur compounds such as H_2S, $S_2O_3^{2-}$, and S^0 are electron donors for energy conservation in sulfur chemolithotrophs. Electrons from these substances enter electron transport chains, yielding a proton motive force. Sulfur chemolithotrophs are also autotrophs and fix CO_2 by the Calvin cycle.

Q Compare and contrast the utilization of H_2S by a purple phototrophic bacterium and by a colorless sulfur bacterium such as *Beggiatoa*. What role does H_2S play in the metabolism of each organism?

14.8 Chemolithotrophic iron bacteria oxidize Fe^{2+} as an electron donor. Most iron bacteria grow at acidic pH and are often associated with acidic pollution from mineral and coal mining. A few chemolithotrophic and phototrophic bacteria can oxidize Fe^{2+} to Fe^{3+} anaerobically.

Q Why do most iron-oxidizing bacteria grow at an acidic pH?

14.9 The ammonia-oxidizing *Bacteria* and *Archaea* produce nitrite from ammonia, which is oxidized by nitrite-oxidizing *Bacteria* to nitrate.

Q Are there any types of *Bacteria* or *Archaea* that are able to oxidize ammonia to nitrate?

14.10 Anoxic ammonia oxidation (anammox) consumes both ammonia and nitrite, forming N_2. The anammox reaction occurs within a membrane-enclosed compartment called the anammoxosome.

Q Contrast classical nitrification with anammox in terms of oxygen requirements, organisms involved, and the need for monooxygenases.

IV • Respiratory Processes Defined by Electron Acceptor

14.11 Nitrate is a common electron acceptor in anaerobic respiration. Nitrate reduction is catalyzed by the enzyme nitrate reductase, reducing NO_3^- to NO_2^-. Denitrification is an anaerobic respiratory process through which NO_3^- is reduced to gaseous nitrogen compounds including NO, N_2O, or N_2.

Q Why is it that denitrifiers that reduce NO_3^- to N_2 can still release significant amounts of N_2O?

14.12 Sulfate-reducing bacteria are obligately anaerobic bacteria that reduce SO_4^{2-} to H_2S in a process in which SO_4^{2-} must first be activated to adenosine phosphosulfate (APS). Some sulfur-reducing organisms can reduce S^0 but not SO_4^{2-} to H_2S.

Q Why is the enzyme hydrogenase useful to *Desulfovibrio* even when it is not grown on H_2 as electron donor?

14.13 Iron-reducing bacteria can use nanowires to reduce insoluble electron acceptors on the outside of the cell. Many different electron-accepting respiratory reactions are possible including the reduction of various metals and metalloids, fumarate and other organic compounds, and various chlorinated compounds.

Q In what way might bacteria alter the toxicity of groundwater contaminated with arsenic, chromium, or even uranium?

V • One-Carbon (C_1) Metabolism

14.14 Acetogens are strict anaerobes that reduce CO_2 to acetate, usually with H_2 as electron donor. The mechanism of acetate formation is the reductive acetyl-CoA pathway, a pathway widely distributed in obligate anaerobes for either autotrophic purposes or acetate oxidation.

Q Describe the mechanism of energy conservation for acetogens growing on $H_2 + CO_2$.

14.15 Methanogenesis is the production of CH_4 from $CO_2 + H_2$ or from acetate or methanol by strictly anaerobic methanogenic *Archaea*. Several unique coenzymes are required for methanogenesis, and energy conservation is linked to either a proton or sodium motive force.

Q Why is it that methanogens that lack cytochromes are unable to grow on methanol?

14.16 Methanotrophy is the use of CH_4 as both carbon source and electron donor. Aerobic methanotrophs use the enzyme methane monooxygenase and oxidize CH_4 to formaldehyde, which is then assimilated by the cell through one of two pathways. Methane can also be oxidized anaerobically by diverse organisms through syntrophic partnerships or by generating O_2 as a catalyst for CH_4 oxidation.

Q Which pathway for C_1 assimilation found in aerobic methanotrophs is most energetically efficient and why? In what ways does *Methylomirabilis oxyfera* resemble denitrifiers and aerobic methanotrophs, and in what ways does it differ?

VI • Fermentation

14.17 In the absence of external electron acceptors, organic compounds can be catabolized anaerobically only by fermentation. In fermentation, most ATP is typically formed by substrate-level phosphorylation. Redox balance is achieved by the excretion of fermentation products.

Q Define the term substrate-level phosphorylation: How does it differ from oxidative phosphorylation? What compound(s) do fermentative bacteria need to synthesize in order to make ATP by substrate-level phosphorylation?

14.18 The lactic acid fermentation is carried out by homofermentative species, where lactate is the sole product, and heterofermentative species, where lactate, ethanol, and CO_2 are produced. The mixed-acid fermentation typical of enteric bacteria yields various acids plus neutral products (ethanol, butanediol), depending on the organism.

Q What are the major fermentation products of *Lactobacillus* and *Escherichia*?

14.19 Clostridia ferment sugars, amino acids, and other organic compounds, with butyric and acetic acids and H_2 being major products. Butyrate production allows for additional ATP to be produced. Energy-converting hydrogenases allow some fermenters to generate a proton or sodium motive force by producing H_2.

Q When fermenting 1.5 glucose to butyrate and acetate, *Clostridium pasteurianum* produces 3 NADH during glycolysis. How does it achieve redox balance?

14.20 The bacterium *Propionibacterium* produces propionate, acetate, and CO_2 in a secondary fermentation of lactate. By contrast, *Clostridium kluyveri* produces butyrate and caproate by oxidizing ethanol and reducing acetate.

Q What is the difference between primary and secondary fermentation?

14.21 Energy conservation in *Propionigenium*, *Oxalobacter*, and *Malonomonas* is linked to decarboxylation reactions that pump Na^+ or H^+ across the membrane; ATPases use the energy in the ion gradient to form ATP. The reactions catalyzed by these organisms yield insufficient free energy to make ATP by substrate-level phosphorylation but sufficient energy to pump ions.

Q Give an example of a fermentation that does not employ substrate-level phosphorylation.

14.22 In syntrophy, two organisms cooperate to degrade a compound that neither can degrade alone. In this process, H_2 produced by one organism is consumed by another. H_2 consumption affects the energetics of the reaction carried out by the H_2 producer, allowing it to make ATP where it otherwise could not.

Q When do syntrophic relationships not involve H_2 transfer?

VII • Hydrocarbon Metabolism

14.23 In addition to its role as a terminal electron acceptor, O_2 can also be introduced directly into other substances. In aerobic metabolism, oxygenases introduce atoms of oxygen from O_2 into hydrocarbons. Once oxygenated, aliphatic hydrocarbons can be further degraded by beta-oxidation and aromatic hydrocarbons by ring splitting and oxidation.

Q How do monooxygenases differ from dioxygenases in terms of the reactions they catalyze? Why are oxygenases necessary for the aerobic catabolism of hydrocarbons?

14.24 Hydrocarbons can be oxidized under anoxic conditions following addition of the dicarboxylic acid fumarate. Aromatic compounds are catabolized anaerobically by ring reduction and cleavage to form intermediates that can be catabolized in the citric acid cycle.

Q How do denitrifying and sulfate-reducing bacteria degrade hydrocarbons anaerobically and without oxygenases?

APPLICATION QUESTIONS

1. The growth rate of the phototrophic purple bacterium *Rhodobacter*, grown *phototrophically* with H_2 as electron donor, is about twice as fast in a medium containing malate as in a medium containing CO_2 as the only carbon source. Discuss the reasons why this is true and list the nutritional class in which we would place *Rhodobacter* when growing under each of the two different conditions.
2. A fatty acid such as butyrate cannot be fermented in pure culture, but in many anoxic habitats, fermentative organisms oxidize it readily. Why can butyrate be fermented in a community of organisms when this process cannot be performed by a pure culture?
3. Compare and contrast the methanogenic pathway for growth on $H_2 + CO_2$ in methanogens that have cytochromes with those that lack cytochromes. Why do acetate-degrading (acetoclastic) methanogens not need to fix CO_2?

CHAPTER GLOSSARY

Acetogenesis energy metabolism in which acetate is produced from either H_2 plus CO_2 or from organic compounds

Anaerobic respiration a form of respiration in which the terminal electron acceptor is not O_2

Anammox anoxic ammonia oxidation

Anoxygenic photosynthesis photosynthesis in which O_2 is not produced

Antenna pigments light-harvesting chlorophylls or bacteriochlorophylls in photocomplexes that funnel energy to the reaction center

Autotroph an organism capable of biosynthesizing all cell material from CO_2 as the sole carbon source

Bacteriochlorophyll the chlorophyll pigment of anoxygenic phototrophs

Calvin cycle the series of biosynthetic reactions by which most phototrophs and most aerobic chemolithotrophs convert CO_2 into organic compounds

Carboxysomes a polyhedral cellular inclusion of crystalline ribulose biphosphate carboxylase (RuBisCO), the key enzyme of the Calvin cycle

Carotenoid a hydrophobic accessory pigment present along with chlorophyll in photosynthetic membranes

Chlorophyll a light-sensitive, Mg-containing porphyrin of phototrophic organisms that initiates the process of photophosphorylation

Chlorosome a cigar-shaped structure present in the periphery of cells of green sulfur and green nonsulfur bacteria and containing the antenna bacteriochlorophylls (*c*, *d*, or *e*)

Conserve energy to convert energy from chemical reactions or light into a form that can do work, such as ATP

Denitrification anaerobic respiration in which NO_3^- or NO_2^- is reduced to nitrogen gases, primarily N_2

Electron bifurcation (confurcation) an exergonic redox reaction including three simultaneous half reactions whereby 4 electrons ($2 \times 2\,e^-$) from a donor are split between an exergonic reduction ($2 \times 1\,e^-$) and an endergonic reduction ($2 \times 1\,e^-$); the reverse reaction is a *confurcation* causing an endergonic reduction involving two e^- that is driven by an exergonic reduction performed with the other two e^-

Energy-converting hydrogenase a membrane-bound enzyme that uses reduced ferredoxin to reduce protons to H_2 and couples this reaction to the generation of a proton or sodium motive force

Fermentation anaerobic catabolism in which an organic compound is both an electron donor and an electron acceptor and ATP is produced by substrate-level phosphorylation

Heterofermentative in reference to lactic acid bacteria, capable of making a mixture of fermentation products typically including lactate, ethanol, and CO_2, from the fermentation of glucose

Homofermentative in reference to lactic acid bacteria, producing only lactic acid as a fermentation product

Hydrogenase an enzyme, widely distributed in anaerobic microorganisms, capable of oxidizing or evolving H_2

Methanogen a methane-producing species of the *Archaea*

Methanogenesis the biological production of CH_4

Methanotroph an organism capable of oxidizing methane (CH_4) as an electron donor in energy metabolism

Methylotroph an organism capable of oxidizing organic compounds that do not contain carbon–carbon bonds; if able to oxidize CH_4 also a methanotroph

Mixotroph an organism that conserves energy from the oxidation of inorganic compounds but requires organic compounds serve as a carbon source

Nanowires electrically conductive type IV pili used to exchange electrons with extracellular objects, either other cells or abiotic structures

Nitrification the microbial oxidation of ammonia to nitrate

Oxygenase an enzyme that catalyzes the incorporation of oxygen from O_2 into organic or inorganic compounds

Oxygenic photosynthesis photosynthesis carried out by cyanobacteria and green plants in which O_2 is evolved

Photosynthesis the series of reactions in which ATP is synthesized by light-driven reactions and CO_2 is fixed into cell material

Phototroph an organism that uses light as its source of energy

Phycobiliprotein the antenna pigment complex in cyanobacteria that contains phycocyanin and allophycocyanin or phycoerythrin coupled to proteins

Phycobilisome an aggregate of phycobiliproteins

Reaction center a photosynthetic complex containing chlorophyll or bacteriochlorophyll and several other components; the initial electron transfer reactions of photosynthetic electron flow occur here

Redox balance having the same number of electrons in the reactants and the products of metabolism

Reducing power the ability to donate electrons in metabolic reactions

Reductive acetyl-CoA pathway a pathway of CO_2 fixation used by obligate anaerobes such as acetogens and methanogens; the pathway reduces CO_2 to acetate and can be reversed to oxidize acetate to CO_2

Reductive dechlorination an anaerobic respiration in which a chlorinated organic compound is used as an electron acceptor, usually with the release of Cl^-

Respiration the process in which an electron donor is oxidized and electrons are passed through electron transport reactions to an external electron acceptor in order to generate a proton motive force and synthesize ATP by oxidative phosphorylation

Reverse citric acid cycle a pathway of CO_2 fixation used in green sulfur bacteria as well as microaerophilic and anaerobic chemolithotrophs

Reverse electron transport a method of generating reducing power, usually in the form of NADH or Fd_{red}, whereby the endergonic reduction of electron carriers is driven by dissipation of a proton motive force

Ribulose monophosphate pathway a reaction series in certain methylotrophs in which formaldehyde is assimilated into cell material using ribulose monophosphate as the C_1 acceptor molecule

RuBisCO ribulose bisphosphate carboxylase, a key enzyme for CO_2 fixation by the Calvin cycle

Secondary fermentation a fermentation in which the substrates are the fermentation products of other organisms

Serine pathway a reaction series in certain methylotrophs in which CH_2O plus CO_2 are assimilated into cell material by way of the amino acid serine

Stickland reaction the fermentation of an amino acid pair

Syntrophy a process whereby two or more microorganisms cooperate to degrade a substance neither can degrade alone

Thylakoids membrane stacks containing the photosynthetic pigments in cyanobacteria or in the chloroplast of eukaryotic phototrophs

15 Ecological Diversity of *Bacteria*

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MICROBIOLOGYNOW

Cyanobacterial Diversity and Environmental Change

Cyanobacteria have had an enormous impact on the Earth for over 2 billion years: They caused Earth's oxygenation and still produce much of the oxygen we breathe today. Cyanobacteria show tremendous ecological diversity, and they are an essential component of healthy ecosystems worldwide. However, the ecological traits that make cyanobacteria so successful also allow them to respond rapidly to environmental change. Human activities alter ecosystem function, and when these activities promote the unrestricted growth of bacteria, the results can be catastrophic.

Intensive agriculture often produces nitrogen and phosphorus runoff that causes ecosystem eutrophication (nutrient enrichment). Phosphorus availability limits cyanobacterial growth in freshwater, and excess phosphorus causes rapid growth of certain cyanobacteria, resulting in harmful blooms. These cyanobacterial blooms release toxins and smother aquatic plants, and subsequent bloom decomposition fuels massive growth of heterotrophic bacteria, causing anoxic conditions that kill aquatic animals. Lake Erie (USA) was once so highly eutrophic it was considered a dead lake, but environmental regulations restricted phosphorus inputs, restoring its health. However, eutrophication is a problem once again, now largely because of nitrogen runoff.

Microcystis is a genus of freshwater cyanobacteria. While many cyanobacteria can fix N_2, *Microcystis* cannot, and hence nitrogen enrichment favors the growth of *Microcystis*. Recent blooms in Lake Erie (see natural-color NASA photo, September 2017) were dominated by *Microcystis* species that produce microcystin, a toxin that can kill animals (and humans). A massive *Microcystis* bloom left the city of Toledo, Ohio (USA) without drinking water in 2014, and a similar bloom in Wuxi, China, affected millions of people in 2007.

Elevated temperatures, CO_2, and nutrient levels associated with climate change are predicted to make such blooms more frequent and more intense in the future. It is thus essential that we understand the ecological diversity of microbes such as cyanobacteria if we are to predict and manage their responses to environmental change.

Source: Steffen, M.M., et al. 2017. Ecophysiological examination of the Lake Erie *Microcystis* bloom in 2014: Linkages between biology and the water supply shutdown of Toledo, OH. *Environ. Sci. Technol. 51*: 6745.

I • Ecological Diversity Among Microorganisms

Diversity in the microbial world can be described in many different ways. Ecological diversity emphasizes the characteristics of microbial groups in the context of their environments and the microbes they interact with.

Microbial diversity defies simple definitions. If we are to make sense of microbial diversity, we must first identify and measure how one organism differs from another. Microbes differ in many ways, but at a fundamental level we define these differences in terms of three aspects: metabolism, ecology, and phylogeny. Ultimately, these three threads weave together in the tapestry that we call microbial diversity, and only by examining each thread can we understand their connections. We explored microbial metabolic diversity in the previous chapter, and we will explore the phylogenetic diversity of microorganisms in the chapters that follow. Now we will consider and define microbial diversity in terms of ecological differences between microorganisms.

15.1 Making Sense of Microbial Diversity

Metabolic diversity, as explored in the last chapter, is defined in terms of the *cellular processes* that support growth. **Ecological diversity**, which we review in the present chapter, is defined in terms of *microbial interactions* between organisms and their environments. Finally, **phylogenetic diversity**, which we will review in the coming chapters, is defined by *evolutionary relationships* between organisms.

Ecological diversity is the component of microbial diversity that deals with how microbes interact with each other and with their environments. In the microbial world, ecological interactions are often defined by metabolic traits. For example, consider lactic acid bacteria (◀ Section 14.18). These fermentative organisms achieve redox balance by excreting lactic acid as a fermentation product. The excretion of lactic acid also lowers the pH of the environment. Lactic acid bacteria grow well in these acidic conditions, and this acidity inhibits their neutrophilic competitors. Hence, lactic acid production allows these bacteria to interact with their environment and their competitors in a way that facilitates their own growth. In addition to transforming the environment, the products of metabolism often serve as microbial substrates for other microbes, leading to complex mutualistic interactions (◀ Section 14.22). For example, the lactic acid just mentioned is still an energy-rich substance and is actively consumed by propionic acid bacteria, secondary fermenters that ferment lactate into propionate (◀ Section 14.20). Hence, in the microbial world, ecology is interdependent with metabolism.

While it is useful to consider ecological diversity in terms of metabolism, it is incorrect to consider ecological diversity solely in terms of metabolism. Microbes that have similar metabolic traits can still differ substantially in their ecology. For example, organisms that have similar metabolic traits can differ with respect to their morphology, motility, growth rate, environmental tolerances, mutualisms and antagonisms, as well as their systems of gene regulation and development. All of these traits can have major impacts on microbial distribution and activity in the environment. These complex traits cannot be defined solely in terms of metabolism and are best understood in terms of ecological interactions.

Most fundamentally, phylogenetic diversity deals with the diversity of evolutionary lineages such as phyla, genera, and species. At its broadest, phylogenetic diversity encompasses the genetic and genomic diversity of evolutionary lineages and so can be defined on the basis of either genes or organisms (◀ Section 13.11). Most commonly, though, phylogenetic diversity is defined on the basis of ribosomal RNA gene phylogeny, which is thought to reflect the phylogenetic history of the entire organism (◀ Sections 13.3 and 13.12). Phylogenetic diversity is the overarching theme of our coverage of microbial diversity in Chapters 16–18.

In many cases, metabolic and ecological characteristics align with phylogenetic groups (for example, with the organisms described in Sections 15.3, 15.4, 15.6, 15.7, and 15.17). Microbial ecological diversity, however, often does not correspond with phylogenetic diversity as defined by the 16S ribosomal RNA gene. We will see many examples in this chapter where metabolic and ecological traits are widely distributed among the *Bacteria* and *Archaea* (**Figure 15.1**), and at least three reasons can account for why metabolic and ecological traits are shared between divergent organisms. The first is *gene loss*, a situation where a trait present in the common ancestor of several lineages is subsequently lost in some lineages but retained in others that over evolutionary time became quite divergent. The second is **convergent evolution**, in which a trait has evolved independently in two or more lineages and is not encoded by homologous genes shared by these lineages. The third is **horizontal gene transfer** (◀ Sections 9.5–9.11 and 13.9–13.11), a situation where genes that confer a particular trait are homologous and have been exchanged between distantly related lineages. Figure 15.1 emphasizes these points by showing how many common metabolisms are dispersed among distinct phylogenetic groups of microbes.

The examples of ecological diversity provided in this chapter are meant to be illustrative and not exhaustive, and we will consider other organisms with important ecological functions in Chapters 16–18 and 20–23. In particular, we will explore the causes and consequences of ecological diversity when we consider the science of microbial ecology in Unit 5 (Chapters 19–23) and bring microbial ecology into a human focus when we consider the human microbiome in Chapter 24.

Check Your Understanding

- Describe a metabolic process that would have a major impact on an organism's ecology.
- What are three reasons that metabolic or ecological traits might not correspond with distinct phylogenetic groups as defined by 16S ribosomal RNA gene sequences?
- Why is it useful to consider microbial diversity in terms of metabolic diversity, ecological diversity, and phylogenetic diversity?

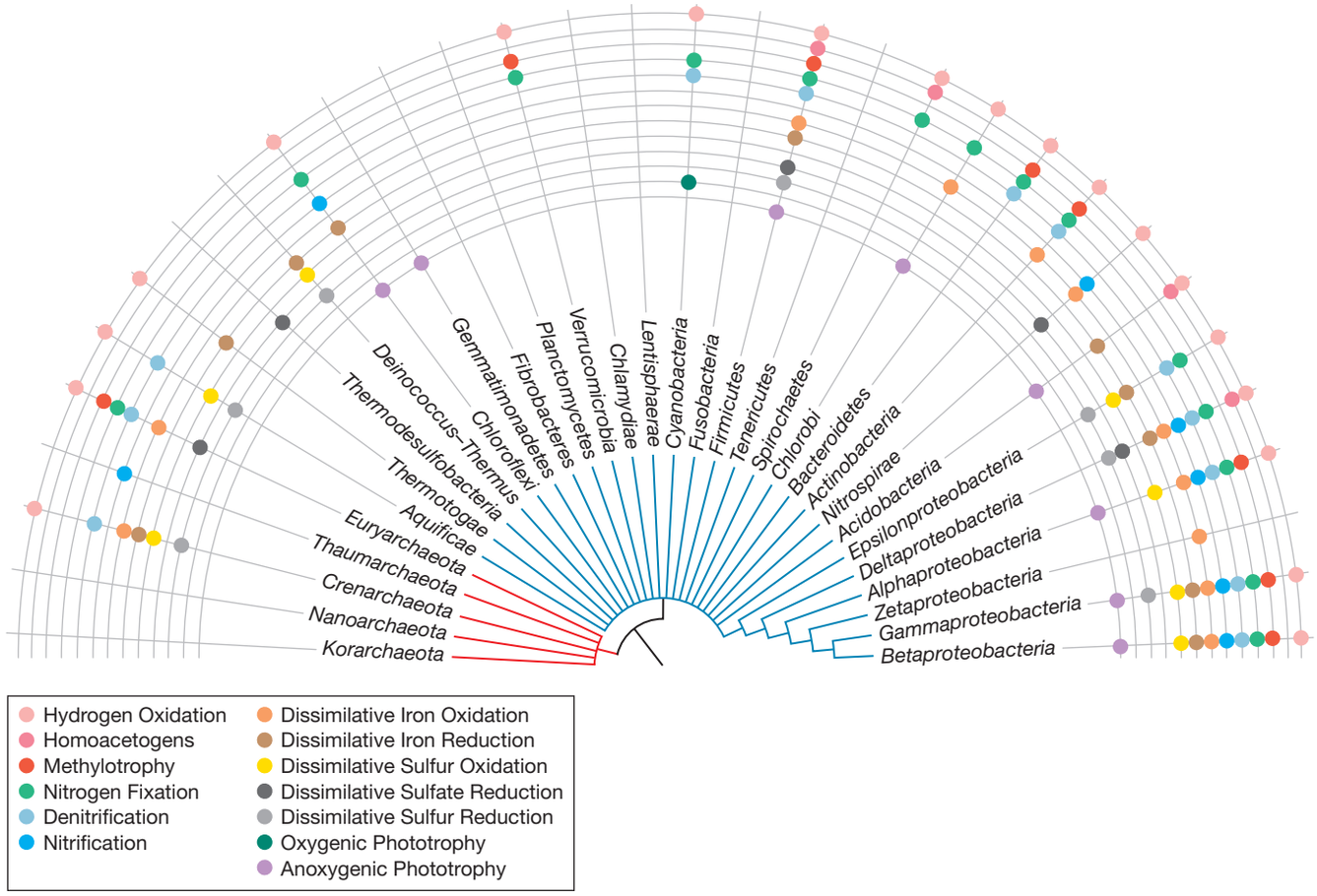


Figure 15.1 Major functional traits mapped across major phyla of *Bacteria* and *Archaea*. The dendrogram shows relationships between microbial phyla as inferred by analysis of 16S ribosomal RNA gene sequences. Blue branches are used to denote phyla of *Bacteria* and red branches denote phyla of *Archaea*. Colored circles indicate phyla that contain at least one species with a functional trait indicated in the color key.

UNIT 4

II • Ecological Diversity of Phototrophic *Bacteria*

Light is a powerful energy source and has been tapped by a wide variety of microorganisms—the phototrophs—whose metabolisms share many key attributes yet differ in major ways.

In this section we consider the ecological diversity of phototrophic microorganisms, those microbes that conserve energy from light. We will see that phototrophy is widespread within the domain *Bacteria* and that several distinct types of phototrophs can be defined on the basis of their metabolic and ecological traits.

15.2 Overview of Phototrophic *Bacteria*

The ability to conserve energy from light evolved early in the history of life, when the Earth was anoxic (◀ Section 13.2). Photosynthesis originated within the *Bacteria*, and the first phototrophic organisms were *anoxygenic phototrophs*, organisms that do not generate O_2 as a product of photosynthesis (◀ Section 14.5). Instead of H_2O, these early phototrophs likely used H_2, ferrous iron (Fe^{2+}), or H_2S as the electron donor for photosynthesis. Anoxygenic photosynthesis is present in six bacterial phyla: the *Proteobacteria*, *Chlorobi*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, and *Gemmatimonadetes*. Oxygenic photosynthesis, by contrast, is known only within the *Cyanobacteria* (Figure 15.1). There is extensive metabolic diversity among the anoxygenic phototrophs, which are found in a wide range of habitats. It is clear that horizontal gene exchange has had a major impact on the evolution of photosynthesis and on the distribution of photosynthetic genes across the phylogenetic tree of *Bacteria*.

Phototrophic bacteria have several common features. All phototrophic bacteria use chlorophyll-like pigments and various accessory pigments to harvest energy from light and transfer this energy to a membrane-bound reaction center where it is used to drive electron transfer reactions that ultimately result in the production of ATP (◀ Sections 14.3–14.5). There are two different types of photosynthetic reaction centers: FeS-type reaction centers, which are found in photosystem I of oxygenic phototrophs, and Q-type reaction centers, which are found in photosystem II of oxygenic phototrophs (◀ Sections 14.3–14.6). Both types of reaction centers are present

in *Cyanobacteria* (◀ Section 14.6), whereas only one type or the other is present in anoxygenic phototrophs. In some cases, photosynthetic pigments are found in the cytoplasmic membrane, but often they are present in intracellular photosynthetic membrane systems that originate from invaginations of the cytoplasmic membrane. These internal membranes allow phototrophic bacteria to increase the amount of pigment they contain for better use of light of low intensities.

Many phototrophic bacteria couple light energy to carbon fixation through a variety of different mechanisms (◀ Section 14.2), but not all phototrophs fix CO_2; some instead either prefer or require organic sources of carbon to support growth. We will see that many of the characteristics of phototrophic bacteria, including their membrane systems and photosynthetic pigments, have evolved as a result of niche adaptation for the light environment.

As we begin our tour of ecological diversity, it can be seen that each of the sections in this and the next three chapters begins with a list of a few "Key Genera." These names are simply meant to familiarize the student with some of the most common microbes in the microbial world and are often the names that appear if one travels beyond this text book to explore the primary scientific literature.

Check Your Understanding

- What form of photosynthesis was most likely the first to appear on Earth?
- What are the different types of photosynthetic reaction centers?
- Why are pigments important to phototrophs?

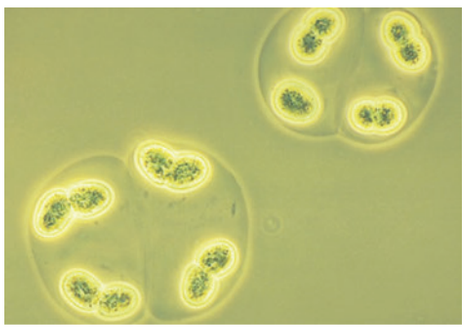
15.3 *Cyanobacteria*

KEY GENERA: *Prochlorococcus, Crocosphaera, Synechococcus, Trichodesmium, Oscillatoria, Anabaena*

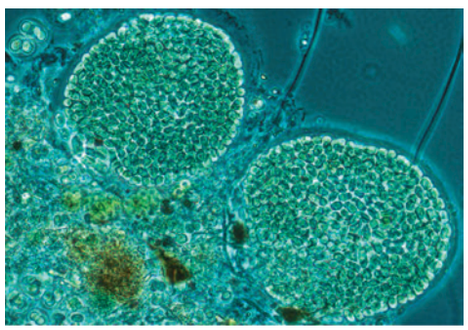
Cyanobacteria comprise a large, morphologically and ecologically heterogeneous group of oxygenic, phototrophic *Bacteria*. As we saw in Section 13.2, these organisms were the first oxygen-evolving phototrophic organisms on Earth, and over billions of years they converted the once anoxic atmosphere of Earth to the oxygenated atmosphere we see today. Though they all share the same form of metabolism, we will see that cyanobacterial species encompass extensive ecological diversity.

Phylogeny and Classification of *Cyanobacteria*

The morphological diversity of the *Cyanobacteria* is impressive (**Figure 15.2**). Both unicellular and filamentous forms exist, comprising considerable variation in morphology. Cyanobacterial cells range in size from 0.5 μm in diameter to cells as large as 100 μm in diameter. *Cyanobacteria* can be divided into five morphological groups: (1) *Chroococcales* are unicellular, dividing by binary fission (Figure 15.2*a*); (2) *Pleurocapsales* are unicellular, dividing by multiple fission (colonial) (Figure 15.2*b*); (3) *Oscillatoriales* are filamentous nonheterocystous forms (Figure 15.2*c*); (4) *Nostocales* are filamentous, divide along a single axis, and are capable of cellular differentiation (Figure 15.2*d*); and (5) *Stigonematales* are morphologically similar to *Nostocales* except that cells divide in multiple planes, forming branching filaments (Figure 15.2*e*). Finally, the **prochlorophytes** are a lineage of unique unicellular *Cyanobacteria* once thought to be distinct but now classified within the *Chroococcales*.


Susan Barns and Norman Pace

(a)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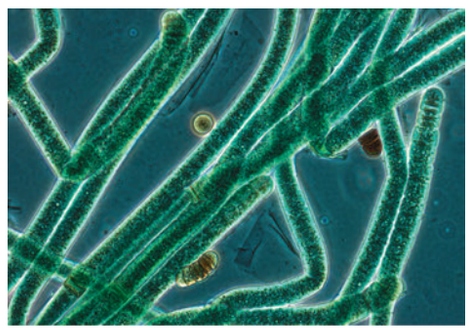
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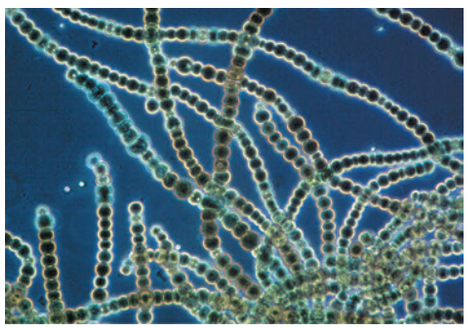
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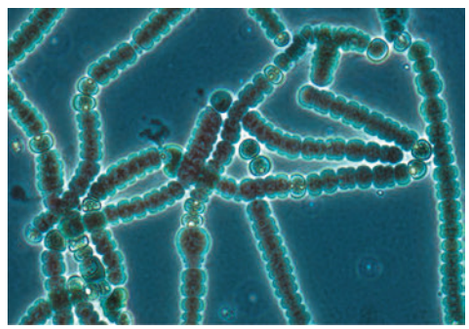

Daniel H. Buckley

(e)

Figure 15.2 The five major morphological types of *Cyanobacteria*. *(a)* Unicellular, *Gloeothece*; a single cell measures 5–6 μm in diameter; *(b)* colonial, *Pleurocapsa*; these structures are >50 μm in diameter and contain hundreds of cells; *(c)* filamentous, *Lyngbya*; a single cell measures about 10 μm wide; *(d)* filamentous heterocystous, *Nodularia*; a single cell measures about 10 μm wide; *(e)* filamentous branching, *Fischerella*; a cell is about 10 μm wide. See how morphological diversity relates to phylogenetic diversity in Figure 15.3.

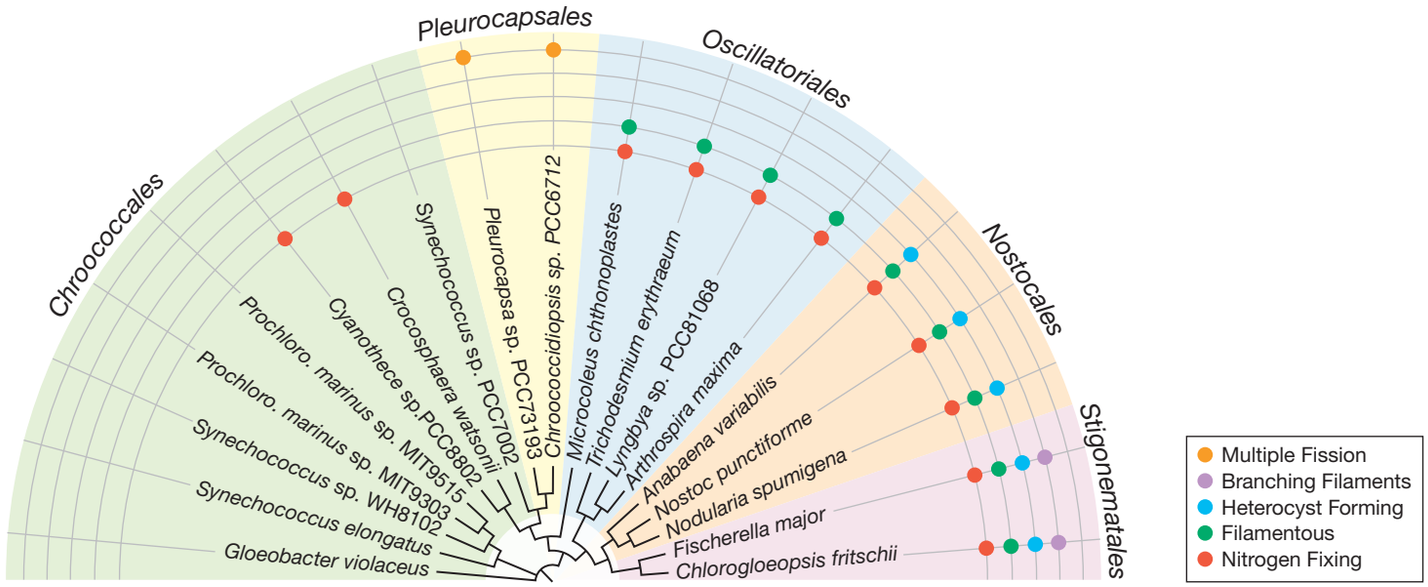


Figure 15.3 **Taxonomically informative traits mapped onto the phylogeny of *Cyanobacteria*.** The dendrogram depicts phylogenetic relationships inferred from analysis of conserved protein families in cyanobacterial genomes. Colored circles are used to indicate species traits as indicated by the key. Color shading is used to indicate taxonomic groupings. "*Prochloro.*" is used to indicate *Prochlorococcus*, which is a distinct group within the *Chroococcales*. Note that the *Chroococcales* and *Oscillatoriales* are not monophyletic in origin, meaning that their shared trait of nitrogen fixation has arisen independently on multiple occasions in the phylogeny.

Some of the major morphological classifications of *Cyanobacteria* correspond to coherent phylogenetic groups, but others do not (Figure 15.3). Species of *Pleurocapsales* form a coherent group within the cyanobacteria, indicating that reproduction by multiple fission arose only once in the evolutionary history of *Cyanobacteria* (Figure 15.3). Likewise, species of the *Nostocales* and *Stigonematales* share a common ancestor and form a coherent phylogenetic group, indicating a single origin of cellular differentiation within the *Cyanobacteria* (Figure 15.3). All *Stigonematales* share a single ancestor within the clade composed of *Nostocales* and *Stigonematales*, indicating that the capacity to form branching filaments arose only once within the lineage of *Cyanobacteria* capable of cellular differentiation (Figure 15.3). In contrast, unicellular and simple filamentous *Cyanobacteria* (*Chroococcales* and *Oscillatoriales*, respectively) are dispersed in the cyanobacterial phylogeny, and these morphological groups do not represent coherent evolutionary lineages (Figure 15.3).

Physiology and Photosynthetic Membranes

Cyanobacteria are oxygenic phototrophs and therefore have both FeS-type and Q-type photosystems. All species are able to fix CO_2 by the Calvin cycle, many can fix N_2, and most can synthesize their own vitamins (◀ Sections 3.12 and 4.1). Cells harvest energy from light and fix CO_2 during the day. During the night, cells generate energy by fermentation or aerobic respiration of carbon storage products such as glycogen. While CO_2 is the predominant source of carbon for most species, some *Cyanobacteria* can assimilate simple organic compounds such as glucose and acetate if light is present, a process called *photoheterotrophy*. A few *Cyanobacteria*, mainly filamentous species, can also grow in the dark on glucose or sucrose, using the sugar as both carbon and energy source. Finally, when sulfide concentrations are high, some *Cyanobacteria* are able to switch from oxygenic photosynthesis to anoxygenic photosynthesis using hydrogen sulfide rather than water as electron donor for photosynthesis (◀ Figure 14.18).

Cyanobacteria have specialized membrane systems called *thylakoids* that increase the ability of cells to harvest light energy (◀ Figure 14.9). Photosynthesis takes place in the thylakoid membrane, a complex and multilayered photosynthetic membrane system containing photopigments and proteins that mediate photosynthesis (◀ Sections 14.3 and 14.4). In most unicellular *Cyanobacteria*, the thylakoid membranes are arranged in regular concentric circles around the periphery of the cytoplasm (Figure 15.4). *Cyanobacteria* produce chlorophyll *a*, and most also have characteristic pigments called **phycobilins** (◀ Figure 14.14), which function as accessory pigments in photosynthesis. One class of phycobilins, *phycocyanins*, are blue and, together with the green chlorophyll *a*, are responsible for the blue-green color of most cyanobacteria. Some *Cyanobacteria* produce *phycoerythrin*, a red phycobilin, and species producing phycoerythrin

Figure 15.4 **Thylakoids in *Cyanobacteria*.** Electron micrograph of a thin section of the cyanobacterium *Synechococcus lividus*. A cell is about 5 μm in diameter. Note thylakoid membranes running parallel to the cell wall.

are red or brown. Photopigments are fluorescent and emit light when visualized using a fluorescence microscope; chlorophyll *a*, for example, fluoresces bright red (**Figure 15.5**). Prochlorophytes, such as *Prochlorococcus* and *Prochloron*, are unique among *Cyanobacteria* in that all members of this group contain chlorophyll *a* and *b* but do not contain phycobilins.

Cellular Structures and Motility

The cell wall of *Cyanobacteria* contains peptidoglycan and is structurally similar to that of other gram-negative bacteria. And, like many bacteria, *Cyanobacteria* possess several mechanisms for motility but do not contain flagella. Many cyanobacteria exhibit gliding motility (◀ Section 2.10). Gliding occurs only when a cell or filament is in contact with a solid surface or with another cell or filament. In some *Cyanobacteria*, gliding is not a simple translational movement but is accompanied by rotations, reversals, and flexing of filaments (◀ Figure 2.37*b*). Most gliding species exhibit directional movement toward light (phototaxis), and chemotaxis (◀ Section 2.11) may occur as well. *Synechococcus* exhibits an unusual form of swimming motility that does not require flagella or any other extracellular organelle. The cell surface of *Synechococcus* has specialized proteins that provide direct thrust by way of a mechanism that has yet to be resolved. Gas vesicles (◀ Section 2.7) are also found in a variety of aquatic *Cyanobacteria* and are important in positioning cells in the water column. The function of gas vesicles is to regulate cell buoyancy such that cells can remain in a position in the water column where light intensity is optimal for photosynthesis.

Cyanobacteria are able to form a variety of structures associated with energy storage, reproduction, and survival. Many *Cyanobacteria* produce extensive mucilaginous envelopes, or sheaths, that bind groups of cells or filaments together (Figure 15.2*a*). Some filamentous cyanobacteria form *hormogonia* (**Figure 15.6**), short, motile filaments that break off from longer filaments to facilitate dispersal in times of stress. Some species also form resting structures called *akinetes* (Figure 15.6*c*), which protect the organism during periods of darkness, desiccation, or cold. Akinetes are cells with thickened outer walls. When conditions improve, akinetes germinate by breaking down their outer wall and initiating growth of a new vegetative filament. Many *Cyanobacteria* also form a structure called *cyanophycin*. This structure is a copolymer of aspartic acid and arginine and is a nitrogen storage product; when nitrogen in the environment becomes deficient, cyanophycin is broken down and used as a cellular nitrogen source. Many species of the *Nostocales* and *Stigonematales* are also able to form heterocysts, as discussed next.

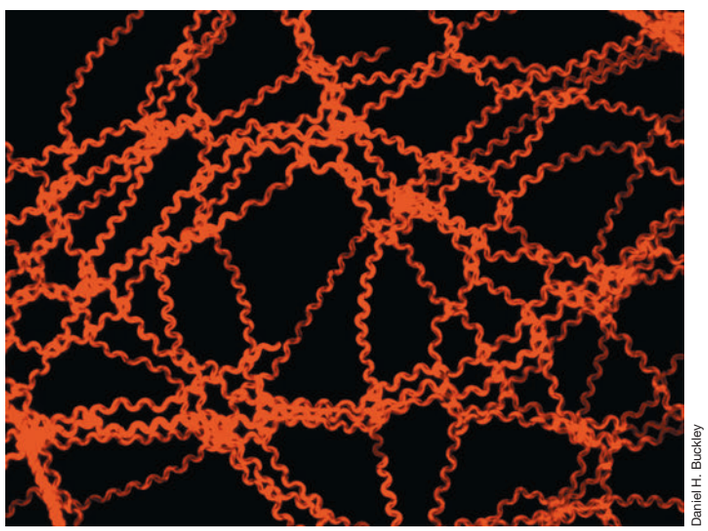


Figure 15.5 **Phycocyanin fluorescence in *Cyanobacteria*.** Fluorescence micrograph of *Spirulina*. Filaments consist of chains of helical cells with each cell approximately 5 μm wide.

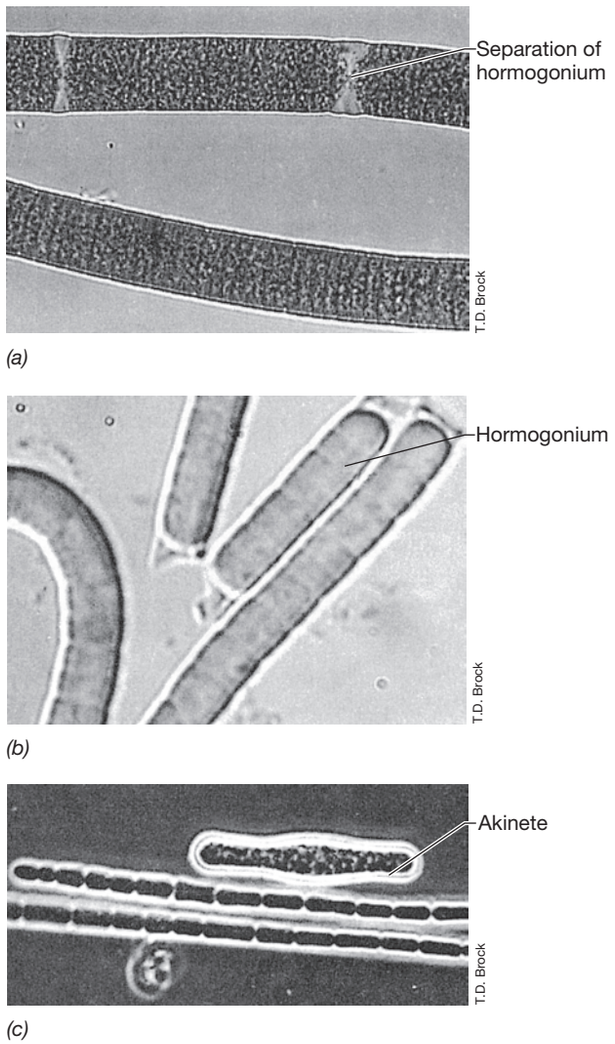
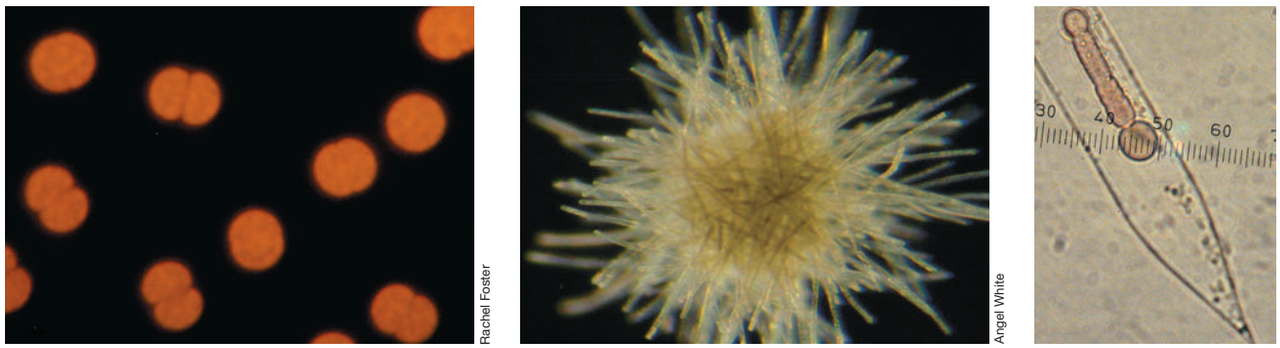


Figure 15.6 **Structural differentiation in filamentous *Cyanobacteria*.** *(a)* Initial stage of hormogonium formation in *Oscillatoria*. Notice the empty spaces where the hormogonium is separating from the filament. *(b)* Hormogonium of a smaller *Oscillatoria* species. Notice that the cells at both ends are rounded. Cells are about 10 μm wide. Differential interference contrast microscopy. *(c)* Akinete (resting spore) of *Anabaena* in a phase-contrast micrograph, cells about 5 μm wide.

Heterocysts and Nitrogen Fixation

Many *Cyanobacteria* are capable of nitrogen fixation (Figure 15.3). The nitrogenase enzyme, however, is inhibited by oxygen, and thus nitrogen fixation cannot occur along with oxygenic photosynthesis (◀ Section 3.12). *Cyanobacteria* have evolved several regulatory mechanisms for separating nitrogenase activity from photosynthesis

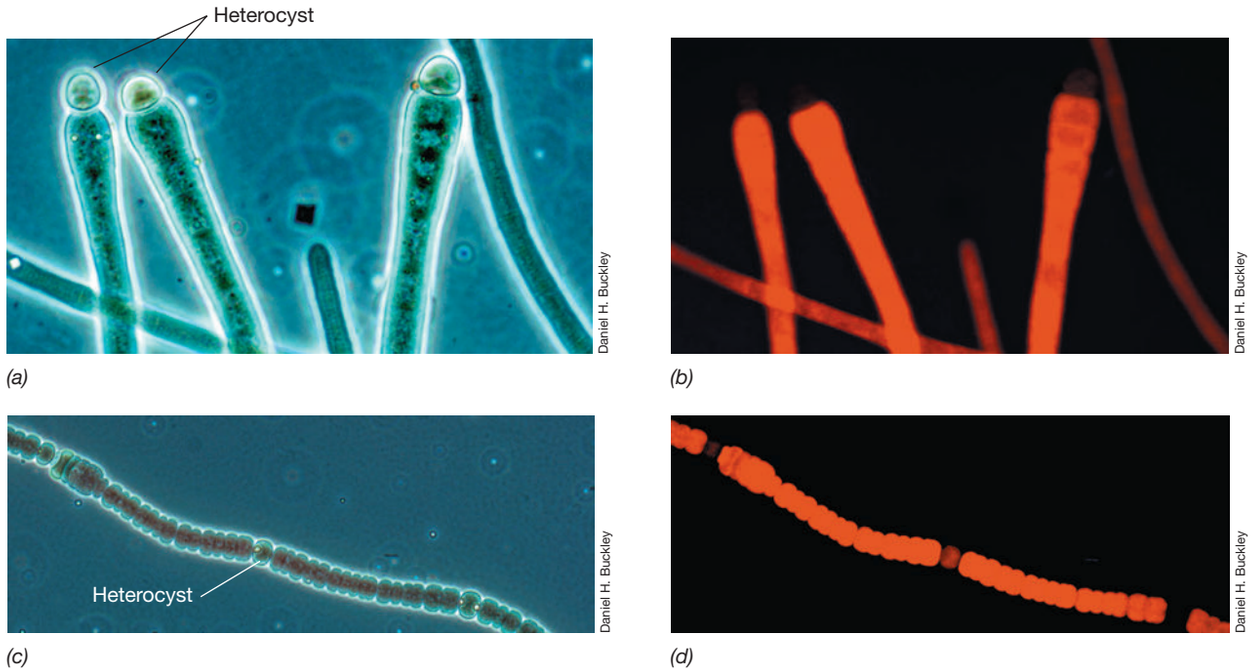


(a) (b) (c)

Figure 15.7 **Marine *Cyanobacteria* that fix N_2.** *(a)* Unicellular *Crocosphaera*-like cells in the process of dividing; cells are approximately 5 μm diameter. *(b)* Colonial "tuft" of *Trichodesmium*. The tuft is composed of many attached, undifferentiated, unbranched filaments and has a diameter of approximately 100 μm. *(c)* A diatom containing the cyanobacterial symbiont *Richelia* (scale in micrometers). The *Richelia* symbiont is an unbranched filament with a terminal heterocyst; cells are about 5 μm wide.

(◀ Section 8.9). For example, many unicellular *Cyanobacteria*, such as *Cyanothece* and *Crocosphaera* (**Figure 15.7*a***), fix nitrogen only at night when photosynthesis does not occur. In contrast, the filamentous cyanobacteria *Trichodesmium* (Figure 15.7*b*) fixes nitrogen only during the day through a mechanism that remains unclear but appears to require transient suppression of photosynthetic activity (and its O_2 production) within filaments. Finally, many filamentous *Cyanobacteria* of the *Nostocales* and *Stigonematales* facilitate nitrogen fixation by forming specialized cells called *heterocysts*, either on the ends of filaments (**Figure 15.8*a*, *b***) or along the filament (Figure 15.8*c*, *d*).

Heterocysts arise from differentiation of vegetative cells and are the sites of nitrogen fixation in heterocystous *Cyanobacteria*. Heterocysts are surrounded by a thickened cell wall that slows the diffusion of O_2 into the cell and permits nitrogenase activity to occur in an anoxic environment. Heterocysts lack photosystem II, the oxygen-evolving photosystem that generates reducing power from H_2O (◀ Section 14.6), and thus do not fluoresce as strongly as vegetative cells (Figure 15.8). Without photosystem II, heterocysts are unable to fix CO_2 and thus lack the necessary electron donor (pyruvate) for nitrogen fixation. However, heterocysts have intercellular connections with adjacent vegetative cells that allow for mutual exchange of materials between these cells. Fixed carbon is imported by the heterocyst from adjacent vegetative cells, and this is oxidized to yield electrons for nitrogen fixation. The products of photosynthesis move from vegetative cells to heterocysts, and fixed nitrogen moves from heterocysts to vegetative cells (◀ Figure 8.21).



(a) (b) (c) (d)

Figure 15.8 **Heterocysts.** Differentiation of heterocysts causes the loss of photopigments and inability to carry out photosynthesis. *(a)* Phase-contrast micrograph of *Calothrix* with terminal heterocysts. *(b)* Fluorescence micrograph of the same *Calothrix* filaments; cells are about 10 μm wide. *(c)* Phase-contrast micrograph of *Fischerella*. *(d)* Fluorescence micrograph of the same *Fischerella* filaments; cells are about 10 μm wide. See how heterocyst formation is regulated at the genetic level in the well-studied cyanobacterium *Anabaena* in Figure 8.21.

Ecology of *Cyanobacteria*

Cyanobacteria are of central importance to the productivity of the oceans. Small unicellular *Cyanobacteria*, such as *Synechococcus* and *Prochlorococcus* (▶ Section 20.11), are the most abundant phototrophs in the oceans. Together these organisms contribute 80% of marine photosynthesis and 35% of all photosynthetic activity on Earth.

Cyanobacterial nitrogen fixation represents the dominant input of new nitrogen into vast segments of Earth's oceans, particularly in oligotrophic tropical and subtropical waters. Marine nitrogen fixation is dominated by two groups of *Cyanobacteria*, the unicellular species, such as *Crocosphaera*, and the filamentous *Trichodesmium*. *Crocosphaera* (Figure 15.7*a*) and relatives dominate nitrogen fixation in most of the Pacific Ocean and are widespread in tropical and subtropical habitats. *Trichodesmium* is the dominant nitrogen-fixer in the North Atlantic Ocean and parts of the Pacific where dissolved iron concentrations are elevated. *Trichodesmium* forms macroscopically visible tufts of filaments (Figure 15.7*b*) and relies on gas vesicles to remain suspended in the photic zone, where it is often observed in dense masses of cells called *blooms*. In addition, other marine nitrogen-fixers including species of *Calothrix* and *Richelia* form symbiotic associations with diatoms (Figure 15.7*c*); these symbiotic associations are often observed in tropical and subtropical oceans. Finally, heterocystous cyanobacteria such as *Nodularia* (Figure 15.2*d*) and *Anabaena* can sometimes dominate nitrogen fixation in cold waters of the Northern Hemisphere and are often observed in the Baltic Sea.

Cyanobacteria are also widely found in terrestrial and freshwater environments. In general, they are more tolerant of environmental extremes, particularly extremes of desiccation, than are eukaryotic algae. *Cyanobacteria* are often the dominant or sole oxygenic phototrophic organisms in hot springs, saline lakes, desert soils, and other extreme environments. In some of these environments, cyanobacterial mats of variable thickness may form (▶ Figure 20.8). Freshwater lakes, especially those rich in inorganic nutrients, often develop blooms of *Cyanobacteria*, especially in late summer when temperatures are warmest (▶ Figures 20.1 and 20.19*b*). A few *Cyanobacteria* are symbionts of liverworts, ferns, and cycads, and a number are phototrophic components of lichens, a symbiosis between a phototroph and a fungus (▶ Section 23.1).

Several metabolic products of *Cyanobacteria* are of considerable practical importance. Some *Cyanobacteria* produce potent neurotoxins, and toxic blooms may form when massive accumulations of *Cyanobacteria* develop. Animals ingesting water containing these toxic products may be killed. Many *Cyanobacteria* are also responsible for the production of earthy odors and flavors in some freshwater, and if such waters are used as drinking water sources, aesthetic problems may arise. The major compound produced is geosmin, a substance also produced by many actinomycetes (▶ Section 16.12).

Check Your Understanding

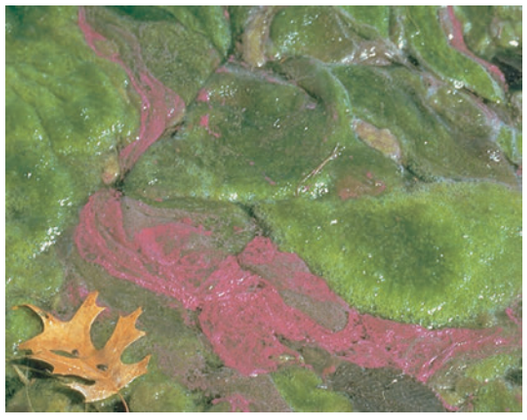
- What are the differentiating properties of the five major morphological groups of *Cyanobacteria*?
- What is a heterocyst and what is its function?
- Why are cyanobacteria such as *Synechococcus* and *Prochlorococcus* so important to the global oxygen budget?

15.4 Purple Sulfur Bacteria

KEY GENERA: *Chromatium, Thermochromatium, Ectothiorhodospira*

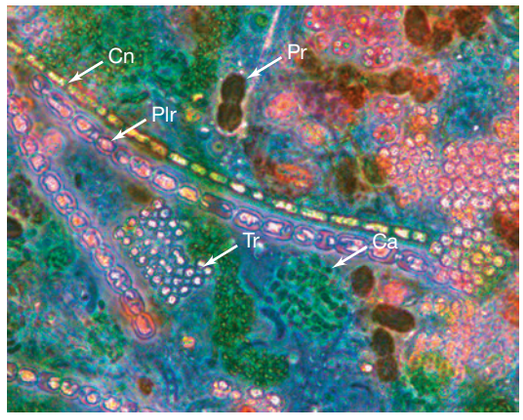
Purple sulfur bacteria are anoxygenic phototrophs that use hydrogen sulfide (H_2S) as an electron donor for photosynthesis (◀ Figure 14.5). Purple sulfur bacteria are a phylogenetically coherent group found within the order *Chromatiales* in the *Gammaproteobacteria*.

Purple sulfur bacteria are generally found in illuminated anoxic zones where H_2S is present. Such habitats occur commonly in lakes, marine sediments, and "sulfur springs," where H_2S produced geochemically or biologically can support the growth of purple sulfur bacteria (**Figure 15.9**).



(a)

(b)



(c)

Figure 15.9 Anoxygenic phototrophs in aquatic environments. Groundwater passing through sedimentary rock can introduce sulfur minerals into lakes and ponds. *(a)* Purple sulfur bacteria, such as *Lamprocystis roseopersicina* (shown here), grow on the sediment of a spring-fed pool and float to the surface (by virtue of their gas vesicles) when disturbed. The purple color is from the photopigments of the purple sulfur bacteria and the green color is from cells of the alga *Spirogyra*. *(b)* Sample of water from a depth of 7 m in Lake Mahoney, British Columbia; the major phototroph is the purple sulfur bacterium *Amoebobacter purpureus*. These phototrophs grow in a dense purple cloud at the chemocline, where sulfide-rich bottom water is exposed to light from the surface. *(c)* Phase-contrast photomicrograph of water from the chemocline of Lake Dagow, Germany, containing several purple and green sulfur bacteria. Abbreviations: Tr, *Thiopedia rosea* (purple sulfur bacteria); Ca, green sulfur bacteria partially disaggregated from "*Chlorochromatium aggregatum*" (a phototrophic consortium, see Section 15.6); Cn, *Chloronema* sp. (green nonsulfur bacteria, see Section 15.7); Pr, "*Pelochromatium roseum*" (a phototrophic consortium, ▶ Section 23.2); Plr, *Planktothrix rubescens* (cyanobacteria, Section 15.3). Gas vesicles (◀ Section 2.7) within Tr, Cn, and Plr cells cause them to appear refractile. Each Cn cell is about 5 μm long.

Purple sulfur bacteria are also commonly found in microbial mats (▶ Section 20.5) and in salt marsh sediments. The characteristic color of purple sulfur bacteria comes from their carotenoids, accessory pigments involved in light harvesting (◀ Section 14.4). These bacteria use a Q-type photosystem (◀ Figure 14.16), contain either bacteriochlorophyll *a* or *b*, and carry out CO_2 fixation by the Calvin cycle (◀ Section 3.12).

During autotrophic growth of purple sulfur bacteria, H_2S is oxidized to elemental sulfur (S^0), which is deposited as sulfur granules (**Figure 15.10**). When sulfide is limiting, the sulfur is used as an electron donor for photosynthesis, resulting in the oxidation of S^0 to sulfate (SO_4^{2-}). Many purple sulfur bacteria can also use other reduced sulfur compounds as photosynthetic electron donors; for example, thiosulfate ($S_2O_3^{2-}$) is commonly used to grow laboratory cultures.

The purple sulfur bacteria form two families: the *Chromatiaceae* and the *Ectothiorhodospiraceae*. Species of the two families are readily distinguished by the location of sulfur granules and by their photosynthetic membranes. *Chromatiaceae*, including the genera *Chromatium* and *Thiocapsa*, store S^0 granules *inside* their cells (in the periplasmic space) and have vesicular intracellular photosynthetic membrane systems (**Figure 15.11*b***). These organisms are common in stratified lakes containing sulfide and in the anoxic sediments of salt marshes.

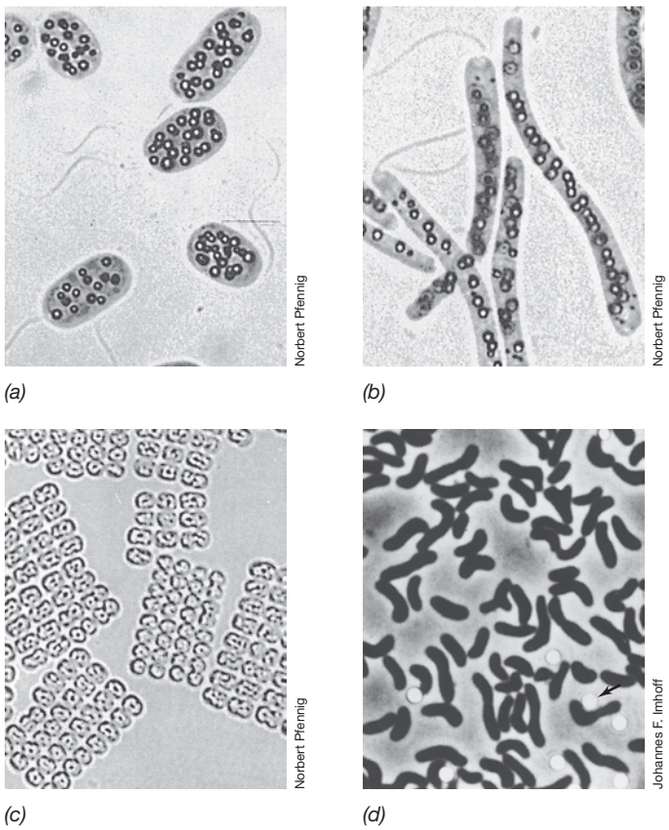


Figure 15.10 Bright-field and phase-contrast photomicrographs of purple sulfur bacteria. *(a) Chromatium okenii*; cells are about 5 μm wide. Note the globules of elemental sulfur inside the cells. *(b) Thiospirillum jenense*, a very large, polarly flagellated spiral; cells are about 30 μm long. Note the sulfur globules. *(c) Thiopedia rosea*; cells are about 1.5 μm wide. *(d)* Phase-contrast micrograph of cells of *Ectothiorhodospira mobilis*; cells are about 0.8 μm wide. Note external sulfur globules (arrow).

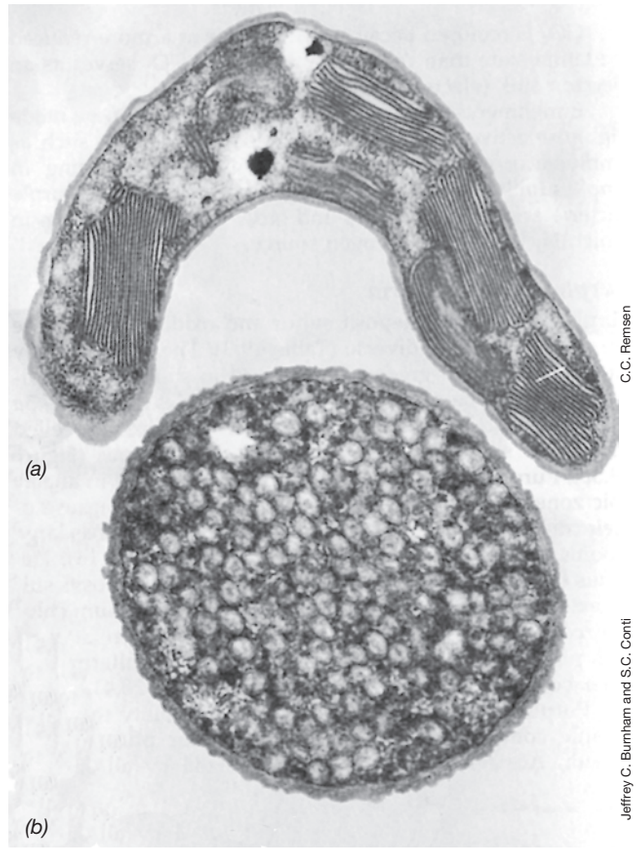


Figure 15.11 Membrane systems of phototrophic purple bacteria as revealed by transmission electron microscopy. *(a) Ectothiorhodospira mobilis*, showing the photosynthetic membranes in flat sheets (lamellae). *(b) Allochromatium vinosum*, showing the membranes as individual, spherical vesicles.

Ectothiorhodospiraceae, including the two main genera *Ectothiorhodospira* and *Halorhodospira*, oxidize H_2S to S^0 that is deposited *outside* the cell (Figure 15.10*d*) and have lamellar intracellular photosynthetic membrane systems (Figure 15.11*a*). These genera are also interesting because many species are extremely halophilic (salt loving) or alkaliphilic (alkalinity loving) and are among the most extreme in these characteristics of all known *Bacteria*. These organisms are typically found in saline lakes, soda lakes, and salterns, where abundant levels of SO_4^{2-} support sulfate-reducing bacteria (▶ Section 21.4 and Section 15.11), the organisms that produce H_2S.

Purple sulfur bacteria are often observed in high density in meromictic (permanently stratified) lakes. Meromictic lakes form layers because they have denser (usually saline) water on the bottom and less dense water (usually freshwater) nearer the surface. If sufficient sulfate is present to support sulfate reduction, sulfide is produced in the sediments and diffuses upward into the anoxic bottom waters. The presence of sulfide and light in the anoxic layers of the lake allows purple sulfur bacteria to form dense cell masses (Figure 15.9*b*, *c*), usually in association with green phototrophic bacteria.

Thermochromatium, a thermophilic purple sulfur bacterium, inhabits sulfidic hot springs where it forms thin microbial biofilms

(◀ Section 4.9 and Figure 4.17*a*). The only known species of *Thermochromatium*, *T. tepidum*, has unique absorption properties and has become a model organism for the study of energy transfer from light-harvesting (antenna) pigments to the photosynthetic reaction center (◀ Section 14.5).

Check Your Understanding

- What is the source of the purple color from which the purple sulfur bacteria get their name?
- Where would you expect to find purple sulfur bacteria in nature?

15.5 Purple Nonsulfur Bacteria and Aerobic Anoxygenic Phototrophs

Purple Nonsulfur Bacteria

KEY GENERA: *Rhodospirillum, Rhodoferax, Rhodopseudomonas, Rhodobacter*

The **purple nonsulfur bacteria** are the most metabolically versatile of all microbes. Despite their name, they are not always purple; these organisms synthesize an array of carotenoids (◀ Section 14.4) that can lend them a variety of spectacular colors (**Figure 15.12**). Together, these pigments give purple bacteria their colors, usually purple, red, or orange. Purple nonsulfur bacteria are typically photoheterotrophs (a condition where light is the energy source and an organic compound is the carbon source), and species are able to use a wide range of carbon sources and electron donors for photosynthesis, including organic acids, amino acids, alcohols, sugars, and even aromatic compounds like benzoate or toluene. Like purple sulfur bacteria, purple nonsulfur bacteria use a Q-type photosystem and contain either bacteriochlorophyll *a* or *b*. The purple nonsulfur bacteria are phylogenetically and morphologically diverse (**Figure 15.13**) and reside within the *Alphaproteobacteria* (e.g., *Rhodospirillum, Rhodobacter, Rhodopseudomonas*) or *Betaproteobacteria* (e.g., *Rubrivivax, Rhodoferax*).

One interesting characteristic shared by all species of purple nonsulfur bacteria (including the aerobic species to be discussed in the next subsection) is the presence of a "photosynthetic gene cluster" (PGC). The PGC is a set of several connected operons whose genes encode the necessary proteins to carry out anoxygenic photosynthesis: pigment biosynthesis proteins, light-harvesting and reaction center proteins, and a variety of regulatory and other photosynthesis proteins (◀ Section 14.5). Gene co-localization enables coordinated regulation of expression, but such co-localization also facilitates horizontal gene transfer. Genome sequence data indicates that horizontal transfer of PGC likely contributed to the evolution of phototrophy in lineages such as *Gemmatimonadetes* and *Chloroflexi*.

Purple nonsulfur bacteria can conserve energy through a variety of metabolic processes. For example, some species can grow photoautotrophically using H_2, low levels of H_2S, or even ferrous iron (Fe^{2+}) as the electron donor for photosynthesis with CO_2 fixation carried out by the Calvin cycle. Most species are also able to grow in darkness by using aerobic respiration of organic or even some

Figure 15.12 Photograph of liquid cultures of phototrophic purple bacteria showing the color of species with various carotenoid pigments. All species contain bacteriochlorophyll *a*. The blue culture is a carotenoidless mutant strain of *Rhodospirillum rubrum* showing that bacteriochlorophyll *a* is actually blue. The bottle on the far right (*Rhodobacter sphaeroides* strain G) lacks one of the carotenoids of the wild type and thus is less red and more green.

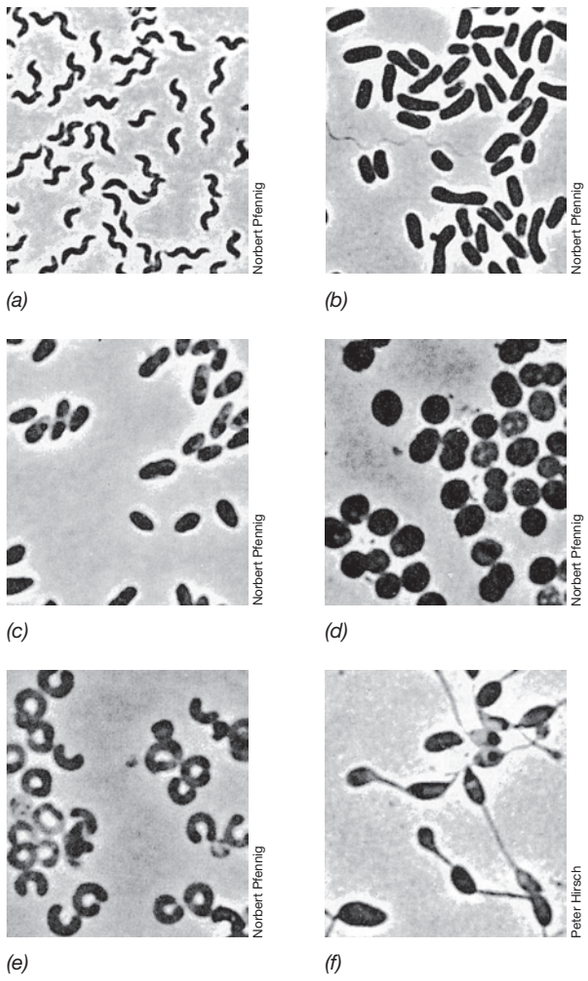


Figure 15.13 Representatives of several genera of purple nonsulfur bacteria. *(a) Phaeospirillum fulvum*; cells are about 3 μm long. *(b) Rhodoblastus acidophilus*; cells are about 4 μm long. *(c) Rhodobacter sphaeroides*; cells are about 1.5 μm wide. *(d) Rhodopila globiformis*; cells are about 1.6 μm wide. *(e) Rhodocyclus purpureus*; cells are about 0.7 μm in diameter. *(f) Rhodomicrobium vannielii*; cells are about 1.2 μm wide.

inorganic compounds; synthesis of the photosynthetic machinery is typically repressed by O_2. Finally, some species can also grow by fermentation or anaerobic respiration using a variety of electron donors and acceptors.

Enrichment and isolation of purple nonsulfur bacteria is easy using a mineral-salts medium supplemented with an organic acid (for example, acetate, malate, or succinate) as carbon source. Such media, inoculated with a mud, lake water, or sewage sample and incubated anaerobically in the light, invariably select for purple nonsulfur bacteria. Enrichment cultures can be made even more selective by omitting fixed nitrogen sources (for example, ammonia) or organic nitrogen sources (for example, yeast extract or peptone) from the medium and supplying a gaseous headspace of N_2. Virtually all purple nonsulfur bacteria can fix N_2 (◀ Section 3.12) and will thrive under such conditions, rapidly outcompeting other bacteria.

Aerobic Anoxygenic Phototrophs

KEY GENERA: *Roseobacter, Erythrobacter*

The **aerobic anoxygenic phototrophs** are obligatory aerobic heterotrophs that use light as a supplemental source of energy to support growth. Like purple nonsulfur bacteria, aerobic anoxygenic phototrophs are phylogenetically diverse and are *Alphaproteobacteria* or *Betaproteobacteria*. Their primary physiological difference from the purple nonsulfur bacteria is that aerobic anoxygenic phototrophs are strict heterotrophs and employ anoxygenic photosynthesis only under *oxic* conditions as a supplemental source of energy. Aerobic anoxygenic phototrophs contain bacteriochlorophyll *a* and a Q-type photosystem but are unable to fix CO_2 and thus rely on organic forms of carbon as their carbon source. Carotenoids of various types lend colors of yellow, orange, or pink to cultures.

Aerobic anoxygenic phototrophs are only able to photosynthesize when grown on a day/night cycle. Under these conditions, bacteriochlorophyll *a* is made only in the dark and then used to conserve energy by photophosphorylation when the light returns. Aerobic anoxygenic phototrophs can account for as much as a quarter of the microbial community inhabiting coastal marine waters and 5% of gross photosynthesis in such systems (▶ Section 20.11). Common genera found in coastal marine habitats include *Roseobacter* and *Erythrobacter*.

Check Your Understanding

- What are some similarities between purple nonsulfur bacteria and aerobic anoxygenic phototrophs? What are the differences between these two groups?
- Where would you expect to find aerobic anoxygenic phototrophs?

15.6 Green Sulfur Bacteria

KEY GENERA: *Chlorobium, Chlorobaculum, Prosthecochloris, "Chlorochromatium"*

Green sulfur bacteria are a phylogenetically coherent group of anoxygenic phototrophs that forms the phylum *Chlorobi*. Green sulfur bacteria have little metabolic versatility, and they are typically nonmotile and strictly anaerobic anoxygenic phototrophic bacteria. The group is also morphologically restricted and includes primarily short to long rods (**Figure 15.14**).

Like purple sulfur bacteria, green sulfur bacteria oxidize hydrogen sulfide (H_2S) as an electron donor for autotrophic growth, oxidizing it first to sulfur (S^0) and then to sulfate (SO_4^{2-}). But unlike most purple sulfur bacteria, the S^0 produced by green sulfur bacteria is deposited only outside the cell (Figure 15.14*a*). Autotrophy is supported not by the reactions of the Calvin cycle, as in purple bacteria, but instead by a reversal of steps in the citric acid cycle (◀ Section 14.2), a unique means of autotrophy in phototrophic bacteria.

Pigments and Ecology

Green sulfur bacteria contain bacteriochlorophyll *c*, *d*, or *e* and house these pigments in unique structures called **chlorosomes**

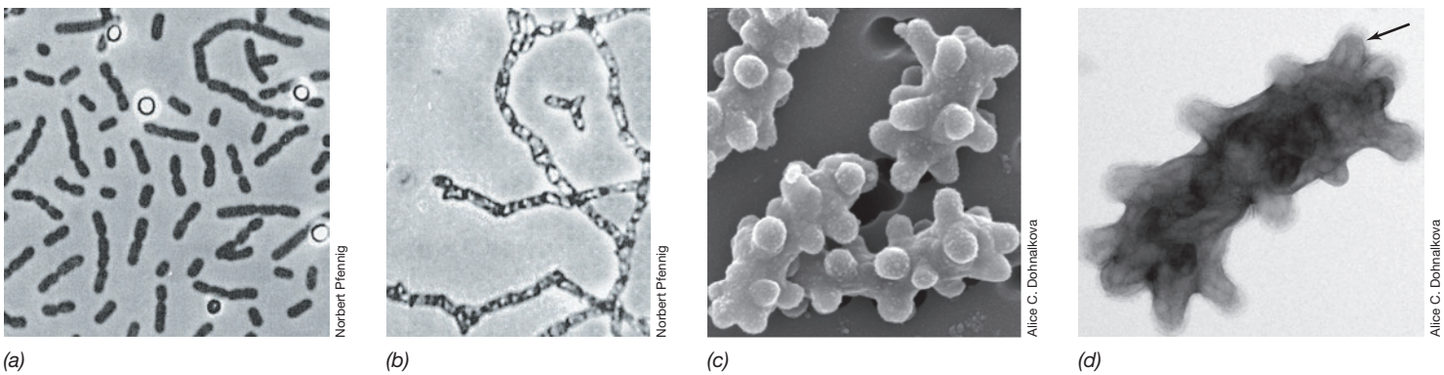


Figure 15.14 Phototrophic green sulfur bacteria. *(a)* Phase-contrast micrograph of *Chlorobium limicola*; cells are about 0.8 μm wide. Note the spherical sulfur granules deposited extracellularly. *(b)* Phase-contrast micrograph of *Chlorobium clathratiforme*, a bacterium forming a three-dimensional network; cells are about 0.8 μm wide. *(c)* Scanning electron micrograph of cells of *Prosthecochloris aestuarii* showing numerous prosthecae on each cell; cells are about 1.2 μm long. *(d)* Transmission electron micrograph of a *P. aestuarii* cell showing chlorosomes (arrow) within prosthecae.

Figure 15.15 The thermophilic green sulfur bacterium *Chlorobaculum tepidum*. Transmission electron micrograph. Note chlorosomes (arrow) in the cell periphery. A cell is about 0.7 μm wide.

(◀ Section 14.3 and **Figure 15.15**). A small amount of bacteriochlorophyll *a* is present in the reaction center and FMO protein, the latter of which connects the chlorosome to the cytoplasmic membrane (◀ Figure 14.11*b*). Chlorosomes are oblong, bacteriochlorophyll-rich bodies bounded by a thin, nonunit membrane and attached to the cytoplasmic membrane in the periphery of the cell (Figure 15.15 and ◀ Figure 14.11). Chlorosomes function to funnel energy into the photosystem, and this eventually leads to ATP synthesis. Unlike purple anoxygenic phototrophs, green sulfur bacteria use an FeS-type photosystem. Both green- and brown-colored species of green sulfur bacteria are known, the brown-colored species containing bacteriochlorophyll *e* and carotenoids that turn dense cell suspensions brown (**Figure 15.16**).

Like purple sulfur bacteria (Section 15.4), green sulfur bacteria live in anoxic, sulfidic, illuminated aquatic environments. However, the chlorosome is a very efficient light-harvesting structure, which allows green sulfur bacteria to grow at light intensities much lower than those required by other phototrophs. Green sulfur bacteria also tend to have a greater tolerance of H_2S than do other anoxygenic phototrophs. As a result, green sulfur bacteria are typically found at the greatest depths of all phototrophic microorganisms in lakes or microbial mats, where light intensities are low and H_2S levels the highest. As an example, a species of green sulfur bacteria isolated from a deep-sea hydrothermal vent (▶ Section 20.16) was found to be growing phototrophically on the weak glow of infrared radiation emitted from the geothermally heated rock.

Although most green sulfur bacteria are freshwater species, some are marine and at least one species is thermophilic. *Prosthecochloris* is a marine species whose cells form extensions of the cytoplasm and cell wall called *prosthecae* (see Section 15.18 and Figure 15.53) that contain chlorosomes (Figure 15.14*c*, *d*). Some *Prosthecochloris* species inhabit saline lakes and appear to be the only green sulfur bacteria to be widespread outside of freshwater habitats. *Chlorobaculum tepidum* (Figure 15.15) is thermophilic and forms dense microbial mats in high-sulfide hot springs. *C. tepidum* also grows rapidly and is amenable to genetic manipulation by both conjugation and transformation (Chapter 9). In addition, *C. tepidum* can use thiosulfate as an electron donor for autotrophic CO_2 fixation, a property not universal among green sulfur bacteria. Because of these features, *C. tepidum* has become the model organism for studying the molecular biology of green sulfur bacteria.

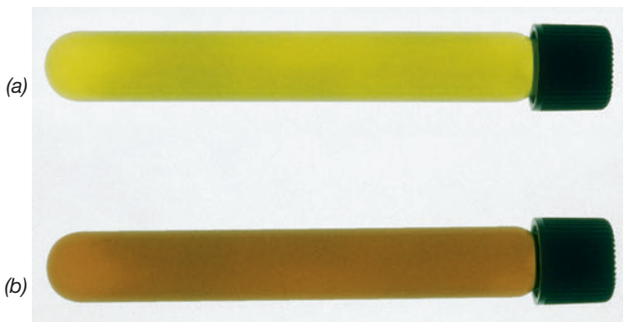


Figure 15.16 Green and brown chlorobia. Tube cultures of *(a) Chlorobaculum tepidum* and *(b) Chlorobaculum phaeobacteroides*. Cells of *C. tepidum* contain bacteriochlorophyll *c* and green carotenoids, and cells of *C. phaeobacteroides* contain bacteriochlorophyll *e* and isorenieratene, a brown carotenoid. The structures of bacteriochlorophylls *c* and *e* and of green bacteria carotenoids were shown in Figures 14.7 and 14.13, respectively.

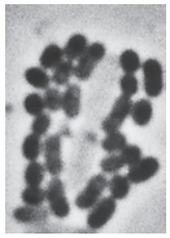
Green Sulfur Bacteria Consortia

Certain species of green sulfur bacteria form an intimate two-membered association, called a **consortium**, with a chemoorganotrophic bacterium. In the consortium, each organism benefits, and thus a variety of such consortia containing different phototrophic and chemotrophic components probably exist in nature. The phototrophic component, called the *epibiont*, is physically attached to the nonphototrophic central cell (**Figure 15.17**) and communicates with it in various ways (▶ Section 23.2).

The name "*Chlorochromatium aggregatum*" (not a formal name because this is a mixed culture) has been used to describe a commonly observed green-colored consortium that is green because the epibionts are green sulfur bacteria that contain green-colored carotenoids (Figure 15.17*b* and Figure 15.9*c*). Evidence that the epibionts are indeed green sulfur bacteria comes from pigment analyses, the presence of chlorosomes (Figure 15.17*d*), and phylogenetic staining (Figure 15.17*c*). A structurally similar consortium called "*Pelochromatium roseum*" is brown because its epibionts produce brown-colored carotenoids (Figure 15.9*c* and ▶ Figures 23.3–23.5). We examine the symbiotic nature of the *Chlorochromatium* consortium in more detail in (▶ Section 23.2).

Check Your Understanding

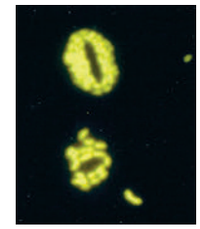
- Which pigments are present in the chlorosome?
- What evidence exists that the epibionts of green bacterial consortia are truly green sulfur bacteria?


Douglas Caldwell

(a)

Jörg Overmann

(b)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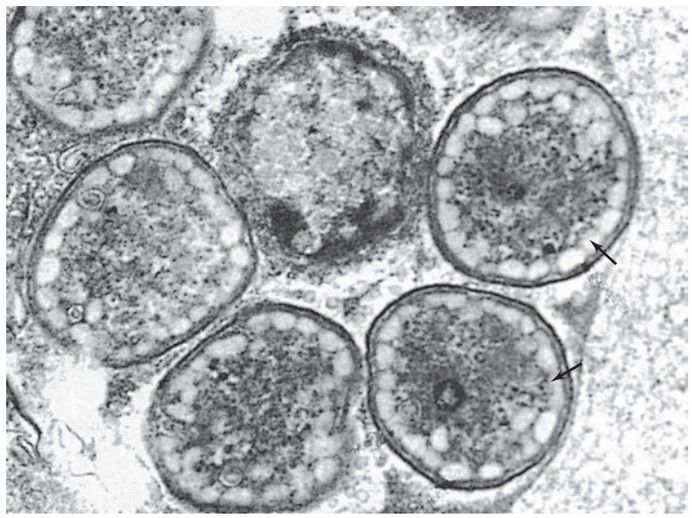
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Figure 15.17 **"*Chlorochromatium aggregatum*."** Consortia of green sulfur bacteria and a chemoorganotroph (see also Figure 15.9 and ▶ Section 23.2). *(a)* In a phase-contrast micrograph, the nonphototrophic central organism is lighter in color than the pigmented phototrophic bacteria. *(b)* Green carotenoids lend their color to the phototrophs in a differential interference contrast micrograph. *(c)* A fluorescence micrograph shows the cells stained with a fluorescent probe specific for green sulfur bacteria. *(d)* Transmission electron micrograph of a cross section through a single consortium; note the chlorosomes (arrows) in the epibionts. The entire consortium is about 3 μm in diameter.

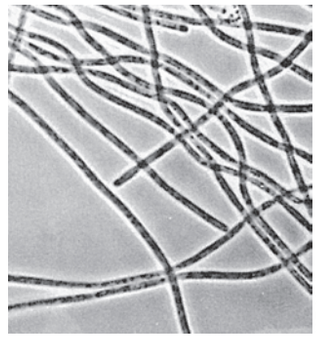
15.7 Green Nonsulfur Bacteria

KEY GENERA: *Chloroflexus, Heliothrix, Roseiflexus*

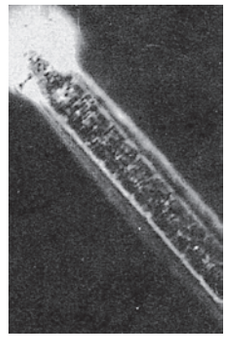
Green nonsulfur bacteria, which are also called *filamentous anoxygenic phototrophs*, are anoxygenic phototrophs of the phylum *Chloroflexi*. The latter contains several distinct lineages, one of which, the class *Chloroflexi*, contains green nonsulfur bacteria. The remainder of the phylum contains metabolically diverse organisms including both aerobic and anaerobic chemoorganotrophs as well as the *Dehalococcoidetes*, a group of dehalogenating bacteria that use halogenated organic compounds as electron acceptors in anaerobic respiration (◀ Section 14.13). Analyses of 16S ribosomal RNA sequences from environmental samples (▶ Sections 19.6 and 19.8) indicate that species of the phylum *Chloroflexi* are widespread and that most species in the phylum have yet to be cultivated in isolation; thus, the extent of the metabolic diversity of this phylum remains unclear.

All cultured representatives of the green nonsulfur bacteria are filamentous bacteria that are capable of gliding motility. *Chloroflexus*, one of the most studied of the green nonsulfur bacteria, forms thick microbial mats in neutral to alkaline hot springs along with thermophilic cyanobacteria (**Figure 15.18**; ▶ Figure 20.8*b*). Green nonsulfur bacteria grow best as photoheterotrophs using simple carbon sources as electron donors for photosynthesis. However, growth also occurs photoautotrophically using H_2 or H_2S as electron donors for photosynthesis. The 3-hydroxypropionate bi-cycle, a pathway of CO_2 incorporation unique to only a few *Bacteria* and *Archaea*, supports autotrophic growth (◀ Section 14.2). Most green nonsulfur bacteria also grow well in the dark by aerobic respiration of a wide variety of carbon sources.

The photosynthetic features of the green nonsulfur bacteria form a "hybrid" between those of both green sulfur bacteria (Section 15.6) and purple phototrophic bacteria (Sections 15.4, 15.5). Green nonsulfur bacteria have reaction centers that contain bacteriochlorophyll *a* and chlorosomes that contain bacteriochlorophyll *c* and in this way are similar to green sulfur bacteria (Figure 15.15). However, in contrast


M.T. Madigan

(a)


V.M. Gorlenko

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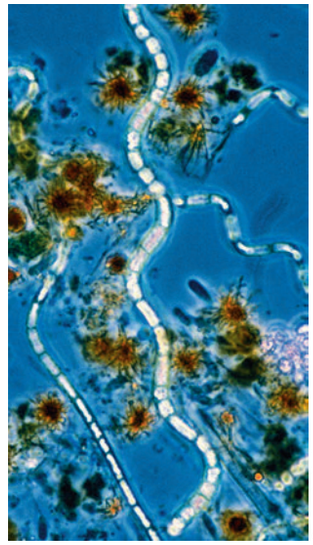
Charles A. Abella

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Deborah O. Jung

(d)

Figure 15.18 **Green nonsulfur bacteria.** *(a)* Phase-contrast micrograph of the anoxygenic phototroph *Chloroflexus aurantiacus*; cells are about 1 μm in diameter. *(b)* Phase-contrast micrograph of the large phototroph *Oscillochloris*; cells are about 5 μm wide. The brightly contrasting material on the top is a holdfast, used for attachment. *(c)* Phase-contrast micrograph of filaments of a *Chloronema* species; the cells are wavy filaments and about 2.5 μm in diameter. *(d)* Tube cultures of *C. aurantiacus* (right) and *Roseiflexus* (left). *Roseiflexus* is yellow because it lacks bacteriochlorophyll *c* and chlorosomes.

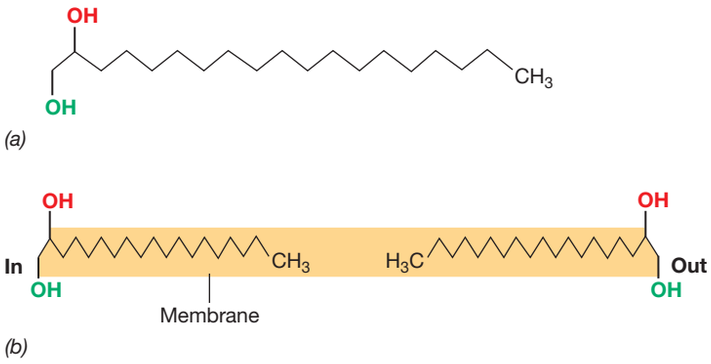


Figure 15.19 The unusual lipids of *Thermomicrobium*. *(a)* Membrane lipids from *Thermomicrobium roseum* contain long-chain diols like the one shown here (1,2-nonadecanediol). Note that unlike the lipids of other *Bacteria* or of *Archaea*, neither ester- nor ether-linked side chains are present. *(b)* To form a bilayer membrane, dialcohol molecules oppose each other at the methyl groups, and the —OH groups are the inner and outer hydrophilic surfaces. Small amounts of the diols have fatty acids esterified to the secondary —OH group (shown in red), whereas the primary —OH group (shown in green) can bond with a hydrophilic molecule like phosphate.

to green sulfur bacteria, green nonsulfur bacteria contain a Q-type photosynthetic reaction center (◀ Section 14.5 and Figure 14.16) and in this respect resemble purple bacteria.

Other *Chloroflexi*

In addition to *Chloroflexus*, other phototrophic green nonsulfur bacteria include the thermophile *Heliothrix* and the large-celled mesophiles *Oscillochloris* (Figure 15.18*b*) and *Chloronema* (Figure 15.18*c*). *Oscillochloris* and *Chloronema* form rather large cells, 2–5 μm wide and up to several hundred micrometers long (Figure 15.18*c*). Species of both genera inhabit freshwater lakes containing H_2S. *Roseiflexus* and *Heliothrix* are similar to *Chloroflexus* in their filamentous morphology and thermophilic lifestyle but differ in a major photosynthetic property. *Roseiflexus* and *Heliothrix* lack bacteriochlorophyll *c* and chlorosomes and thus more closely resemble purple phototrophic bacteria (Sections 15.4, 15.5) than *Chloroflexus*. This can be seen in cultures of *Roseiflexus* that are yellow-orange instead of green from their extensive carotenoid pigments and lack of bacteriochlorophyll *c* (Figure 15.18*d*).

Thermomicrobium is a nonphototrophic genus of *Chloroflexi* and a strictly aerobic, gram-negative rod, growing optimally in complex media at 75°C. Besides its phylogenetic properties, *Thermomicrobium* is also of interest because of its membrane lipids (**Figure 15.19**). Recall that the lipids of *Bacteria* and *Eukarya* contain fatty acids esterified to *glycerol* (◀ Section 2.1). By contrast, the lipids of *Thermomicrobium* are formed on *1,2-dialcohols* instead of glycerol and have neither ester *nor* ether linkages (Figure 15.19; ◀ Section 2.1). In addition, cells of *Thermomicrobium* contain only small amounts of peptidoglycan (◀ Section 2.3), and the cell wall is composed primarily of protein.

Check Your Understanding

- In what ways do *Chloroflexus* and *Roseiflexus* resemble *Chlorobium*? *Rhodobacter*?
- What is unique about *Thermomicrobium*?

15.8 Other Phototrophic *Bacteria*

Heliobacteria

KEY GENERA: *Heliobacterium, Heliorestis*

Heliobacteria are a phylogenetically coherent group of phototrophic gram-positive *Bacteria* found within the phylum *Firmicutes* (▶ Section 16.8). The heliobacteria are anoxygenic phototrophs that have an FeS-type photosystem (◀ Section 14.5) and that produce a unique pigment, bacteriochlorophyll *g* (◀ Figure 14.7). Heliobacteria grow photoheterotrophically using a narrow range of organic compounds including pyruvate, lactate, acetate, or butyrate, and the group contains five genera: *Heliobacterium*, *Heliophilum*, *Heliorestis*, *Heliomonas*, and *Heliobacillus*. All known heliobacteria form rod-shaped or filamentous cells (**Figure 15.20**), although *Heliophilum* is unusual because its cells form into bundles (Figure 15.20*b*) that are motile as a unit.

Heliobacteria are strict anaerobes, but in addition to phototrophic growth, they can grow chemotrophically in darkness by pyruvate fermentation (as can many clostridia, close relatives of the heliobacteria). Heliobacteria produce endospores, the highly resistant structures produced by certain gram-positive bacteria (◀ Section 2.8). Like the endospores of *Bacillus* or *Clostridium* species, the endospores of heliobacteria (Figure 15.20*c*) contain elevated calcium (Ca^{2+}) levels and the signature molecule of the endospore, *dipicolinic acid*. Heliobacteria reside in soil, especially paddy (rice) field soils, where

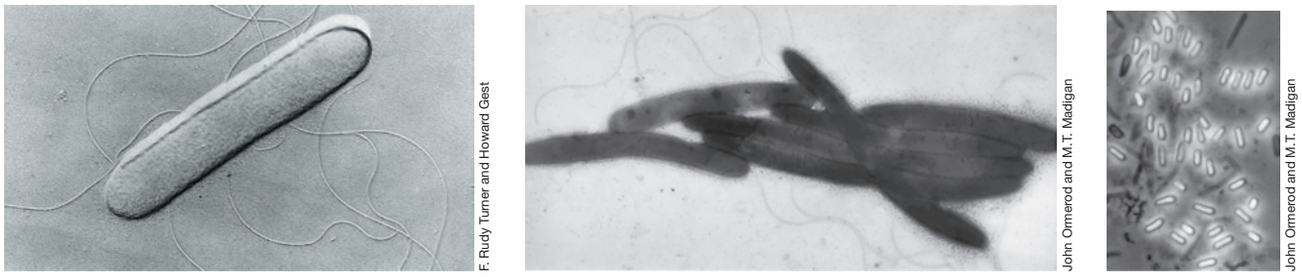


Figure 15.20 Cells and endospores of heliobacteria. *(a)* Electron micrograph of *Heliobacillus mobilis*, a peritrichously flagellated species. *(b) Heliophilum fasciatum* cell bundles as observed by electron microscopy. *(c)* Phase-contrast micrograph of endospores from *Heliobacterium gestii*. Most heliobacteria cells are about 1–2 μm in diameter.

their nitrogen fixation activities may benefit rice productivity. A large diversity of heliobacteria have also been found in highly alkaline environments, such as soda lakes and surrounding alkaline soils.

Phototrophic *Acidobacteria*

KEY GENUS: *Chloracidobacterium*

A novel group of anoxygenic phototrophs has been discovered growing in photosynthetic microbial mats of certain thermal springs in Yellowstone National Park (USA). *Chloracidobacterium thermophilum* is a thermophilic oxygen-tolerant anoxygenic phototroph of the phylum *Acidobacteria* (▶ Section 16.21). Like green sulfur bacteria, *C. thermophilum* produces bacteriochlorophyll *a* and *c*, the latter in chlorosomes (**Figure 15.21**), and uses an FeS-type photosystem. However, unlike green sulfur bacteria, *C. thermophilum* can also grow aerobically, as is true for the aerobic anoxygenic phototrophs (Section 15.5). In terms of its carbon metabolism, *C. thermophilum* is a photoheterotroph that uses short-chain fatty acids as carbon sources, but unlike green sulfur or green nonsulfur bacteria, it is incapable of autotrophy.

Phototrophic *Gemmatimonadetes*

KEY GENUS: *Gemmatimonas*

Another novel group of anoxygenic phototrophs has been discovered in a freshwater lake in the western Gobi desert (part of China and Mongolia). *Gemmatimonas phototrophica* is an aerobic facultative photoheterotroph of the phylum *Gemmatimonadetes*. It gains most of its energy through the aerobic respiration of organic compounds, whether in the light or in the dark. However, in the light, *G. phototrophica* uses photophosphorylation to supplement energy generated by aerobic respiration. *G. phototrophica* cannot grow as an obligate phototroph, it cannot fix CO_2, and it cannot grow anaerobically. *G. phototrophica* contains a photosynthetic gene cluster that resembles those of aerobic anoxygenic phototrophs (Section 15.5), and it produces bacteriochlorophyll *a* and a Q-type reaction center, both of which are characteristic properties of purple bacteria (◀ Section 14.5). It thus seems likely that *G. phototrophica* acquired its photosynthetic gene cluster and the ability to perform photophosphorylation as the result of an ancient horizontal gene transfer event.

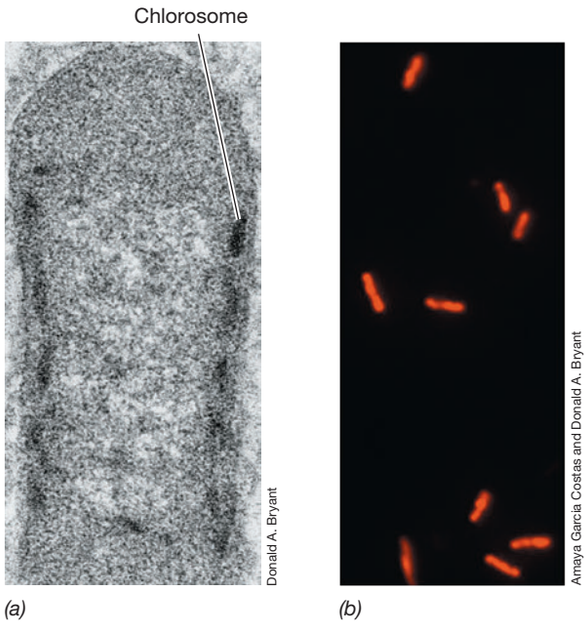


Figure 15.21 **Chlorosomes in *Chloracidobacterium thermophilum*, a phototrophic member of the phylum *Acidobacteria*.** *(a)* Electron micrograph of *C. thermophilum* showing chlorosomes. (*b*) Fluorescence photomicrograph of *C. thermophilum*. The red color is the fluorescence of bacteriochlorophyll *c* present in chlorosomes. A cell of *C. thermophilum* is about 0.8 μm wide.

Check Your Understanding

- What types of anoxygenic phototrophs contain chlorosomes?
- What kind of phototrophic bacteria make endospores?

III • Diversity of *Bacteria* Defined by Metabolic Traits

Some microbial metabolisms, such as those where sulfur or iron are oxidized to yield energy, are so characteristic of a specific group of microbes that the metabolism becomes the defining characteristic of the group.

Certain types of microbial metabolism have major impacts on biogeochemical cycling of elements such as nitrogen and sulfur (see Chapter 21). Hence, it is useful to define the diversity of these organisms in terms of their metabolism. All cells must assimilate nitrogen, sulfur, and some metals for growth, and thus all organisms catalyze certain assimilatory reactions. The *Bacteria* and *Archaea*, however, are the only domains containing organisms that can conserve energy from the dissimilative metabolism of inorganic nitrogen, sulfur, and metallic compounds.

In this part of the chapter, we focus on the diversity of microorganisms defined by metabolic traits. We will see that considerable ecological and phylogenetic diversity often exists within any metabolic group and that ecological characteristics often govern the distribution and activity of species within each metabolic group (for example, see Section 15.12). We start our tour of the nitrogen cycle by considering those microbes that reduce atmospheric nitrogen: the nitrogen fixers.

15.9 Diversity of Nitrogen Fixers

KEY GENERA: *Mesorhizobium, Desulfovibrio, Azotobacter*

Diazotrophs are microorganisms that fix dinitrogen gas (N_2) into NH_3, which can be assimilated as a source of nitrogen for cells. [The term *diazotroph* is derived from a combination of the French *azote*, which means "nitrogen," the Greek *troph*, which means "eating," and *di*, which means two. Thus, a diazotroph is an organism that "eats two nitrogens" (N_2)] . Nitrogen fixation is an assimilative process and requires ATP and the enzyme nitrogenase (◀ Section 3.12). Diazotrophs typically fix N_2 only when other forms of N are absent, and nitrogenase expression is inhibited when NH_3 is available to cells. Nitrogenase is irreversibly inhibited by O_2, and this is one

cause of ecological diversification among diazotrophs; we will see that different organisms have evolved different solutions to protect nitrogenase from O_2.

Nitrogen fixation is widespread among *Bacteria* and is also found in a few *Archaea*. It is thought to be an ancient process that was present in the last universal common ancestor (◀ Section 13.1). The *nifH* gene encodes the dinitrogenase reductase component of nitrogenase (◀ Section 3.12) and can be used as a measure of diazotroph diversity. More than 30,000 unique *nifH* gene sequences have been described, spanning nine bacterial phyla and one archaeal phylum (Figure 15.1). The phylogenetic distribution of nitrogenase in the tree of life has been influenced strongly by horizontal gene exchange. As a result, the phylogeny of *nifH* is largely inconsistent with the 16S ribosomal RNA gene phylogeny (**Figure 15.22**). We consider here the diversity of both symbiotic and free-living diazotrophic *Bacteria*.

Symbiotic Diazotrophs

Diazotrophs form several symbiotic relationships with plants, animals, and fungi. These relationships typically consist of a host that provides a hospitable environment, a source of carbon and energy,

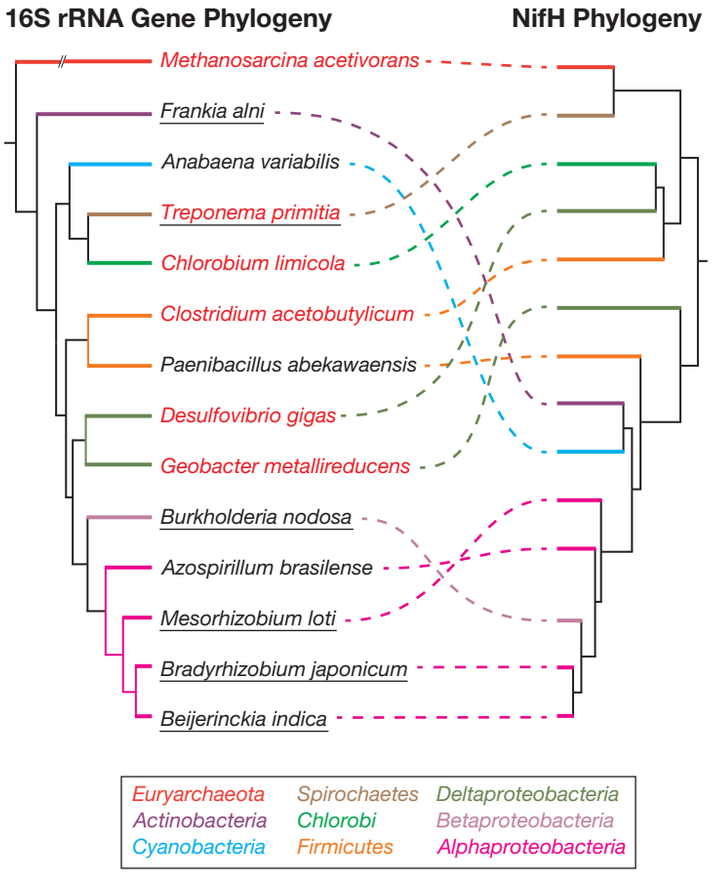


Figure 15.22 Relationships among diazotrophic (nitrogen-fixing) bacteria as inferred from 16S ribosomal RNA gene sequences and NifH amino acid sequences. Branches in each tree are colored to indicate phyla. The dashed lines indicate branches shared between the two trees. The incongruence between the two trees has resulted from multiple horizontal transfer events of the *nifH* gene. Red text denotes obligate anaerobes and underlined text indicates species that form symbioses with *Eukarya*.

and a system for regulating O_2 concentrations, and a microbial symbiont that provides a supply of fixed nitrogen to the host.

The symbiosis between rhizobia and leguminous plants is one of the best-characterized nitrogen-fixing symbiotic associations (▶ Section 23.4). Root-nodule-forming bacteria are *Alphaproteobacteria* (e.g., *Mesorhizobium, Bradyrhizobium, Sinorhizobium*), *Betaproteobacteria* (e.g., *Burkholderia*), or *Actinobacteria* (e.g., *Frankia*). Other genera of symbiotic diazotrophs are found in association with shipworms (*Teredinibacter*), termite guts (*Treponema*) (▶ Section 23.9), endomycorrhizal fungi (*Glomeribacter*) (▶ Sections 18.12 and 23.5), and several fungi, algae, and plants (*Cyanobacteria*) (▶ Sections 23.1 and 23.4). These different symbioses have evolved independently multiple times as a result of convergent evolution (Figure 15.22).

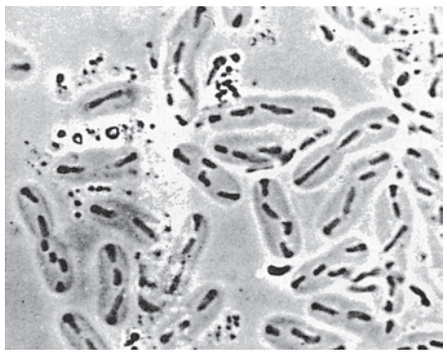
Free-Living Diazotrophs

Free-living diazotrophs need a mechanism for protecting nitrogenase from oxygen (◀ Section 3.12 and Section 15.3). The simplest solution to this problem is to grow only in anoxic environments. The origin of nitrogen fixation predates the origin of oxygenic photosynthesis, which means the first nitrogen-fixing organisms were free-living anaerobes. Obligately anaerobic free-living diazotrophs are common in anoxic environments including marine and freshwater sediments and microbial mats. Obligately anaerobic free-living diazotrophs are found in the bacterial phyla *Firmicutes* (e.g., *Clostridium*), *Chloroflexi* (e.g., *Oscillochloris*), *Chlorobi* (e.g., *Chlorobium*), *Spirochaetes* (e.g., *Spirochaeta*), and *Proteobacteria* (e.g., *Desulfovibrio, Chromatium, Rhodobacter*) and in the archaeal phylum *Euryarchaeota* (e.g., *Methanosarcina*). *Desulfovibrio* species occur in anoxic salt marsh sediments dominated by *Spartina* grass, and their N_2 fixation is an important nitrogen source to plants that live in this ecosystem.

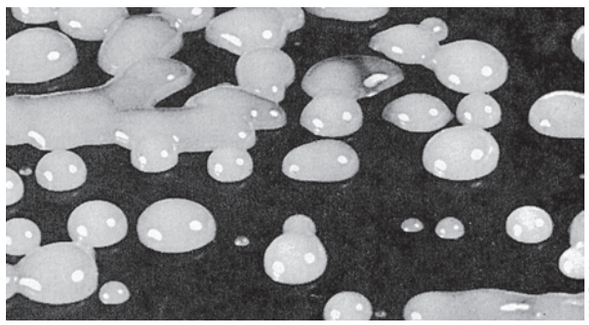
Other simple mechanisms for protecting nitrogenase from oxygen include fixing N_2 only at times when oxygen is absent or present in low concentration. For example, facultative aerobes will often fix N_2 only while growing anaerobically (e.g., *Klebsiella*). Some aerobic nitrogen-fixers are *microaerophiles*; these organisms fix nitrogen only in environments where oxygen is present at low concentration (typically less than 2%). However, some organisms have evolved more complex mechanisms for protecting nitrogenase from oxygen and are able to grow in the presence of air.

Obligately aerobic free-living diazotrophs include the *Cyanobacteria* (which have evolved a variety of mechanisms of protecting nitrogenase from oxygen, see Section 15.3) and several unicellular free-living chemoorganotrophic bacteria. Obligately aerobic free-living diazotrophs include *Azotobacter*, *Azospirillum*, and *Beijerinckia*. *Azotobacter* cells are large rods or cocci with diameters of 2–4 μm or more. When they are growing on N_2 as a nitrogen source, extensive capsules or slime layers are typically produced (**Figure 15.23** and ◀ Figures 2.16 and 3.29*a, b*). It is thought that the high respiratory rate characteristic of *Azotobacter* cells and the abundant capsular slime they produce help protect nitrogenase from O_2. *Azotobacter* is able to grow on many different carbohydrates, alcohols, and organic acids, and metabolism is strictly oxidative.

Azotobacter can form resting structures called *cysts* (**Figure 15.24*b***). Like bacterial endospores, *Azotobacter* cysts show negligible endogenous respiration and are resistant to desiccation, mechanical disintegration,



(a)



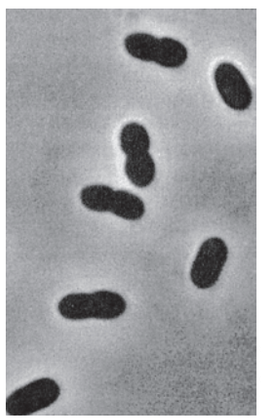
(b)

Figure 15.23 Examples of slime production by free-living N_2-fixing bacteria. *(a)* Cells of *Derxia gummosa* encased in slime. Cells are about 1–1.2 μm wide. *(b)* Colonies of *Beijerinckia* species growing on a carbohydrate-containing medium. Note the raised, glistening appearance of the colonies due to abundant capsular slime.

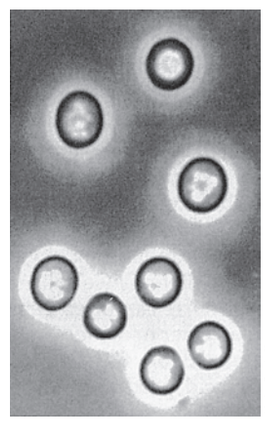
and ultraviolet and ionizing radiation. In contrast to endospores, however, cysts are not very heat resistant, and they are not completely dormant because they rapidly oxidize carbon sources if supplied.

Azotobacter and Alternative Nitrogenases

We considered the important process of biological N_2 fixation in Section 3.12 and discussed the central importance of the metals molybdenum (Mo) and iron (Fe) to the enzyme nitrogenase. The species *Azotobacter chroococcum* was the first nitrogen-fixing bacterium shown to grow on N_2 in the absence of molybdenum. This is because either of two "alternative nitrogenases" are formed when Mo limitation prevents the MoFe nitrogenase from being synthesized. These nitrogenases are less efficient than the MoFe nitrogenase and contain either vanadium (V) or Fe in place of Mo. The three different types of nitrogenase (MoFe, VFe, and FeFe) are encoded by paralogous genes and likely arose as the result of gene duplications (◀ Section 13.8). Subsequent investigations of other nitrogen-fixing bacteria have shown that these genetically distinct "backup" nitrogenases are widely distributed among nitrogen-fixing microbes, in particular in the *Cyanobacteria* and *Archaea*.



(a)



(b)

Figure 15.24 ***Azotobacter vinelandii.*** *(a)* Vegetative cells and *(b)* cysts visualized by phase-contrast microscopy. A cell measures about 2 μm in diameter and a cyst about 3 μm.

Check Your Understanding

- What mechanisms do free-living diazotrophs use to protect nitrogenase from oxygen?
- Where might you expect to find nitrogen-fixing bacteria?

15.10 Diversity of Nitrifiers and Denitrifiers

Microorganisms that grow by the anaerobic respiration of inorganic nitrogen (NO_3^-, NO_2^-) to the gaseous products NO, N_2O, and N_2 are called **denitrifiers** (◀ Section 14.11). These organisms are typically facultative aerobes and chemoorganotrophs that use organic carbon as both carbon source and electron donor.

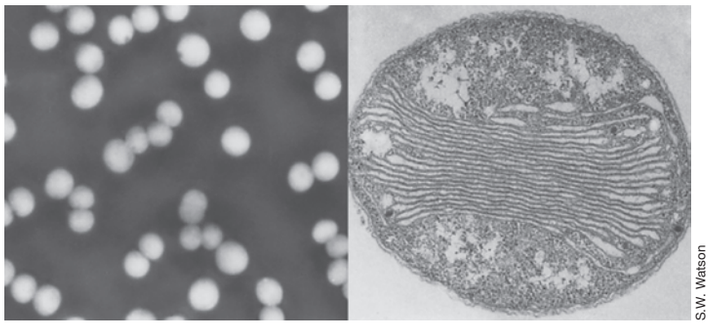
Microorganisms able to grow chemolithotrophically at the expense of reduced inorganic nitrogen compounds (NH_3, NO_2^-) are called **nitrifiers** (Figure 15.25; ◀ Section 14.11). These organisms are typically obligate aerobes that can also grow autotrophically; most species fix CO_2 by the Calvin cycle. A few species have also been shown to grow mixotrophically by assimilating organic carbon in addition to CO_2.

Physiology of Nitrifying *Bacteria* and *Archaea*

Nitrification often results from the sequential activities of two physiological groups of organisms, the *ammonia oxidizers* (which oxidize NH_3 to nitrite, NO_2^-) (Figure 15.25*a*) and the *nitrite oxidizers*, the actual nitrate-producing microorganisms, which oxidize NO_2^- to NO_3^- (Figure 15.25*b*). Ammonia oxidizers typically have genus names beginning in *Nitroso-*, whereas genus names of nitrate producers begin with *Nitro-*. However, certain microbes within the genus *Nitrospira* are able to carry out both ammonia oxidation and nitrite oxidation and are therefore able to oxidize ammonia all the way to nitrate.

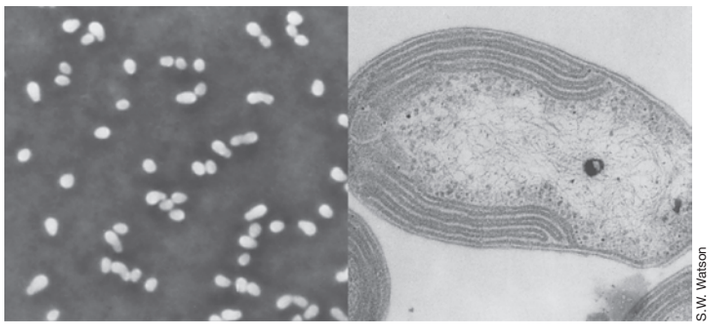
Many species of nitrifiers have internal membrane stacks (Figure 15.25) that closely resemble the photosynthetic membranes found in their close phylogenetic relatives, the purple phototrophic bacteria (Section 15.4) and the methane-oxidizing (methanotrophic) bacteria (Section 15.15). The membranes are the location of key enzymes in nitrification: *ammonia monooxygenase*, which oxidizes NH_3 to hydroxylamine (NH_2OH), and *nitrite oxidoreductase*, which oxidizes NO_2^- to NO_3^- (◀ Section 14.9).

Enrichment cultures of nitrifying bacteria can be achieved using mineral salts media containing NH_3 or NO_2^- as electron donors and bicarbonate (HCO_3^-) as the sole carbon source. Because these organisms produce very little ATP from their electron donors



Reaction: $NH_3 + 1\frac{1}{2}\ O_2 \longrightarrow NO_2^- + H^+ + H_2O$

(a)



Reaction: $NO_2^- + \frac{1}{2}\ O_2 \longrightarrow NO_3^-$

(b)

Figure 15.25 **Nitrifying bacteria.** *(a)* Phase-contrast photomicrograph (left) and electron micrograph (right) of the ammonia-oxidizing bacterium *Nitrosococcus oceani*. A single cell is about 2 μm in diameter. *(b)* Phase-contrast photomicrograph (left) and electron micrograph (right) of the nitrite-oxidizing bacterium *Nitrobacter winogradskyi*. A cell is about 0.7 μm in diameter. Beneath each panel is the energy-conserving reaction that each organism catalyzes. The distinct internal membranes of each species are sites of key enzymes of nitrification.

(◀ Section 14.9), visible turbidity may not develop in cultures even after extensive nitrification has occurred. An easy means of monitoring growth is thus to assay for the production of NO_2^- (with NH_3 as electron donor) or NO_3^- (with NO_2^- as electron donor).

Nitrifying *Bacteria* and *Archaea*: Ammonia Oxidizers

KEY GENERA: *Nitrosomonas, Nitrosospira, Nitrosopumilus*

Ammonia oxidizers are found in the *Betaproteobacteria* (e.g., *Nitrosomonas, Nitrosospira, Nitrosolobus, Nitrosovibrio*) and *Gammaproteobacteria* (*Nitrosococcus*), in the phylum *Nitrospirae*, and in the archaeal phylum *Thaumarchaeota* (*Nitrosopumilus, Nitrosocaldus, Nitrosoarchaeum, Nitrososphaera*).

Ammonia oxidizers are widespread in soil and water. Bacterial ammonia-oxidizers are present in highest numbers in habitats where NH_3 is abundant, such as sites with extensive protein decomposition (ammonification), and also in sewage treatment facilities (▶ Sections 22.6 and 22.7). Nitrifying bacteria develop especially well in lakes and streams that receive inputs of sewage or other wastewaters because these are frequently high in NH_3. *Nitrosomonas* is often observed in the activated sludge present in aerobic wastewater treatment facilities. Bacterial ammonia-oxidizers are also common in soils (e.g., *Nitrosospira, Nitrosovibrio*) and in the oceans (e.g., *Nitrosococcus*).

Archaeal ammonia-oxidizers (*Thaumarchaeota*, ▶ Section 17.5) appear to be most common in habitats where NH_3 is present in low concentration. These organisms are thought to be the dominant ammonia-oxidizers in the oceans where ammonia levels are very low (▶ Sections 20.10 and 20.12). Archaeal ammonia-oxidizers are also common in soils, and in some soils they outnumber bacterial ammonia-oxidizers by several orders of magnitude. The availability of NH_3 relative to NH_4^+ declines with pH (the substrate for the enzyme that oxidizes ammonia, ammonia monooxygenase, is NH_3, not NH_4^+) and thus acid soils (pH < 6.5), which are common, may favor organisms able to grow at low NH_3 concentration.

Nitrifying Bacteria: Nitrite Oxidizers

KEY GENERA: *Nitrospira, Nitrobacter*

Nitrite oxidizers are found in the classes *Alpha-* (*Nitrobacter*), *Beta-* (*Nitrotoga*), *Gamma-* (*Nitrococcus*), and *Deltaproteobacteria* (*Nitrospina*), as well as in the phylum *Nitrospirae* (genus *Nitrospira*) (▶ Section 16.21).

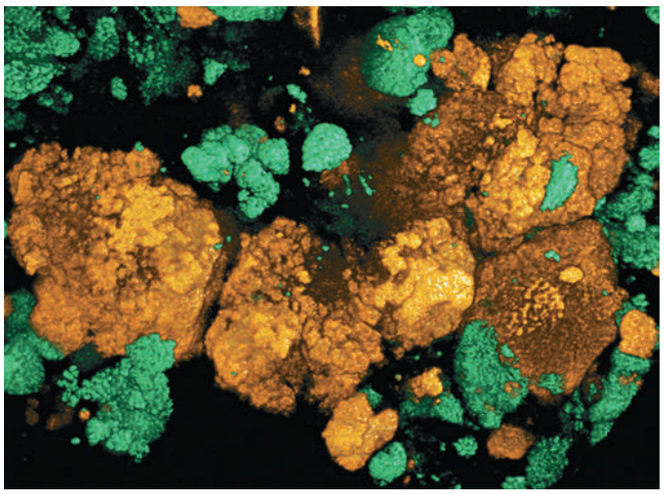
Like nitrite-oxidizing *Proteobacteria*, *Nitrospira* oxidizes nitrite (NO_2^-) to nitrate (NO_3^-) and grows autotrophically (**Figure 15.26**). However, *Nitrospira* lacks the extensive internal membranes found in species of nitrifying *Proteobacteria*. Nevertheless, *Nitrospira* inhabits many of the same environments as nitrite-oxidizing *Proteobacteria* such as *Nitrobacter*, so it has been suggested that its capacity for NO_2^- oxidation may have been acquired by horizontal gene flow from nitrifying *Proteobacteria* (or vice versa). As we know, this mechanism for acquiring physiological traits has been widely exploited in the bacterial world (Chapter 9 and ◀ Section 13.9). However, environmental surveys for the presence of nitrifying bacteria in nature have shown *Nitrospira* to be much more abundant than *Nitrobacter*; thus most of the NO_2^- oxidized in natural environments is probably due to the activities of *Nitrospira*.

Denitrifying *Bacteria* and *Archaea*

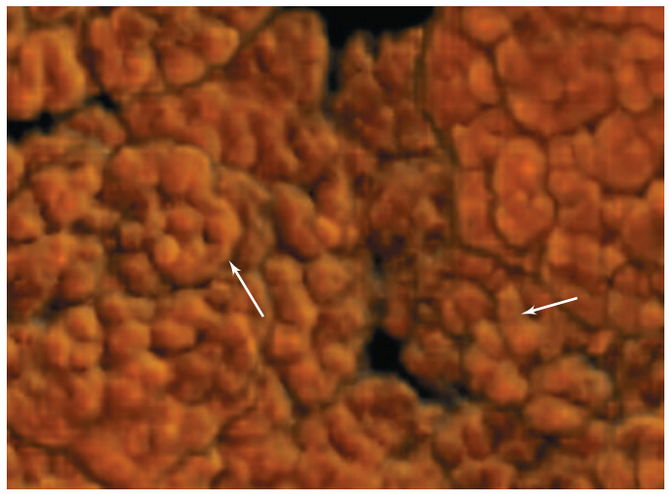
KEY GENERA: *Paracoccus, Pseudomonas*

Denitrifiers are capable of growth by the anaerobic respiration of NO_3^- or NO_2^- to the gaseous products NO, N_2O, and N_2 (◀ Section 14.11). Nearly all denitrifiers are chemoorganotrophs that use organic carbon as both carbon source and electron donor. Exceptions include the denitrifying sulfur-oxidizers discussed in Section 15.12. Denitrifiers are typically facultative aerobes, and in nearly all cases they will grow preferentially as aerobes if O_2 is present. Denitrifiers are of great importance in agricultural soils where they cause the loss of nitrogen fertilizers and the production of N_2O, which is a dominant component of greenhouse gases produced by agricultural soils (▶ Section 21.9).

Denitrifiers are phylogenetically and metabolically diverse and include two archaeal phyla (▶ Section 17.10) and six bacterial phyla, including five classes of *Proteobacteria* (Figure 15.1). One of the best-characterized denitrifiers is *Paracoccus denitrificans* (*Alphaproteobacteria*). Denitrification of NO_3^- to N_2 requires several key enzymatic steps (◀ Section 14.11), and the genes that encode these enzymes are present throughout the tree of life, indicating the strong influence of horizontal gene exchange. However, many nitrate reducers possess only part of the denitrification pathway and are thus unable to reduce NO_3^- completely to N_2, producing final products such as NO_2^-, NO, or N_2O.



(a)



(b)

Figure 15.26 Nitrifying bacteria. *Nitrospira* are a genus of nitrifying bacteria often found in biofilms and aggregates. The cell aggregates shown were obtained from activated sludge in a wastewater treatment facility. *(a)* Multiple cell aggregates (about 100 μm in diameter and $>10^6$ cells each) containing "*Candidatus* Nitrospira inopinata" (yellow), a species capable of completely oxidizing ammonia to nitrite. The biofilm was imaged by confocal laser microscopy using a phylogenetic stain specific for "*Ca.* N. inopinata" (▶ Section 19.5). (*b*) A single aggregate of *Nitrospira* cells. Individual cells are curved (arrows) and group into tetrads, which then cluster together to form the larger aggregate. A single cell of *Nitrospira* is about 0.3 × 1–2 μm.

Check Your Understanding

- Under what conditions would you expect microorganisms to grow as a result of denitrification?
- Which traits are shared among ammonia oxidizers and nitrite oxidizers?

15.11 Dissimilative Sulfur- and Sulfate-Reducers

Sulfur metabolism may have fueled the earliest forms of life on our planet (◀ Section 13.1 and Figure 13.5), and the sulfur cycle (▶ Section 21.4) continues to support an enormous diversity of microorganisms today. In this section, we consider the diversity of organisms capable of *dissimilative sulfur metabolism*; that is, organisms that conserve energy through the oxidation or reduction of sulfur compounds (◀ Sections 14.7 and 14.12).

The remarkable diversity of *Bacteria* and *Archaea* capable of dissimilative sulfur metabolism is in part a function of the chemical diversity in which sulfur occurs in the biosphere. Sulfur has eight oxidation states that range from its most oxidized form, sulfate (SO_4^{2-}, oxidation state of +6), to thiosulfate ($S_2O_3^{2-}$, oxidation state of +2), to elemental sulfur (S^0, oxidation state of 0), and finally to hydrogen sulfide (H_2S, oxidation state of −2), its most reduced form. In addition, sulfur compounds can take on diverse chemical forms including inorganic sulfur compounds, organosulfur compounds, and metal sulfides.

Dissimilative Sulfate-Reducers

KEY GENERA: ***Desulfovibrio, Desulfobacter***

Dissimilative sulfate-reducers gain energy by coupling the oxidation of H_2 or organic compounds to the reduction of SO_4^{2-} (anaerobic respiration, ◀ Section 14.12). The more than 30 known genera of sulfate reducers are found across five phyla of *Bacteria* and *Archaea* (**Figure 15.27**). Most sulfate reducers reside in the *Deltaproteobacteria*, though sulfate reducers are also found in the *Firmicutes* (e.g., *Desulfotomaculum* and *Desulfosporosinus*), *Thermodesulfobacteria* (e.g., *Thermodesulfobacterium*), and *Nitrospirae* (e.g., *Thermodesulfovibrio*). Sulfate reduction also occurs in *Archaeoglobus*, a genus of the archaeal phylum *Euryarchaeota*.

Physiology of Sulfate-Reducing Bacteria

Sulfate-reducing bacteria are morphologically and biochemically diverse. The biochemistry of sulfate reduction was discussed in Section 14.12, so here we consider some of the more general physiological properties of this group. Sulfate reducers are generally obligate anaerobes, and strict anoxic techniques must be used in their cultivation (**Figure 15.28*g***).

Sulfate reducers use H_2 or organic compounds as electron donors for growth, and the range of organics used is broad. Lactate and pyruvate are almost universally used, and many species also oxidize short-chain alcohols (ethanol, propanol, and butanol) as electron donors. Some species, such as *Desulfosarcina and Desulfonema*, grow chemolithotrophically and autotrophically with H_2 as an electron donor, SO_4^{2-} as an electron acceptor, and CO_2 as the sole carbon source. A few sulfate reducers can oxidize hydrocarbons as electron donors (◀ Section 14.24).

There are two physiological types of dissimilative sulfate-reducers, the *complete oxidizers*, which can oxidize acetate and other fatty acids completely to CO_2, and the *incomplete oxidizers*, which are unable to oxidize acetate to CO_2. The latter group includes the best studied of the sulfate-reducing bacteria, *Desulfovibrio* (Figure 15.28*a*), along with *Desulfomonas*, *Desulfotomaculum*, and *Desulfobulbus* (Figure 15.28*c*). The acetate oxidizers include *Desulfobacter* (Figure 15.28*d*), *Desulfococcus*, *Desulfosarcina* (Figure 15.28*e*), and

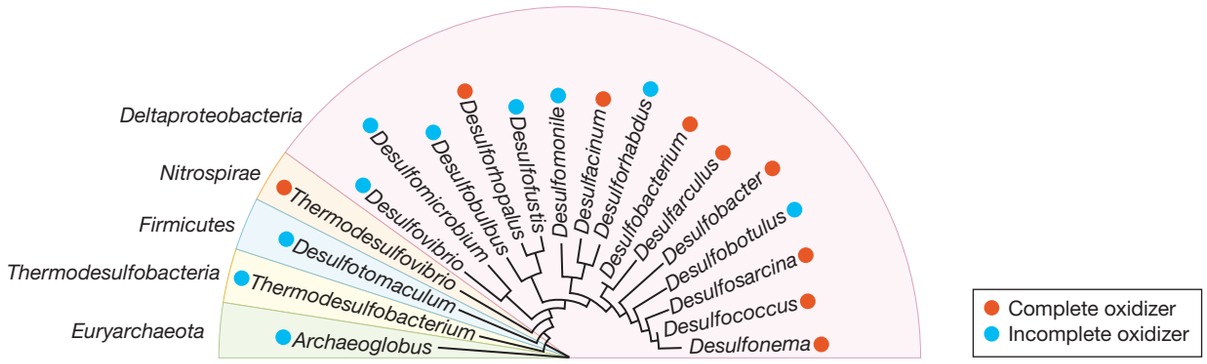


Figure 15.27 Dissimilative sulfate-reducers. The dendrogram depicts phylogenetic relationships among some genera of sulfate reducers as inferred by analysis of their 16S ribosomal RNA gene sequences. Color shading is used to differentiate the five main phyla that contain genera of sulfate reducers. Colored circles indicate whether species are complete oxidizers, which are able to oxidize acetate to CO_2, or incomplete oxidizers, which cannot oxidize acetate. The physiology of sulfate-reducing bacteria is considered in Section 14.12 and their role in the sulfur cycle in Section 21.4.

Desulfonema (Figure 15.28*b*), among many others. These bacteria specialize in the oxidation of fatty acids (in particular acetate) to CO_2 and reducing SO_4^{2-} to H_2S. These two physiological groups are not phylogenetically coherent but instead are distributed widely across the phylogeny of sulfate-reducing bacteria (Figure 15.27).

Some sulfate-reducing bacteria can exploit alternative metabolic pathways. In addition to SO_4^{2-} or S^0, some sulfate reducers can also reduce nitrate and sulfonates (such as isethionate, $HO—CH_2—CH_2—SO_3^-$). Sulfate-reducing bacteria can also ferment certain organic compounds such as pyruvate. Moreover, although generally obligate anaerobes, a few sulfate-reducing bacteria are quite O_2-tolerant (primarily strains that coexist with O_2-producing cyanobacteria in microbial mats). At least one species, *Desulfovibrio oxyclinae*, can actually grow with O_2 as the electron acceptor under microaerophilic conditions.

Ecology of Sulfate-Reducing Bacteria

Sulfate reducers are widespread in aquatic and terrestrial environments that contain SO_4^{2-} and become anoxic as a result of microbial decomposition. Sulfate reducers are abundant in marine sediments, and the H_2S they generate is responsible for the pungent smell (like that of rotten eggs) often encountered in decaying vegetation near coastal ecosystems. *Desulfotomaculum*, phylogenetically a species of *Firmicutes* (gram-positive *Bacteria*), consists of endospore-forming rods found primarily in soil. Growth and reduction of SO_4^{2-} by *Desulfotomaculum* in certain canned foods leads to a type of spoilage called *sulfide stinker*. Species of *Thermodesulfobacterium*, *Thermodesulfovibrio*, and *Archaeoglobus* (an archaeon) are all thermophilic and found in geothermally heated environments such as hot springs, hydrothermal vents, and oil reserves. The remaining genera of sulfate reducers are indigenous to anoxic marine and freshwater environments and can occasionally be isolated from the mammalian gut.

The enrichment of *Desulfovibrio* species is straightforward in an anoxic lactate–sulfate medium containing ferrous iron (Fe^{2+}). A reducing agent, such as thioglycolate or ascorbate, is required to achieve a low reduction potential (E_0') in the medium. When sulfate-reducing bacteria grow, the H_2S they form combines with the ferrous iron to form black, insoluble ferrous sulfide (Figure 15.28*g*). Purification can be accomplished by diluting the culture in molten agar tubes (▶ Section 19.2 and Figure 19.3*b*). Upon solidification, individual cells of sulfate-reducing bacteria become distributed throughout the agar and grow to form black colonies (Figure 15.28*g*) that can be removed aseptically to yield pure cultures.

Dissimilative Sulfur-Reducers

KEY GENERA: *Desulfuromonas, Wolinella*

Here we consider the **dissimilative *sulfur*-reducers**, microorganisms that are able to use the respiratory reduction of S^0 to conserve energy. Dissimilative sulfur-reducing bacteria can reduce S^0 and other oxidized forms of sulfur (such as SO_3^{2-}) to H_2S but are unable to reduce SO_4^{2-}. There are more than 25 genera of dissimilative sulfur-reducers spread across five bacterial and archaeal phyla (Figure 15.1).

Most sulfur-reducing bacteria are *Proteobacteria*, primarily *Deltaproteobacteria* (e.g., *Desulfuromonas*, *Pelobacter*, *Desulfurella*, *Geobacter*), with some genera residing in the *Epsilonproteobacteria* (e.g., *Wolinella* and *Sulfurospirillum*) and *Gammaproteobacteria* (e.g., *Shewanella* and *Pseudomonas mendocina*). Other sulfur-reducing bacteria are species of *Firmicutes* (e.g., *Desulfitobacterium* and *Ammonifex*), *Aquificae* (e.g., *Desulfurobacterium* and *Aquifex*), *Synergistetes* (e.g., *Dethiosulfovibrio*), *Deinococcus–Thermus* (e.g., *Thermus*) or *Deferribacteres* (e.g., *Geovibrio*). The sulfur-reducing *Archaea*—of which there are many—are all genera of the phylum *Crenarchaeota* (e.g., *Acidianus*, *Pyrodictium*, and *Thermodiscus*).

Physiology and Ecology of Sulfur-Reducing Bacteria

The physiology of sulfur reducers is more diverse than that of sulfate reducers. Most sulfur reducers are obligate anaerobes, but facultatively aerobic species are also common. Sulfur reducers are often able to reduce electron acceptors such as nitrate, ferrous iron, or thiosulfate as alternatives to S^0. Like sulfate reducers, the physiology of sulfur reducers is characterized by whether they completely oxidize acetate and other fatty acids to CO_2. Species of *Desulfuromonas* (Figure 15.28*f*) are complete oxidizers that grow anaerobically by coupling the oxidation of acetate, succinate, ethanol, or propanol to the reduction of S^0.

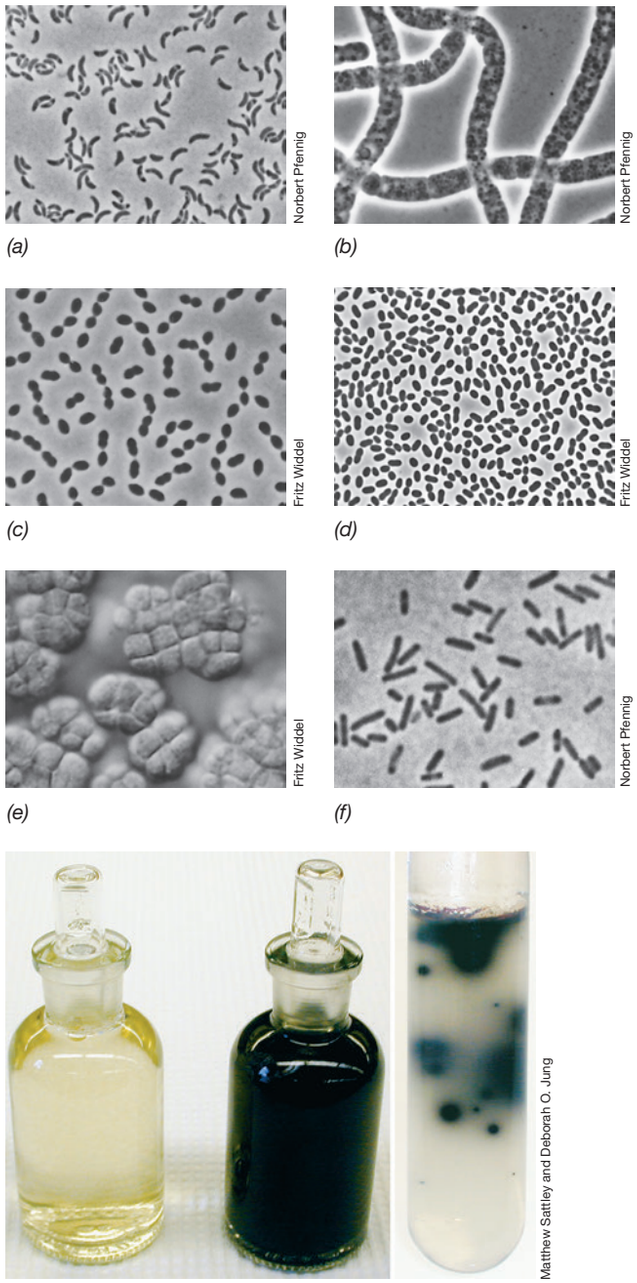


Figure 15.28 **Representative sulfate-reducing and sulfur-reducing bacteria.** *(a) Desulfovibrio desulfuricans*; cell diameter about 0.7 μm. *(b) Desulfonema limicola*; cell diameter 3 μm. *(c) Desulfobulbus propionicus*; cell diameter about 1.2 μm. *(d) Desulfobacter postgatei*; cell diameter about 1.5 μm. *(e) Desulfosarcina variabilis*; cell diameter about 1.25 μm. *(f) Desulfuromonas acetoxidans*; cell diameter about 0.6 μm. *(g)* Enrichment culture of sulfate-reducing bacteria. Left, sterile medium; center, a positive enrichment showing black FeS; right, colonies of sulfate-reducing bacteria in a dilution tube (▶ Section 19.2 and Figure 19.3*b*). Photos *a–d* and *f* are phase-contrast photomicrographs; part *e* is a differential interference contrast micrograph.

In contrast, *Sulfospirillum* and *Wolinella* are incomplete oxidizers and cannot use acetate as an electron donor. *Sulfospirillum* can reduce S^0 using either H_2 or formate as electron donor.

Dissimilative sulfur-reducing bacteria reside in many of the same habitats as dissimilative sulfate-reducing bacteria and often form associations with bacteria that oxidize H_2S to S^0, such as green sulfur bacteria (Section 15.6). The S^0 produced from H_2S oxidation is then reduced back to H_2S during metabolism of the sulfur reducer, completing an anoxic sulfur cycle (▶ Section 21.4).

Check Your Understanding

- What are the typical electron donors used by dissimilative sulfur-reducers?
- What are the typical electron donors used by dissimilative sulfate-reducers?
- What bacterial phyla are known to contain dissimilative sulfate-reducers?

15.12 Dissimilative Sulfur-Oxidizers

KEY GENERA: ***Thiobacillus, Achromatium, Beggiatoa***

Dissimilative sulfur-oxidizers are **chemolithotrophs** that oxidize reduced sulfur compounds such as H_2S, S^0, thiosulfate, or thiocyanate (SCN^-) as electron donors in energy conservation, typically with O_2 as electron acceptor. These organisms are common in environments such as marine sediments, sulfur springs, and hydrothermal systems where H_2S produced by sulfate- or sulfur-reducing bacteria (Section 15.11), or abiotically by geothermal reactions, is released into oxygenated waters (**Figure 15.29**). The sulfur oxidizers are found in three phyla of *Bacteria* (*Proteobacteria*, *Aquificae*, *Deinococcus–Thermus*) and one of *Archaea* (*Crenarchaeota*) (Figure 15.1). Most sulfur-oxidizing bacteria are *Beta-* (*Thiobacillus*), *Gamma-* (*Achromatium*, *Beggiatoa*), or *Epsilonproteobacteria* (*Thiovulum*, *Sulfurimonas*).

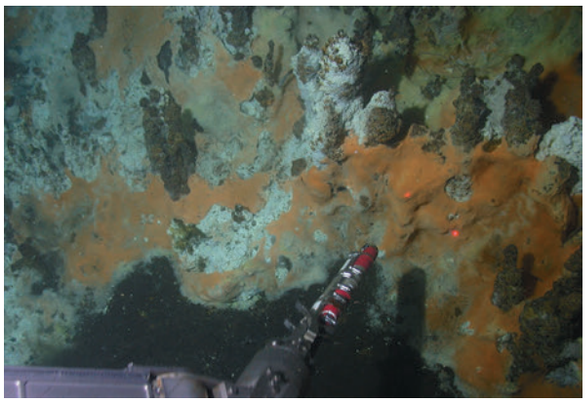
Physiological Diversity of Sulfur-Oxidizing Bacteria

The morphological and physiological diversity of sulfur oxidizers is significant. Cells can be less than 1 micrometer in diameter (e.g., *Sulfurimonas denitrificans*) or as large as 750 micrometers in diameter (e.g., *Thiomargarita namibiensis*). Most sulfur oxidizers are obligate aerobes; however, species of *Thiomargarita* and *Sulfurimonas* can also reduce NO_3^- in denitrification (◀ Section 14.11 and Section 15.10). Many species oxidize H_2S to elemental sulfur (S^0), which they deposit as either intracellular or extracellular granules for later use as an electron donor (◀ Figure 14.19) if H_2S becomes limiting.

Some sulfur chemolithotrophs are *obligate chemolithotrophs*, locked into a lifestyle of using inorganic instead of organic compounds as electron donors. When growing in this fashion, they are also autotrophs, converting CO_2 into cell material by reactions of the Calvin cycle. **Carboxysomes** are often present in cells of obligate chemolithotrophs (**Figure 15.30*a***). These structures contain high levels of Calvin cycle enzymes and probably increase the rate at which these organisms fix CO_2 (◀ Section 14.2).

Other sulfur chemolithotrophs are *facultative chemolithotrophs*, facultative in the sense that they can grow *either* chemolithotrophically (and thus, also as autotrophs) *or* chemoorganotrophically. Most species of *Beggiatoa* can obtain energy from the oxidation of inorganic sulfur compounds but lack enzymes of the Calvin cycle. They thus require organic compounds as carbon sources. Organisms that use a mix of carbon and energy sources, for example those that simultaneously assimilate carbon from both CO_2 and organic sources, are called **mixotrophs**.

(a)



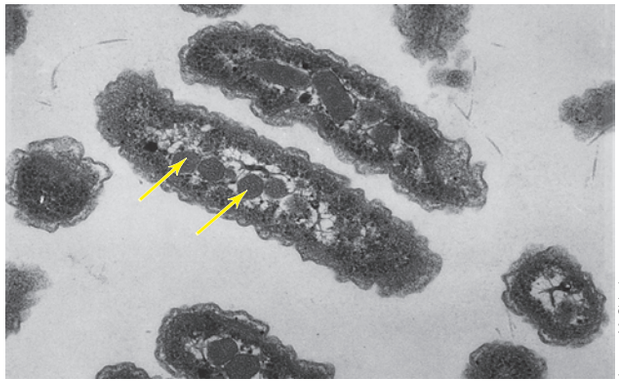
(b)

Figure 15.29 Habitats of sulfur oxidizers. *(a)* A sulfidic artesian spring in Florida (USA). The outside of the spring is coated with a mat of *Thiothrix* (see Figure 15.31*b*). The mat is about 1.5 m in diameter. *(b)* Hydrothermal chimneys at Cathedral Hill in the Guaymas Basin (Mexico), 2000-m depth. Sulfide-rich waters vent from the chimneys, which are covered by mats composed of orange, white, and yellow cells of *Beggiatoa*.

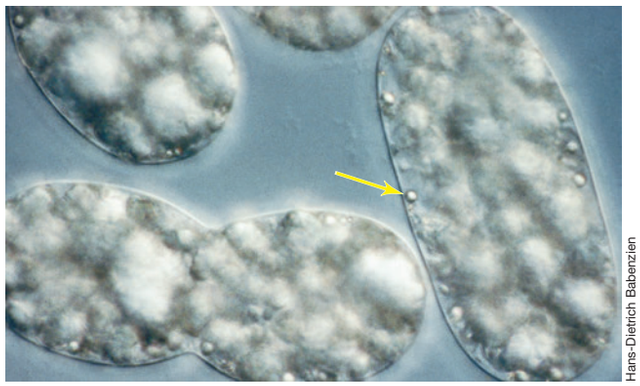
Thiobacillus and *Achromatium*

The genus *Thiobacillus* and related genera include several gram-negative, rod-shaped *Betaproteobacteria* (Figure 15.30*a*) that are the best studied of the sulfur chemolithotrophs. The oxidation of H_2S, S^0, or thiosulfate by *Thiobacillus* generates sulfuric acid (H_2SO_4), and thus thiobacilli are often acidophilic. One highly acidophilic species, *Acidithiobacillus ferrooxidans*, can also grow chemolithotrophically by the oxidation of Fe^{2+} and is a major biological agent for the oxidation of this metal. Iron pyrite (FeS_2) is a major natural source of ferrous iron as well as of sulfide. The oxidation of FeS_2, especially in mining operations, can be both beneficial (because leaching of the ore releases the iron from the sulfide mineral) and ecologically disastrous (the environment can become acidic and contaminated with toxic metals such as aluminum, cadmium, and lead) (▶ Sections 22.1 and 22.2).

Achromatium is a spherical sulfur-oxidizing chemolithotroph that is common in freshwater sediments of neutral pH containing H_2S. Cells of *Achromatium* are large cocci that can have diameters of 10–100 μm (Figure 15.30*b*). *Achromatium* is a species of *Gammaproteobacteria* and is specifically related to purple sulfur bacteria, such as its phototrophic counterpart *Chromatium* (Section 15.4 and Figure 15.10*a*). Like *Chromatium*, cells of *Achromatium* store S^0 internally (Figure 15.30*b*); the granules later disappear as S^0 is oxidized to SO_4^{2-}. Cells of *Achromatium* also store large granules of calcite



(a)



(b)

Figure 15.30 Nonfilamentous sulfur chemolithotrophs. *(a)* Transmission electron micrograph of cells of the chemolithotrophic sulfur-oxidizer *Halothiobacillus neapolitanus*. A single cell is about 0.5 μm in diameter. Note the polyhedral bodies (carboxysomes) distributed throughout the cell (arrows) (◀ Figure 14.3). *(b) Achromatium*. Cells photographed by differential interference contrast microscopy. The small globular structures near the periphery of the cells (arrow) are elemental sulfur, and the large granules are calcium carbonate. A single *Achromatium* cell is about 25 μm in diameter.

($CaCO_3$) (Figure 15.30*b*), possibly as a carbon source (in the form of CO_2) for autotrophic growth. The physiology of chemolithotrophic sulfur-oxidizers is discussed in Section 14.7.

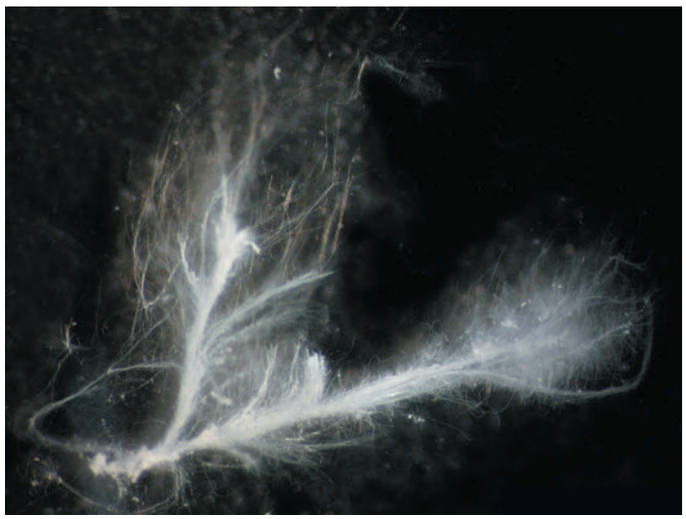
Ecological Diversity and Strategies of Sulfide-Oxidizing Bacteria

Aerobic sulfide-oxidizers provide a case study that demonstrates the degree of ecological diversification that can occur among microbes that share the same basic metabolic features. The chemical oxidation of H_2S to H_2SO_4 is spontaneous and rapid in the presence of O_2. Hence, aerobic H_2S-oxidizers have evolved diverse ecological strategies that allow them to metabolize two molecules that otherwise react with each other spontaneously. We consider here six different strategies used by aerobic sulfide-oxidizers to cope with the chemical instability of H_2S in the presence of O_2.

- *Thiothrix* is a filamentous sulfur chemolithotroph that forms filaments that group together at their ends by way of a holdfast to form cell arrangements called *rosettes* (**Figure 15.31**). The ecological strategy of *Thiothrix* is to use its holdfast to position itself in

high-flow environments downstream from a source of H_2S. Such environments are common near sulfur springs and in creeks draining sulfidic salt marshes where abundant H_2S is produced and carried away in waters rich with O_2 (Figure 15.31*a*). Physiologically, *Thiothrix* is an obligately aerobic mixotroph, and in this and most other respects, it resembles *Beggiatoa*.

- *Beggiatoa* are filamentous, gliding, sulfur-oxidizing bacteria that are usually large in both diameter and length, consisting of many short cells attached end to end (**Figure 15.32*a***). Filaments can flex and twist so that many filaments become intertwined to form a complex tuft. *Beggiatoa* is found primarily in microbial mats, sediments, sulfur springs, and hot springs. The ecological strategy of *Beggiatoa* is to use gliding motility to position itself at the point where H_2S and O_2 co-occur in an environment. For example, *Beggiatoa* in microbial mats can move vertically by as much as several centimeters per day in response to cyanobacterial O_2 production, moving up to obtain O_2 when photosynthesis ceases at night and down during the day when cyanobacterial O_2 production at the mat surface causes H_2S to be found deeper in the mat.
- The genus *Thiomargarita* contains some of the largest bacteria yet observed, with diameters that can be as much as 0.75 millimeter (**Figure 15.33**). *Thiomargarita* is nonmotile, and its ecological strategy is to separate in time the oxidation of H_2S from the reduction of O_2. To accomplish this, *Thiomargarita* contains a giant vacuole (Figure 15.33*b*) that it fills with high concentrations of nitrate (NO_3^-). This vacuole can fill almost the entire volume of the cell. Cells live in sulfide-rich marine sediments that are mixed occasionally with O_2-rich waters, such as that in salt marshes and in ocean upwelling zones. When buried in sediment, cells oxidize H_2S to S^0 anaerobically by reducing NO_3^- stored in the vacuole to ammonium (NH_4^+). They then store the S^0 as intracellular granules (Figure 15.33*a*). When turbulent waters mix the cells into the water column where H_2S is lacking, they switch to the aerobic oxidation of stored S^0. The energy they gain from S^0 oxidation is used to refill their vacuole with NO_3^- from the water column so they will be able to survive the next period of anoxia.



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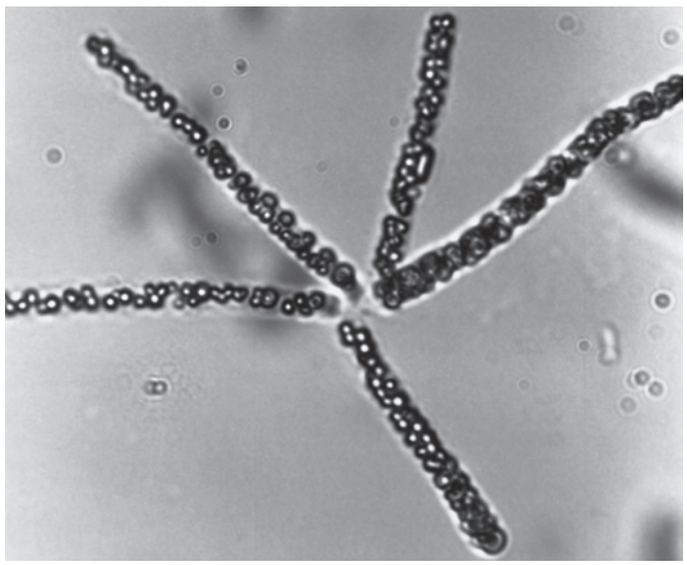
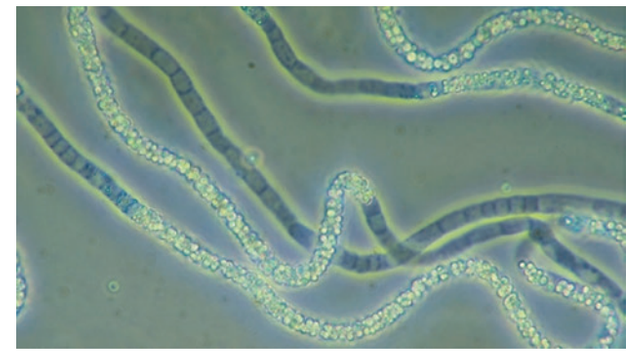


(b)

Figure 15.31 ***Thiothrix.*** *(a)* Filaments of *Thiothrix* attached to plant material found in the outwash stream of a sulfidic cave in Frasassi, Italy. From the plant branch point, the longest branch is about 4 mm long. *(b)* Phase-contrast photomicrograph of a rosette of cells of *Thiothrix* isolated from the sulfide-containing artesian spring shown in Figure 15.29*a*. Note the internal sulfur globules produced from the oxidation of sulfide. Each filament is about 4 μm in diameter.



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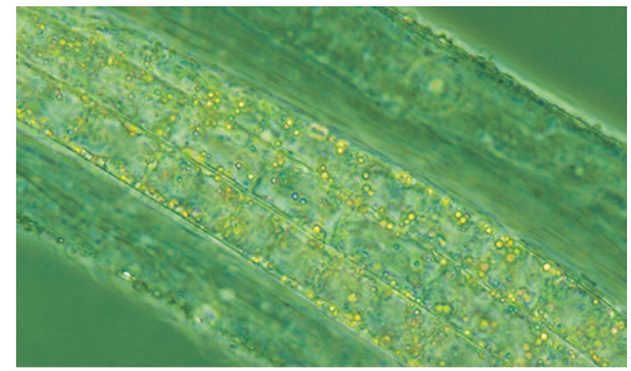


(b)

Figure 15.32 **Filamentous sulfur-oxidizing bacteria.** *(a)* Phase-contrast photomicrograph of a *Beggiatoa* species isolated from a sewage treatment plant. Note the abundant elemental sulfur granules in some of the cells. *(b)* Cells of a large marine *Thioploca* species. Cells contain sulfur granules (yellow) and are about 40–50 μm wide.

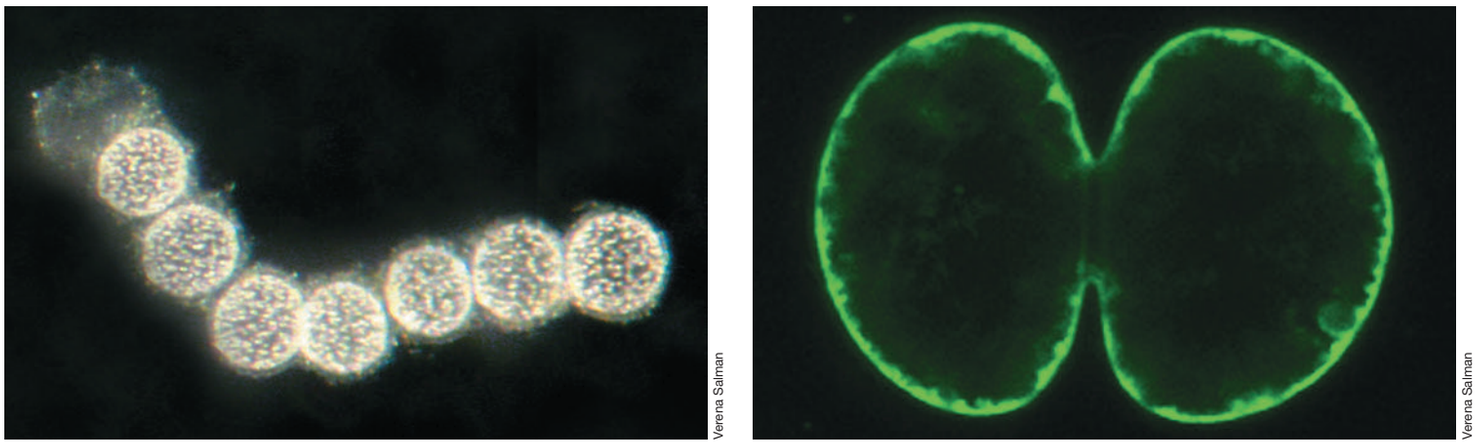


Figure 15.33 **The giant sulfide-oxidizing bacterium, *Thiomargarita*.** *(a) Thiomargarita namibiensis* recovered from the Namibian upwelling (off the Namibian coast, southwest Africa). Cells are about 100 μm in diameter. *(b)* Dividing cells of vacuole-containing sulfide-oxidizers recovered from the same location. The fluorescence micrograph shows ribosomes of *Thiomargarita* stained with a fluorescent nucleic acid probe. Ribosomes are found in the cytoplasm, which is present as a thin layer along the outer edge of the cells. The cytoplasm is squeezed between the cell wall and the large central vacuole, which appears dark in the image. Cells are about 50 μm wide.

- *Thioploca* are large filamentous bacteria that use a strategy similar to that of *Thiomargarita*. *Thioploca* also have intracellular S^0 granules and large vacuoles filled with NO_3^- (Figure 15.32*b*). However, filaments of *Thioploca* are motile by gliding and they occur in large sheaths that can be filled with many parallel filaments (Figure 15.32*b*). Sheaths are arranged vertically in the sediments and filaments glide up and down in the sheaths, going down to anaerobically respire H_2S using stored NO_3^- as electron acceptor and going up to aerobically respire S^0 and to refill their vacuoles with NO_3^- (▶ Figure 20.10).
- *Thiovulum* are found in freshwater and marine habitats in which sulfide-rich muds interface with oxic zones (**Figure 15.34**). *Thiovulum* cells are fairly large (10–20 μm) cocci, and when motile, they swim at exceptionally high speed, perhaps the fastest of all known bacteria (~0.6 mm/sec). The ecological strategy of *Thiovulum* is to actually control the flow of nutrients to cells. *Thiovulum* cells secrete a slime that links cells together in a veil-like structure that can be centimeters in diameter (Figure 15.34*a*). The veils, composed of many *Thiovulum* cells (Figure 15.34*b*), are formed over a source of H_2S. Cells have long flagella that attach to the veil and to solid surfaces. Since the terminal end of the flagellum is attached and immobile, flagellar rotation causes cells to rotate along their flagellar axis (Figure 15.34*c*). The simultaneous unidirectional rotation of all the *Thiovulum* cells in the veil creates a flow of water through the veil, allowing the cells (Figure 15.34*b*, *d*) to generate and regulate the gradients of H_2S and O_2 they require to generate energy (Figure 15.34*c*).
- The final ecological strategy of sulfur chemolithotrophs is for the organism to form a symbiotic association with a eukaryote. There are diverse symbiotic associations in which the host provides a mechanism for regulating H_2S and O_2 levels and the sulfide-oxidizing symbiont fixes CO_2 and provides a source of carbon and energy to the host. The best example is the tube worm *Riftia*, which contains sulfide-oxidizing endosymbionts and lives at deep-sea hydrothermal vents (▶ Section 23.11). A variety of other such symbiotic associations are present at hydrothermal vent

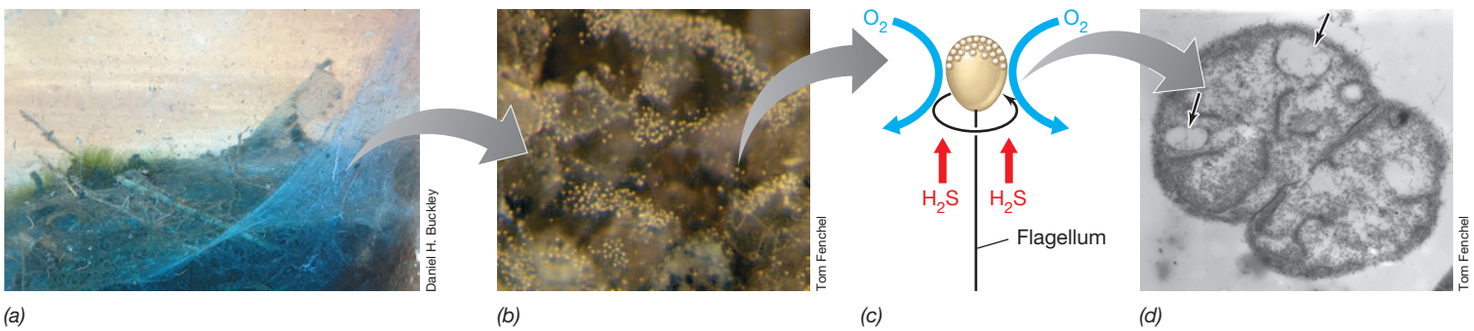


Figure 15.34 **The sulfide-oxidizing bacterium *Thiovulum*.** *(a)* Photo of a *Thiovulum* veil forming over marine sediment. The veil, composed of many cells within a polysaccharide matrix, appears like a spider web covering the sediment surface. Under the veil, H_2S is released from the sediment. *(b)* Close-up photograph of *Thiovulum* cells (yellow dots) within their veil. *(c) Thiovulum* cells attach to the sediment with a long holdfast and begin to rotate. The collective rotation of the many cells within the veil cause O_2-rich water above the veil to be pumped down across the veil. Hence, through collective action, *Thiovulum* cells manipulate their environment to optimize local concentrations of O_2 and H_2S. *(d)* Transmission electron micrograph of a section from a dividing cell of *Thiovulum*. Arrows point to sulfur (S^0) globules. Single cells of *Thiovulum* are typically 10–20 μm in diameter.

ecosystems, including symbionts living in the gill tissue of the giant clam *Calyptogena magnifica* and on the surface of the yeti crab, which farms sulfide-oxidizing bacteria by waving its claws over sulfide-rich vent fluid. Symbioses involving invertebrates are also common in the sulfide-rich marine sediments of shallow coastal systems. For example, bivalves in the family *Solemyidae* burrow into sulfide-rich sediments and pump sulfide- and oxygen-rich water over gills that contain sulfide-oxidizing bacteria.

From these examples it should be clear how ecological diversity drives bacteria that carry out the same energy metabolism—in this case sulfide oxidation—to best exploit the different environments they inhabit. In each case, the goal of the organism is the same, to obtain the electron donor and acceptor it needs. But also in each case, the strategy to accomplish this is unique and the best fit to both the properties of the organism and the habitat it exploits.

Check Your Understanding

- Describe the energy and carbon metabolism of *Thiobacillus* in terms of how ATP and new cell material are made.
- What are some ecological strategies that sulfur oxidizers use to compete with the spontaneous (chemical) oxidation of H_2S?

15.13 Dissimilative Iron-Reducers

KEY GENERA: *Geobacter, Shewanella*

Dissimilative iron-reducers couple the reduction of oxidized metals or metalloids to cellular growth. These organisms need to overcome the fundamental obstacle of using an insoluble solid material as an electron acceptor in respiration (◀ Section 14.13). A variety of microorganisms are able to enzymatically reduce metals as a consequence of either fermentation reactions or sulfur or sulfate reduction, but such organisms do not conserve energy from metal reduction. In contrast, dissimilative iron-reducers carry out metal respiration by coupling the oxidation of H_2 or organic compounds to the reduction of ferric iron (Fe^{3+}) (**Figure 15.35*a***) or manganese (Mn^{6+}).

Dissimilative iron-reducers are phylogenetically diverse (Figure 15.1). Iron-reducing bacterial genera are found in the *Proteobacteria* (*Geobacter*, *Shewanella*), *Acidobacteria* (*Geothrix*), *Deferribacteres* (*Geovibrio*), *Deinococcus–Thermus* (*Thermus*), *Thermotogae* (*Thermotoga*), and *Firmicutes* (*Bacillus*, *Thiobacillus*), while archaeal genera are found in the *Crenarchaeota* (*Pyrobaculum*). Iron respiration likely evolved early in the history of life, and its wide distribution may be due to its presence in the universal ancestor coupled with subsequent gene loss in some lineages and horizontal gene transfer to others.

Physiology

Dissimilative iron-reducers specialize in using insoluble external electron acceptors, and these organisms are typically extremely versatile at anaerobic respiration. Dissimilative iron-reducers are unusual in that they possess outer membrane cytochromes that facilitate electron transfer with insoluble minerals (◀ Figure 14.31). Most species are able to use either iron oxides or manganese oxides as electron acceptors, and various species are also able to use nitrate, fumarate, and oxidized inorganic sulfur, cobalt, chromium, uranium, tellurium, selenium, arsenic, and humic compounds (◀ Section 14.13). Most genera of iron-reducing bacteria are obligate anaerobes, but some, such as

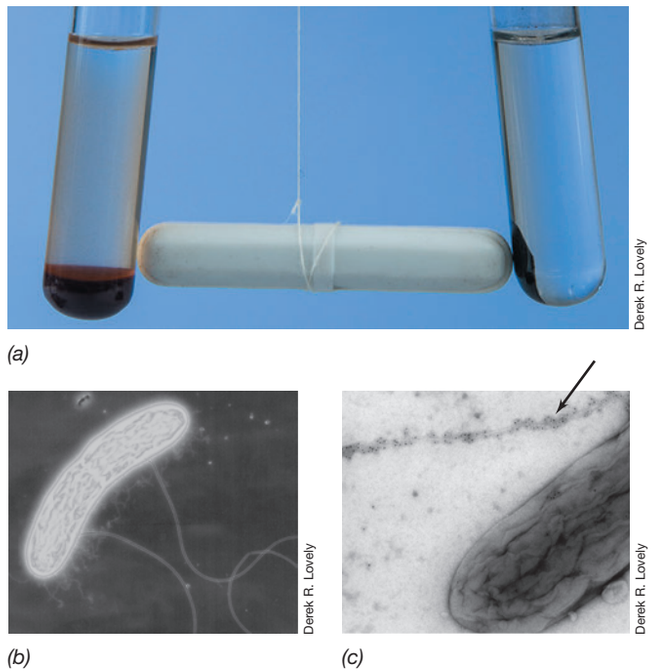


Figure 15.35 The dissimilative iron-reducing bacterium *Geobacter*. *(a)* The uninoculated tube (left) contains an anoxic medium that includes acetate and ferrihydrite, a poorly magnetic iron oxide. Following growth of *Geobacter* (right tube) the ferrihydrite is reduced to magnetite, which is magnetic. *(b)* Transmission electron micrograph of *Geobacter sulfurreducens* showing flagella and pili (◀ Figure 14.31). The cell is about 0.7 × 3.5 μm. *(c)* Transmission electron micrograph of *G. sulfurreducens* showing immunogold labeling of cytochrome OmcS on the pili (arrow).

Shewanella and relatives, are facultative aerobes. Electron donors are typically organic compounds such as fatty acids, alcohols, sugars, and in certain cases, even aromatic compounds. Many species are also able to use H_2 as an electron donor, but they are generally unable to grow autotrophically, requiring a source of organic carbon to support growth.

The family *Geobacteraceae* in the *Deltaproteobacteria* contains four genera of dissimilative iron-reducing bacteria (*Geobacter, Desulfuromonas, Desulfuromusa, Pelobacter*) that aptly demonstrate the physiological diversity of the obligately anaerobic metal reducers. *Geobacter*, *Desulfuromonas*, and *Desulfuromusa* can all use acetate as an electron donor as well as a diversity of other small organics, and they oxidize these substrates completely to CO_2. These genera typically specialize in anaerobic respiration. *Geobacter* in particular can use a wide range of electron donors and acceptors. *Geobacter* produce pili (Figure 15.35*b*) that contain cytochromes (Figure 15.35*c*), and these pili facilitate electron transfer to the surface of iron oxide minerals. *Pelobacter*, in contrast, are primarily fermentative organisms having a more limited respiratory capacity. For example, *Pelobacter carbinolicus* can only use lactate as the electron donor and can only use ferric iron or S^0 as the electron acceptors. *Pelobacter* species are unable to oxidize their carbon substrates completely to CO_2.

Shewanella and its relatives *Ferrimonas* and *Aeromonas* in the *Gammaproteobacteria* are facultative aerobes and will grow aerobically when O_2 is available. *Shewanella* are able to use a wide diversity of electron donors and acceptors in addition to ferric iron and manganese. However, like *Pelobacter* species, they are unable to oxidize their carbon substrates completely to CO_2 and are unable to oxidize acetate as an electron donor for anaerobic respiration.

Ecology

Dissimilative iron-reducers are common in anoxic freshwater and marine sediments. These organisms are thought to play an important role in organic matter oxidation in many anoxic habitats. Dissimilative iron-reducers are also common in shallow aquifers as well as in the deep subsurface environment (▶ Section 20.8). In addition, several thermophilic and hyperthermophilic iron-reducing species are known (e.g., *Thermus*, *Thermotoga*) and are often found in hot springs and other geothermally heated systems, including the deep subsurface.

Check Your Understanding

- In what phylogenetic groups are *Geobacter* and *Shewanella* found?
- Which genera of dissimilative iron-reducers contain facultative aerobes?

15.14 Dissimilative Iron-Oxidizers

KEY GENERA: *Acidithiobacillus, Gallionella*

The ability to couple the oxidation of ferrous iron (Fe^{2+}) to cell growth is widespread in the tree of life and thought to be a trait that evolved early in Earth's history. Genera capable of using ferrous iron as an electron donor to support growth are spread across five bacterial and two archaeal phyla (Figure 15.1).

Aerobic iron-oxidizer diversity and distribution are influenced strongly by pH and O_2. Ferrous iron oxidizes spontaneously to form insoluble precipitates in the presence of O_2 at neutral to alkaline pH (pH $>$ 7) but is stable either under anoxic conditions or aerobically at acidic pH (pH $<$ 4). Iron oxidizers can be divided into four functional groups on the basis of their physiology: acidophilic aerobic iron-oxidizers, neutrophilic aerobic iron-oxidizers, anaerobic chemotrophic iron-oxidizers, and anaerobic phototrophic iron-oxidizers.

Acidophilic Aerobic Iron-Oxidizing Bacteria

The growth of iron-oxidizing bacteria is favored in iron-rich acidic environments where soluble ferrous iron is present. Aerobic iron-oxidizers are often abundant in acid mine drainage generated from abandoned coal or iron mines or from mine tailings (▶ Sections 22.1 and 22.2). Acidophilic aerobic iron-oxidizers also inhabit iron-rich acidic springs in volcanic areas. In these environments, sulfur is often present along with ferrous iron, and many acidophilic aerobic iron-oxidizers are able to oxidize both elemental sulfur and ferrous iron. Species can be either autotrophic or heterotrophic, and commonly observed genera include *Acidithiobacillus* (*Gammaproteobacteria*), *Leptospirillum* (*Nitrospirae*), and *Ferroplasma* (*Euryarchaeota*). Other acidophilic aerobic iron-oxidizers can be found in the *Actinobacteria* and *Firmicutes*.

Neutrophilic Aerobic Iron-Oxidizing Bacteria

Neutrophilic aerobic iron-oxidizers are organisms adapted to a specialized niche (◀ Section 14.8 and see Microbiology Now on page 514). This is because ferrous iron is relatively insoluble at neutral pH and its chemical oxidation is spontaneous and rapid in the presence of air. Furthermore, at neutral pH, iron oxidation at the cell surface causes the formation of an iron oxide crust that can effectively entomb growing cells. Neutrophilic aerobic iron-oxidizers therefore thrive where iron-rich anoxic waters are exposed to air. Such habitats are common near wetlands or soils where anoxic groundwater forms a spring, but iron oxidizers also inhabit the rhizosphere of wetland plants and certain submarine hydrothermal systems.

Few genera of neutrophilic aerobic iron-oxidizers have been described and they all belong to the *Proteobacteria*. Those species found in freshwater habitats belong to a set of closely related genera in the *Betaprotobacteria*, while species found in marine habitats belong to the *Zetaproteobacteria*. The metabolism of these organisms is fairly narrow. Species are typically microaerophiles and obligate chemolithotrophs, though in certain cases mixotrophy has been observed. The genera *Leptothrix* and *Sphaerotilus* are exceptions (Section 15.19). *Leptothrix* and *Sphaerotilus* are common in freshwater environments containing neutrophilic aerobic iron-oxidizers. They catalyze the oxidation of both iron and manganese but do not appear to conserve energy from these reactions, conserving energy instead from the oxidation of organic matter.

Characteristic species of neutrophilic aerobic iron-oxidizers are found in the genus *Gallionella* (freshwater) and the marine genus *Mariprofundus* (marine)(▶ Section 21.6 and Figure 21.19). Species of *Gallionella* and *Mariprofundus* each form a twisted stalklike structure containing $Fe(OH)_3$ from the oxidation of ferrous iron (**Figure 15.36**). The iron-encrusted stalk contains an organic matrix on which $Fe(OH)_3$ accumulates as it is excreted from the cell surface. Stalk formation is presumably an adaptation that prevents cells from becoming entombed in an iron oxide crust.

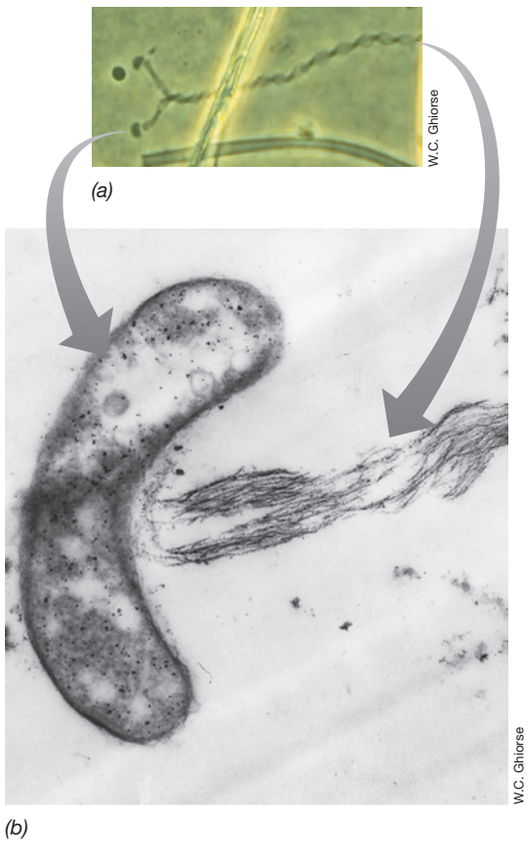


Figure 15.36 The neutrophilic ferrous iron-oxidizer *Gallionella ferruginea*, from an iron seep near Ithaca, New York. *(a)* Photomicrograph of two bean-shaped cells with stalks that combine to form one twisted mass. *(b)* Transmission electron micrograph of a thin section of a *Gallionella* cell with stalk. Cells are about 0.6 μm wide.

Gallionella is common in the waters draining bogs, iron springs, and other habitats where ferrous iron is present. *Mariprofundus* was first isolated from Lō'ihi Seamount, a submarine volcano near Hawaii (▶ Section 21.5 and Figure 21.18). *Gallionella* and *Mariprofundus* are both autotrophic chemolithotrophs containing enzymes of the Calvin cycle (◀ Sections 3.12 and 14.2).

Anaerobic Iron-Oxidizing Bacteria

Anaerobic ferrous iron oxidation can be mediated by both chemotrophic and phototrophic bacteria. These groups are common in anoxic sediments and wetlands. Anoxic conditions promote the solubility of ferrous iron across a wide range of pH and so, unlike the aerobic iron-oxidizing bacteria, growth of anaerobic iron-oxidizers is not strictly limited to neutral pH. These groups contain organisms that are metabolically diverse and able to grow by using a variety of different electron donors and acceptors.

Phototrophic iron oxidation occurs in select species of purple nonsulfur bacteria of the *Alphaproteobacteria* (e.g., *Rhodopseudomonas palustris*), select species of purple sulfur bacteria of the *Gammaproteobacteria* (◀ Figure 14.23), and select species of green sulfur bacteria found in the *Chlorobi* (*Chlorobium ferrooxidans*). In all cases ferrous iron is one of several compounds that these organisms can use as an electron donor in photosynthesis.

Anaerobic chemotrophic iron-oxidizers couple the oxidation of ferrous iron to nitrate reduction, producing either NO_2^- or nitrogen gases (denitrification). These organisms are *Alpha-*, *Beta-*, *Gamma-*, or *Deltaproteobacteria*, and most are also able to use various organic electron donors in nitrate reduction; many can also grow aerobically. The bacterial genera *Acidovorax*, *Aquabacterium*, and *Marinobacter* all contain anaerobic iron-oxidizers. While most species are mixotrophs when growing with ferrous iron as electron donor, species such as *Marinobacter aquaeolei* and *Thiobacillus denitrificans* are able to grow autotrophically as iron-oxidizing chemolithotrophs.

Check Your Understanding

- What habitat characteristics govern the diversity and distribution of iron oxidizers?
- How do aerobic neutrophilic iron-oxidizers keep their cells from becoming entombed in a crust of iron?

15.15 Methanotrophs and Methylotrophs

Methylotrophs are organisms that grow using organic compounds lacking C—C bonds as electron donors in energy metabolism and as carbon sources. Methylotrophy occurs in the bacterial phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and in the archaeal phylum *Euryarchaeota* (Figure 15.1). **Methanotrophs** are a subset of methylotrophs defined by their ability to use methane as a substrate for growth (◀ Section 14.16).

Aerobic methylotrophs are common in soil and aquatic environments where O_2 is present. Anaerobic methylotrophs are common in anoxic environments, particularly in marine sediments. Many anaerobic methylotrophs are methanogenic *Archaea*. In addition, a consortium of methanogenic *Archaea* and sulfate-reducing bacteria combine to oxidize methane from gas hydrates found in deep-sea sediments (◀ Section 14.16 and Figure 14.41). We consider here only the aerobic methylotrophs.

Aerobic Facultative Methylotrophs

KEY GENERA: ***Hyphomicrobium, Methylobacterium***

Aerobic facultative methylotrophs are unable to use methane but can use many other methylated compounds. They are species of *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes*. Facultative methylotrophs are metabolically diverse and, in addition to methylated substrates, most species can grow aerobically using other organic compounds, such as organic acids, ethanol, and sugars. When growing as methylotrophs, most species can grow aerobically with methanol and some can also metabolize methylated amines, methylated sulfur compounds, and halomethanes. Most are obligate aerobes, though some species are capable of denitrification.

The genus *Hyphomicrobium* provides an example of the metabolic versatility of the aerobic facultative methylotrophs. Certain species of *Hyphomicrobium* can grow as aerobic methylotrophs using methanol, methylamine, or dimethyl sulfide. Species of *Hyphomicrobium* can also grow as anaerobic methylotrophs using methanol as an electron donor coupled to denitrification. Finally, *Hyphomicrobium* can grow aerobically on a range of C_2 and C_4 compounds.

Aerobic Methanotrophs

KEY GENERA: ***Methylomonas, Methylosinus***

Aerobic methanotrophs are methylotrophs that can use methane as an electron donor and typically can use it as a carbon source as well. Table 15.1 gives a taxonomic overview of the methanotrophs. Most methanotrophs are *Proteobacteria* and are classified into two major groups based on their internal cell structure, phylogeny, and carbon assimilation pathway. *Type I methanotrophs* assimilate one-carbon compounds via the ribulose monophosphate cycle and are *Gammaproteobacteria*. By contrast, *type II methanotrophs* assimilate C_1 intermediates via the serine pathway and are *Alphaproteobacteria* (Table 15.1). We discussed the biochemical details of these pathways in Section 14.16.

TABLE 15.1 Some characteristics of methanotrophic *Bacteria*

Organism	Internal membranes[a]	Carbon assimilation pathway[b]
Gammaproteobacteria		
Methylomonas	I	Ribulose monophosphate
Methylomicrobium	I	Ribulose monophosphate
Methylobacter	I	Ribulose monophosphate
Methylococcus	I	Ribulose monophosphate and Calvin cycle
Alphaproteobacteria		
Methylosinus	II	Serine
Methylocystis	II	Serine
Methylocella[c]	II	Serine
Verrucomicrobiaceae		
Methylacidiphilum[c]	Membrane vesicles	Calvin cycle

[a]Internal membranes: type I, bundles of disk-shaped vesicles distributed throughout the organism; type II, paired membranes running along the periphery of the cell. See Figure 15.37.
[b]See Figure 14.40.
[c]Acidophiles. For the properties of *Verrucomicrobiaceae*, see Section 16.17.

Most methanotrophs are metabolically specialized for aerobic growth on methane, though some can grow on either methane or methanol. Methanotrophs are typically obligate methylotrophs; however, the methanotrophic genus *Methylocella* contains species that can also grow on acetate or organic acids such as pyruvate and succinate.

In addition to the proteobacterial methanotrophs described above, the phylum *Verrucomicrobia* contains the bacterium *Methylacidiphilum*. Genome analysis has shown that species of *Methylacidiphilum* lack key enzymes of both the ribulose monophosphate and serine pathways. Instead, *Methylacidiphilum* uses the Calvin cycle to assimilate carbon from CO_2.

Physiology

Methanotrophs possess a key enzyme, *methane monooxygenase*, that catalyzes the incorporation of an atom of oxygen from O_2 into CH_4, forming methanol (CH_3OH, ◀ Section 14.16). The requirement for O_2 as a reactant in the initial oxygenation of CH_4 explains why these methanotrophs are obligate aerobes. Methane monooxygenase is located in extensive internal membrane systems that are the site of methane oxidation. Membranes in type I methanotrophs are arranged as bundles of disk-shaped vesicles distributed throughout the cell (**Figure 15.37*b***). Type II species possess paired membranes running along the periphery of the cell (Figure 15.37*a*). Verrucomicrobial methanotrophs possess membrane vesicles. Methylotrophs unable to use methane lack these internal membrane arrays.

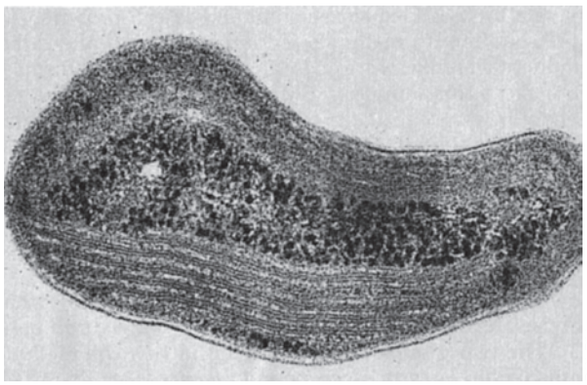
Methanotrophs are virtually unique among bacteria in possessing relatively large amounts of sterols. Sterols are rigid planar molecules found in the cytoplasmic and other membranes of eukaryotes but are absent from most bacteria. Sterols may be an essential part of the complex internal membrane system for methane oxidation (see Figure 15.37). The only other group of bacteria in which sterols are widely distributed is the mycoplasmas, bacteria that lack cell walls and thus probably require a tougher cytoplasmic membrane (▶ Section 16.9). Many methylotrophs contain various carotenoid pigments and high levels of cytochromes in their membranes, and these features often render colonies of aerobic methylotrophs pink.

Ecology

Aerobic methylotrophs are found in the open ocean, in soils, in association with plant roots and leaf surfaces, and at the oxic interface of many anoxic environments. Methanol is produced during the breakdown of plant pectin and this is likely an important substrate for methylotrophs in terrestrial ecosystems. In addition, soils contain aerobic methanotrophs that consume atmospheric methane and are an important biological sink for atmospheric methane. Aerobic methanotrophs are also common at the oxic interface of anoxic environments found in lakes, sediments, and wetlands where methanogens provide a constant source of methane. These methanotrophs play an important role in the global carbon cycle by oxidizing CH_4 and converting it into cell material and CO_2 before it reaches the atmosphere (CH_4 is a strong greenhouse gas).

Methanotrophs also form a variety of symbioses with eukaryotic organisms. For example, some marine mussels live in the vicinity of hydrocarbon seeps on the seafloor, places where CH_4 is released in substantial amounts. Methanotrophic symbionts reside within the animal's gill tissue (**Figure 15.38**), which ensures effective gas exchange with seawater. Assimilated CH_4 is distributed throughout the animal by the excretion of organic compounds by the methanotrophs. These methanotrophic symbioses are therefore conceptually similar to those that develop between sulfide-oxidizing chemolithotrophs and hydrothermal vent tube worms and giant clams (▶ Section 23.11).

Methylomirabilis oxyfera is a methanotroph isolated from anoxic waters in the Black Sea, and it was the first isolate obtained from the unique bacterial phylum NC-10. *M. oxyfera* is an obligate anaerobe; however, it uses the O_2-dependent enzyme of aerobic methanotrophs (methane monooxygenase) to oxidize methane to CO_2. *M. oxyfera* accomplishes this by reducing nitrite to nitric oxide (NO),



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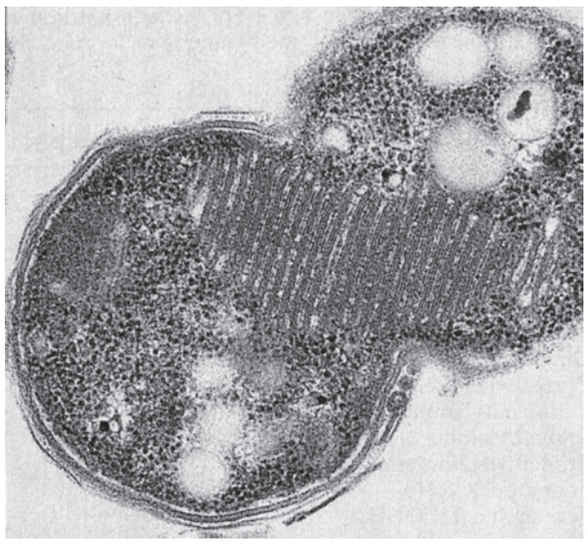
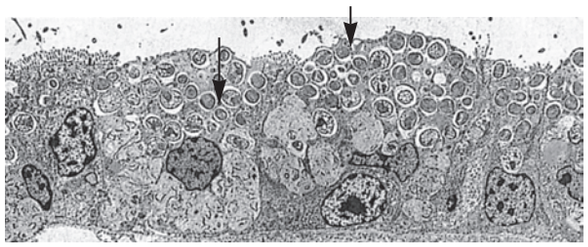


(b)

Figure 15.37 Methanotrophs. *(a)* Electron micrograph of a cell of *Methylosinus*, illustrating a type II membrane system. Cells are about 0.6 μm in diameter. *(b)* Electron micrograph of a cell of *Methylococcus capsulatus*, illustrating a type I membrane system. Cells are about 1 μm in diameter. Compare with Figure 15.25.



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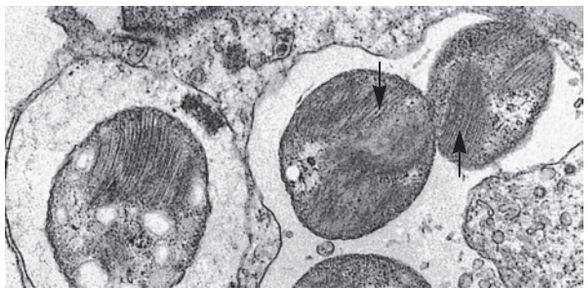


(b)

Figure 15.38 Methanotrophic symbionts of marine mussels. *(a)* Electron micrograph of a thin section at low magnification of gill tissue from a marine mussel living near hydrocarbon seeps in the Gulf of Mexico. Note the symbiotic methanotrophs (arrows) in the tissues. *(b)* High-magnification view of gill tissue showing methanotrophs with type I membrane bundles (arrows). Cells of the methanotrophs are about 1 μm in diameter. Compare with Figure 15.37*b*.

which is then dismutated to N_2 and O_2 ($2\,NO \rightarrow N_2 + O_2$). The O_2 produced by this pathway is then consumed by methane monooxygenase during the oxidation of CH_4 (◀ Section 14.16). Like the methanotroph *Methylacidiphilum*, *M. oxyfera* assimilates C_1 units as CO_2, probably by the Calvin cycle.

Mastering Microbiology
Art Activity: Figure 15.40 Developmental cycle of the bacterial predator *Bdellovibrio bacteriovorus*

Check Your Understanding

- What is the difference between a methanotroph and a methylotroph?
- What is unique about the methanotroph *Methylomirabilis*?

IV • Morphologically and Ecologically Distinctive *Bacteria*

Some *Bacteria* are so morphologically distinct that they can be instantly recognized, even in natural samples. Other *Bacteria* display some unique behavioral property—such as predation or motility controlled by magnetic particles—that sets them apart from all other microbes in an ecological sense.

Many microbes have distinctive lifestyles or morphological characteristics that differentiate them from other organisms. These traits are often associated with specific ecological properties. For example, some chemoorganotrophs obtain their carbon and energy by consuming other organisms. We begin this section by discussing microbial predators.

15.16 Microbial Predators

KEY GENERA: *Bdellovibrio, Myxococcus*

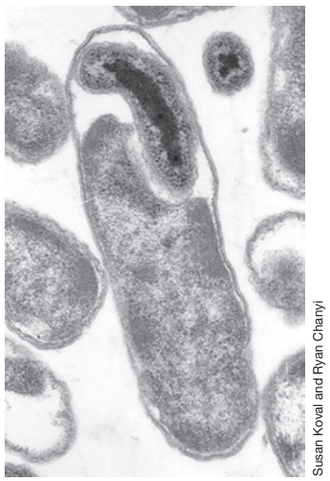
Some bacteria are predators that consume other bacteria. Known bacterial predators reside among several classes of *Proteobacteria* and in the *Bacteroidetes*, *Chloroflexi*, and *Melainabacteria*. Several different methods of predation have been observed. Some predators, such as *Vampirococcus* (phylogeny unknown), *Micavibrio* (*Alphaproteobacteria*), and the green algal predator *Vampirovibrio* (*Melainabacteria*) are *epibiotic predators*; they attach to the surface of their prey and acquire nutrients from its cytoplasm or periplasm. Other predators, such as *Daptobacter* (*Epsilonproteobacteria*), are *cytoplasmic predators*, as they invade their host cells and replicate in the cytoplasm, consuming their prey from the inside out. *Bdellovibrio* have a similar lifestyle as *periplasmic predators*; they invade and replicate within the periplasmic space of their prey cells. Finally, predators such as *Lysobacter* (*Gammaproteobacteria*) and *Myxococcus* (*Deltaproteobacteria*) are *social predators*. These gliding bacteria use swarming behavior to find prey, which they lyse and feed upon collectively. *Bdellovibrio* and *Myxococcus* are the most thoroughly described genera of bacterial predators.

Bdellovibrio

Bdellovibrio are small, highly motile and curved bacteria that prey on other bacteria, using the cytoplasmic constituents of their hosts as nutrients (*bdello* is a prefix meaning "leech"). After attachment of a *Bdellovibrio* cell to its prey, the predator penetrates the cell wall of the prey and replicates in the periplasmic space, eventually forming a spherical structure called a *bdelloplast*. Two stages of penetration are shown in electron micrographs in **Figure 15.39** and diagrammatically in **Figure 15.40*b***. A wide variety of gram-negative prey bacteria can be attacked by *Bdellovibrio*, but gram-positive cells are not attacked.

Bdellovibrio is an obligate aerobe, obtaining its energy from the oxidation of amino acids and acetate. In addition, *Bdellovibrio* assimilates nucleotides, fatty acids, peptides, and even some intact proteins directly

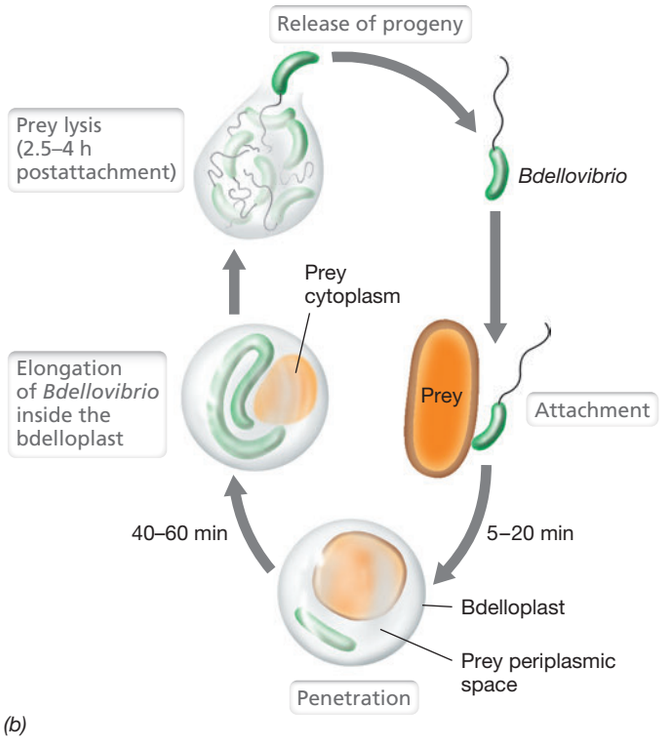
(a)



(b)

Figure 15.39 Attack on a prey cell by *Bdellovibrio*. Thin-section electron micrographs of *Bdellovibrio* attacking a cell of *Delftia acidovorans*. *(a)* Entry of the predator cell. *(b) Bdellovibrio* cell inside the host. The *Bdellovibrio* cell is enclosed in the bdelloplast and replicates in the periplasmic space. A *Bdellovibrio* cell measures about 0.3 μm in diameter.

(a)



(b)

Figure 15.40 **Developmental cycle of the bacterial predator *Bdellovibrio bacteriovorus*.** *(a)* Electron micrograph of a cell of *Bdellovibrio bacteriovorus*; note the thick flagellum. A cell is 0.3 μm wide. *(b)* Events in predation. Following primary contact with a gram-negative bacterium, the highly motile *Bdellovibrio* cell attaches to and penetrates into the prey periplasmic space. Once inside the periplasmic space, *Bdellovibrio* cells elongate, and within 4 h progeny cells are released. The number of progeny cells released varies with the size of the prey; 5–6 bdellovibrios are released from *Escherichia coli* and 20–30 for a larger prey cell, such as *Aquaspirillum*.

from its host without first hydrolyzing them. Prey-independent derivatives of *Bdellovibrio* can be isolated and grown on complex media, however, showing that predation is not an obligatory lifestyle.

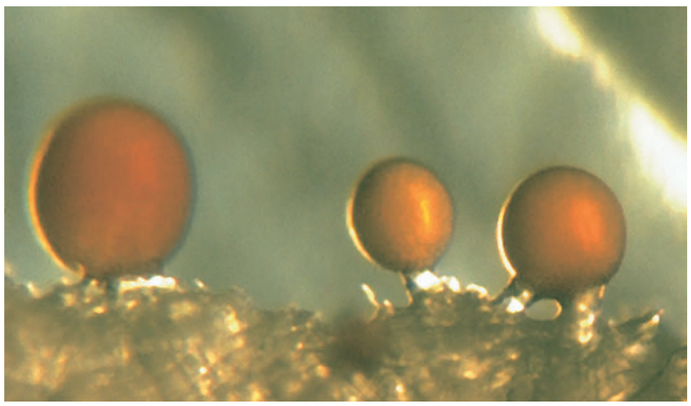
Phylogenetically, bdellovibrios are *Deltaproteobacteria*, and they are widespread in aquatic habitats. Procedures for their isolation are similar to those used to isolate bacterial viruses (◀ Section 5.3). Prey bacteria are spread on the surface of an agar plate, forming a lawn, and the surface is inoculated with a small amount of soil suspension that has been filtered through a membrane filter; the filter retains most bacteria but allows the small *Bdellovibrio* cells to pass. On incubation of the agar plate, plaques analogous to bacteriophage plaques (◀ Figure 5.10*b*) are formed at locations where *Bdellovibrio* cells are multiplying. Pure cultures of *Bdellovibrio* can then be isolated from these plaques. *Bdellovibrio* are widely distributed, as cultures have been obtained from many soils and from sewage.

Myxobacteria

Myxobacteria exhibit the most complex behavioral patterns of all known bacteria. The life cycle of myxobacteria results in the formation of multicellular structures called *fruiting bodies*. The fruiting bodies are often strikingly colored and morphologically elaborate (**Figure 15.41**), and these can often be seen with a hand lens on moist pieces of decaying wood or plant material. The fruiting myxobacteria are classified on morphological grounds using characteristics of the vegetative cells, the myxospores, and fruiting body structure.

The life cycle of a typical myxobacterium is shown in **Figure 15.42**. The vegetative cells of the myxobacteria are simple, nonflagellated, gram-negative rods (Figure 15.42) that glide across surfaces and obtain their nutrients primarily by using extracellular enzymes to lyse other bacteria and use the released nutrients. A vegetative cell

Mastering Microbiology
Art Activity: Figure 15.42 Life cycle of *Myxococcus xanthus*



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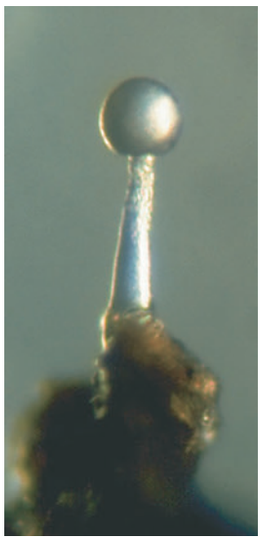

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(c)

Figure 15.41 **Fruiting bodies of three species of fruiting myxobacteria.** *(a) Myxococcus fulvus* (125 μm high). *(b) Myxococcus stipitatus* (170 μm high). *(c) Chondromyces crocatus* (560 μm high).

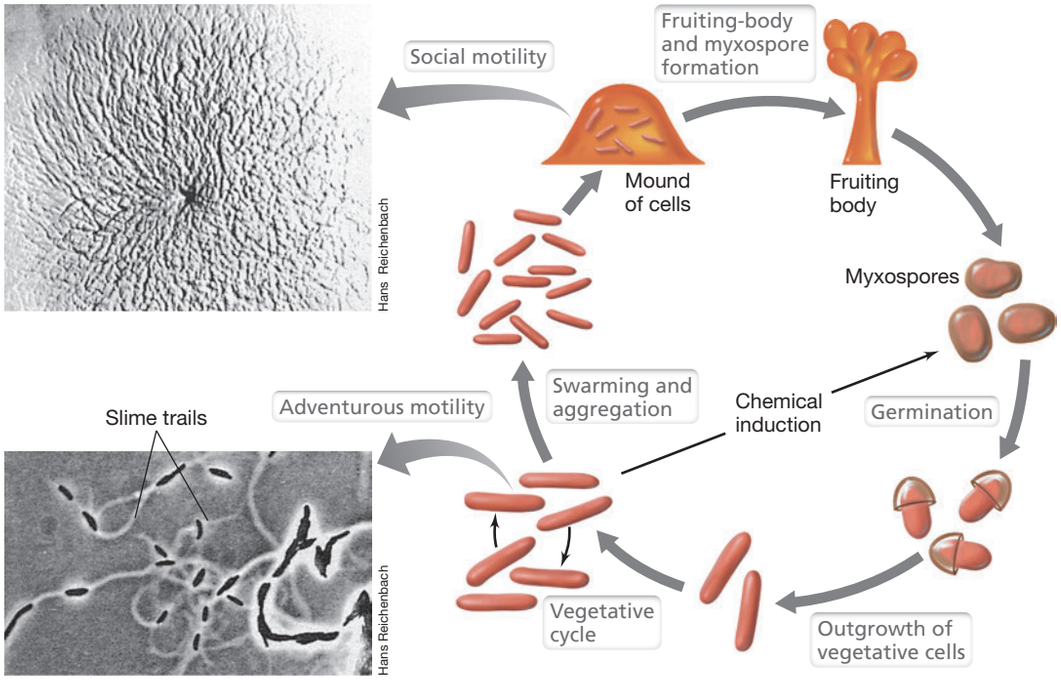


Figure 15.42 Life cycle of *Myxococcus*. Dormant myxospores germinate under favorable conditions to yield vegetative cells. Vegetative cells exhibit adventurous motility characterized by gliding (lower inset, *Myxococcus fulvus* cells, 0.8 μm diameter). When nutrients are limiting, cells aggregate into swarming colonies (upper inset, *Myxococcus xanthus* colony on agar, 10 mm diameter) exhibiting social motility mediated by type IV pili and twitching. Cells eventually aggregate to form fruiting bodies, within which some vegetative cells develop into myxospores.

excretes slime, and as it moves across a solid surface, it leaves behind a slime trail (Figure 15.42). The vegetative cells form a swarm that exhibits self-organizing behavior, and this allows them to behave as a single coordinated entity in response to environmental cues.

Upon nutrient exhaustion, vegetative cells of myxobacteria begin to migrate toward each other, aggregating together in mounds or heaps (**Figure 15.43**). Aggregation is likely mediated by chemotactic or quorum-sensing responses (◀ Sections 7.6 and 7.7). As the cell masses become higher, they begin to differentiate into fruiting bodies (**Figure 15.44**) containing *myxospores*. Myxospores are specialized cells that are resistant to drying, ultraviolet radiation, and heat, but the degree of heat resistance is much less than that of the bacterial endospore (◀ Section 2.8). Fruiting bodies can be simple, consisting of masses of myxospores embedded in slime, or complex, consisting of a stalk and heads (Figure 15.44). The fruiting body stalk is composed of slime within which a few cells are trapped. The majority of the cells migrate to the fruiting body head, where they undergo differentiation into myxospores (Figure 15.42).

Check Your Understanding

- What environmental conditions trigger fruiting body formation in myxobacteria?
- What are the different ways in which species of *Myxococcus* and *Bdellovibrio* kill their prey?

15.17 Spirochetes

KEY GENERA: *Spirochaeta, Treponema, Cristispira, Leptospira, Borrelia*

Spirochetes are morphologically unique bacteria found only within the bacterial phylum *Spirochaetes*. Spirochetes are gram-negative, motile, tightly coiled *Bacteria*, typically slender and flexuous in shape (**Figure 15.45**). Spirochetes are widespread in aquatic sediments and in animals. Some cause diseases, including syphilis, an important human sexually transmitted disease. Spirochetes are classified into eight genera (**Table 15.2**) primarily on the basis of habitat, pathogenicity, phylogeny, and morphological and physiological characteristics.

Spirochetes have an unusual mode of motility conveyed by their unusual morphology. Spirochetes contain *endoflagella*, which resemble normal flagella but are found in the cell periplasm (**Figure 15.46**). The endoflagella are anchored at the cell poles and extend back along the length of the cell. Both the endoflagella and the protoplasmic cylinder are surrounded by a flexible membrane called the *outer sheath* (Figure 15.46*b*). Endoflagella rotate, as do typical bacterial

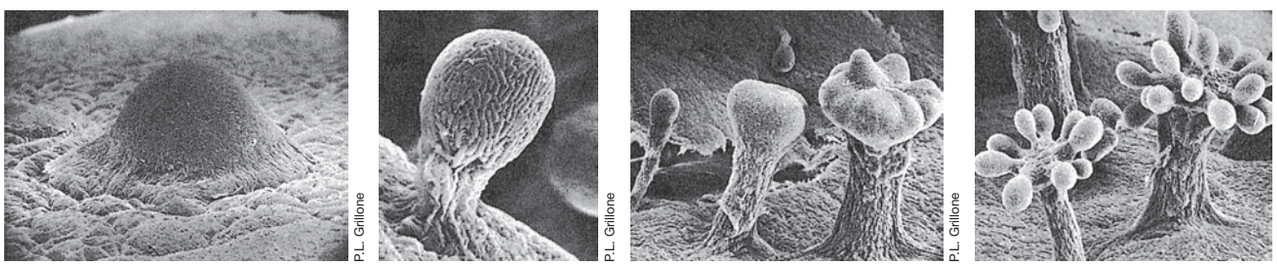


Figure 15.43 Scanning electron micrographs of fruiting body formation in *Chondromyces crocatus*. *(a)* Early stage, showing aggregation and mound formation. *(b)* Initial stage of stalk formation. Slime formation in the head has not yet begun and so the cells that compose the head are still visible. *(c)* Three stages in head formation. Note that the diameter of the stalk also increases. *(d)* Mature fruiting bodies. The entire fruiting structure is about 600 μm in height (compare with Figure 15.41*c*).

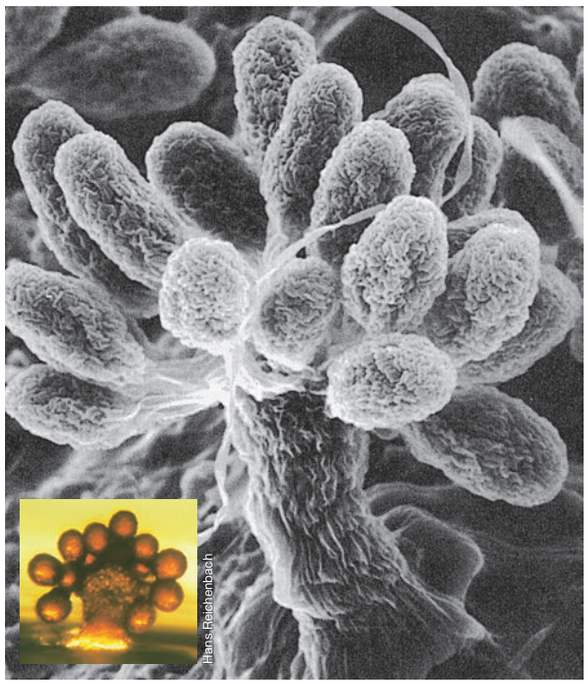


Figure 15.44 **The myxobacterium *Stigmatella aurantiaca*.** Scanning electron micrograph of a fruiting body growing on a piece of wood. Note the individual cells visible in each fruiting body. Inset: Phase-contrast photomicrograph of a single fruiting body about 150 μm high. The color is due to the production of glucosylated carotenoid pigments.

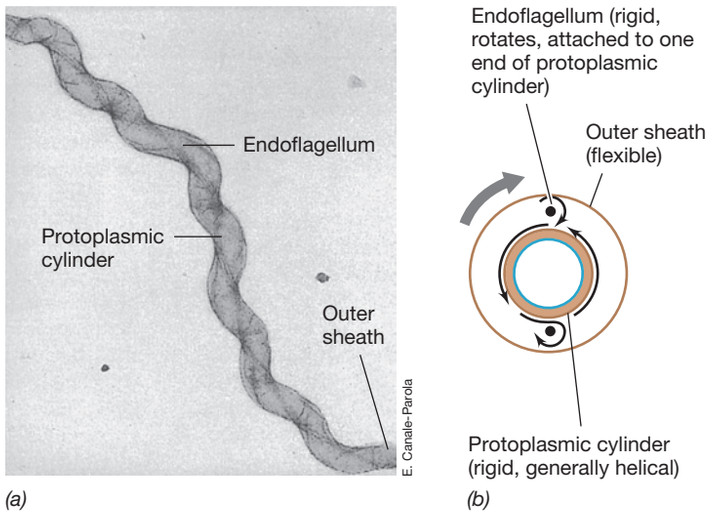


Figure 15.46 **Motility in spirochetes.** *(a)* Electron micrograph of a negatively stained cell of *Spirochaeta zuelzerae*, showing the position of the endoflagellum; the cell is about 0.3 μm in diameter. *(b)* Diagram of a spirochete cell, showing the arrangement of the protoplasmic cylinder, endoflagella, and external sheath, and how rotation of the endoflagellum generates rotation of both the protoplasmic cylinder and the external sheath.

flagella. However, when both endoflagella rotate in the same direction, the protoplasmic cylinder rotates in the opposite direction, placing torsion on the cell (Figure 15.46*b*). This torsion causes the spirochete cell to flex, resulting in a corkscrew-like motion that allows cells to burrow through viscous materials or tissues.

Spirochetes are often confused with spirilla. **Spirilla** are helically curved rod-shaped cells, usually motile by means of polar flagella (**Figure 15.47**). The word *spirillum* refers to a general cell shape that is widespread among *Bacteria* and *Archaea*. The number of helical turns in a single spirillum may vary from less than one complete turn (in which case the organism looks like a vibrio) to many turns. In addition, spirilla that divide terminally, such as the cyanobacterium *Spirulina* (Figure 15.5), can form long helical filaments that superficially resemble spirochetes. Spirilla, however, lack the outer sheath, endoflagella, and corkscrew-like motility of spirochetes. In addition, spirilla are typically fairly rigid cells while spirochetes are highly flexible and quite thin (< 0.5 μm).

Spirochaeta and *Cristispira*

The genus *Spirochaeta* includes free-living, anaerobic, and facultatively aerobic spirochetes. These organisms, of which several species are known, are common in aquatic environments such as freshwater and sediments, and also in the oceans. *Spirochaeta plicatilis* (Figure 15.45*b*) is a large spirochete found in sulfidic freshwater and marine habitats. The 20 or so endoflagella inserted at each pole of *S. plicatilis* are arranged in a bundle that winds around the coiled protoplasmic cylinder. Another species, *Spirochaeta stenostrepta* (Figure 15.45*a*), is an obligate anaerobe commonly found in H_2S-rich black muds. It ferments sugars to ethanol, acetate, lactate, CO_2, and H_2.

Cristispira (**Figure 15.48**) is a unique spirochete found in nature only in the crystalline style of certain molluscs, such as clams and oysters. The crystalline style is a flexible, semisolid rod seated in a sac and rotated against a hard surface of the digestive tract, thereby mixing and grinding the small particles of food taken in by the animal. *Cristispira* lives in both freshwater and marine molluscs, but not all species of molluscs possess this organism. Unfortunately, *Cristispira* has not been cultured, and so the physiological rationale for its restriction to this unique habitat is unknown.

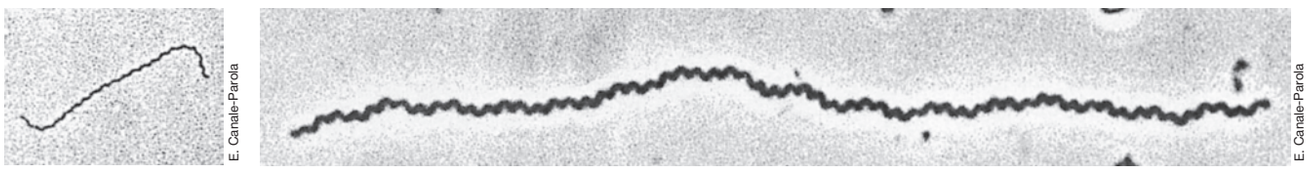


Figure 15.45 **Morphology of spirochetes.** Two spirochetes at the same magnification, showing the wide size range in the group. *(a) Spirochaeta stenostrepta,* by phase-contrast microscopy. A single cell is 0.25 μm in diameter. *(b) Spirochaeta plicatilis.* A single cell is 0.75 μm in diameter and can be up to 250 μm (0.25 mm) in length.

TABLE 15.2 Genera of spirochetes and their characteristics

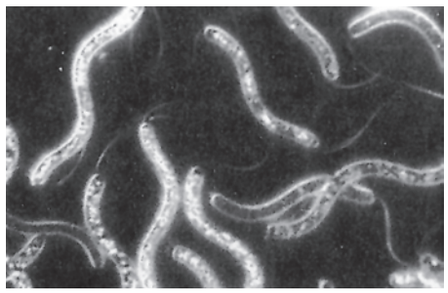
Genus	Dimensions (μm)	General characteristics	Number of endoflagella	Habitat	Diseases
Cristispira	30–150 × 0.5–3.0	3–10 complete coils; bundle of endoflagella visible by phase-contrast microscopy	~100	Digestive tract of molluscs; has not been cultured	None known
Spirochaeta	5–250 × 0.2–0.75	Anaerobic or facultatively aerobic; tightly or loosely coiled	2–40	Aquatic, free-living, freshwater and marine	None known
Treponema	5–15 × 0.1–0.4	Microaerophilic or anaerobic; helical or flattened coil amplitude up to 0.5 μm	2–32	Commensal or parasitic in humans, other animals	Syphilis, yaws, swine dysentery, pinta
Borrelia	8–30 × 0.2–0.5	Microaerophilic; 5–7 coils of approximately 1 μm amplitude	7–20	Humans and other mammals, arthropods	Relapsing fever, Lyme disease, ovine and bovine borreliosis
Leptospira	6–20 × 0.1	Aerobic, tightly coiled, with bent or hooked ends; requires long-chain fatty acids	2	Free-living or parasitic in humans, other mammals	Leptospirosis
Leptonema	6–20 × 0.1	Aerobic; does not require long-chain fatty acids	2	Free-living	None known
Brachyspira	7–10 × 0.35–0.45	Anaerobe	8–28	Intestine of warm-blooded animals	Causes diarrhea in chickens and swine
Brevinema	4–5 × 0.2–0.3	Microaerophile; forms deep branch in spirochete lineage as assessed by 16S rRNA sequence analysis	2	Blood and tissue of mice and shrews	Infectious for laboratory mice

Treponema and *Borrelia*

Anaerobic or microaerophilic host-associated spirochetes that are commensals or pathogens of humans and animals reside in the genus *Treponema*. *T. pallidum*, the causal agent of syphilis (▶ Section 31.13), is the best-known species of *Treponema*. It differs in morphology from other spirochetes in that the *Treponema* cell is not helical but flat and wavy. The *T. pallidum* cell is remarkably thin, measuring only 0.2 μm in diameter. Because of this, dark-field microscopy has long been used to examine exudates from suspected syphilitic lesions (▶ Figure 31.13).

Other species of *Treponema* are also often found as commensals in humans and other animals. For example, *Treponema denticola* is common in the human oral cavity and is associated with gum disease. It ferments amino acids such as cysteine and serine, forming acetate as the major fermentation acid, as well as CO_2, NH_3, and H_2S. Spirochetes are also common in the rumen, the digestive forestomach of ruminant animals (▶ Section 23.15). For instance, *Treponema saccharophilum* (**Figure 15.49*a***) is a large, pectinolytic spirochete found in the bovine rumen where it ferments pectin, starch, inulin, and other plant polysaccharides. *Treponema primitia* can be found in the hindgut of certain termites. In the termite gut, fermentation of cellulose causes production of H_2 and CO_2. *T. primitia* is an acetogen (◀ Section 14.14) that grows on H_2 plus CO_2, forming acetate, which is an important component of the insect's nutrition. *Treponema azotonutricium* is also found in the termite hindgut and is capable of nitrogen fixation (◀ Section 3.12).

The majority of species of *Borrelia* are animal or human pathogens. *Borrelia burgdorferi* (Figure 15.49*b*) is the causative agent of the tick-borne *Lyme disease*, which infects humans and animals (▶ Section 32.4). *B. burgdorferi* is also of interest because it is one of the few bacteria that has a linear (as opposed to a circular) chromosome (◀ Sections 6.2 and 10.3). Other species of *Borrelia* are primarily of veterinary importance, causing diseases in cattle, sheep, horses, and birds. In most cases, the bacterium is transmitted to the animal host from the bite of a tick.



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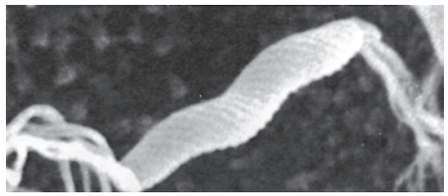

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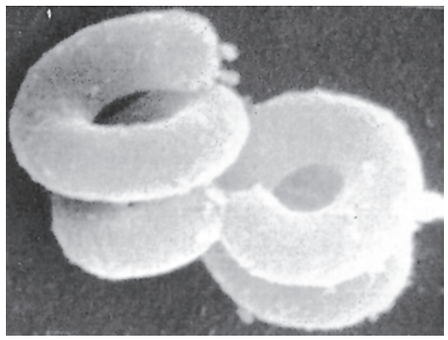


(c)

Figure 15.47 Spirilla. *(a) Spirillum volutans*, visualized by dark-field microscopy, showing flagellar bundles and volutin (polyphosphate) granules. Cells are about 1.5 × 25 μm. *(b)* Scanning electron micrograph of an intestinal spirillum. Note the polar flagellar tufts and the spiral structure of the cell surface. *(c)* Scanning electron micrograph of cells of *Ancylobacter aquaticus*. Cells are about 0.5 μm in diameter.

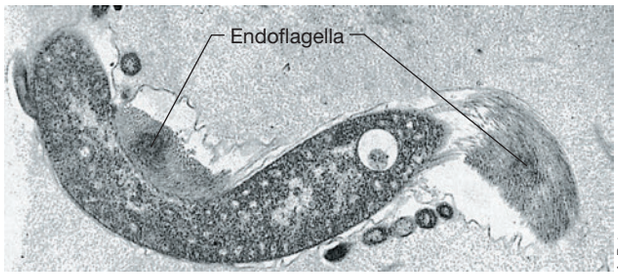
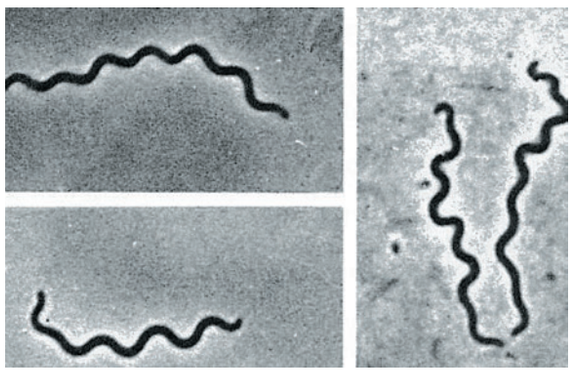


Figure 15.48 ***Cristispira.*** Electron micrograph of a thin section of a cell of *Cristispira*. This large spirochete is about 2 μm in diameter. Notice the numerous endoflagella.

Leptospira and *Leptonema*

The genera *Leptospira* and *Leptonema* contain strictly aerobic spirochetes that oxidize long-chain fatty acids (for example, the C_{18} fatty acid oleic acid) as electron donors and carbon sources. With few exceptions, these are the only substrates utilized for growth. Leptospiras are thin, finely coiled, and usually bent at each end into a semicircular hook. At present, several species are recognized in this group, some free-living and many parasitic. Two major species of *Leptospira* are *L. interrogans* (parasitic) and *L. biflexa* (free-living). Strains of *L. interrogans* are parasitic for humans and animals. Rodents are the natural hosts of most leptospiras, although dogs and pigs are also important carriers of certain strains.

In humans the most common leptospiral syndrome is *leptospirosis*, a disorder in which the organism localizes in the kidneys and can cause renal failure or even death. Leptospiras ordinarily enter the body through the mucous membranes or through breaks in the skin during contact with an infected animal. After a transient multiplication in various parts of the body, the organism localizes in the kidneys and liver, causing nephritis and jaundice. Domestic animals such as dogs are vaccinated against leptospirosis with a killed virulent strain in the combined distemper–leptospira–hepatitis vaccine.



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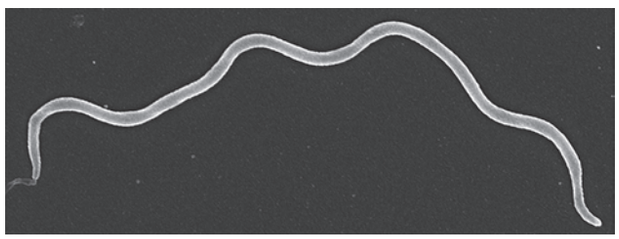

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Figure 15.49 ***Treponema* and *Borrelia*.** *(a)* Phase-contrast micrographs of *Treponema saccharophilum*, a large pectinolytic spirochete from the bovine rumen. A cell measures about 0.4 μm in diameter. Left, regularly coiled cells; right, irregularly coiled cells. *(b)* Scanning electron micrograph of a cell of *Borrelia burgdorferi*, the causative agent of Lyme disease.

Check Your Understanding

- What are the major differences between spirochetes and spirilla?
- Name two diseases of humans caused by spirochetes.

15.18 Budding and Prosthecate/ Stalked *Bacteria*

KEY GENERA: *Hyphomicrobium, Caulobacter*

The growth of most bacteria is coupled to cell division by the well-known process of binary fission (◀ Section 4.6 and Figure 4.8). In this section, we consider organisms that grow and divide in different ways, including budding and the formation of appendages. Budding and appendaged species often have life cycles that are distinct among bacteria.

UNIT 4

Budding Division

As we learned in Section 4.10, budding bacteria divide as a result of unequal cell growth. Cell division in stalked and budding bacteria forms a totally new daughter cell, with the mother cell retaining its original identity (◀ Figure 4.18). In contrast, binary fission produces two equivalent cells.

A fundamental difference between budding bacteria and bacteria that divide by binary fission is the formation of new cell wall material from a single point (polar growth) rather than throughout the whole cell (intercalary growth) as in binary fission (Chapter 8). Several genera not normally considered to be budding bacteria show polar growth without differentiation of cell size (◀ Figure 4.18). An important consequence of polar growth is that internal structures, such as membrane complexes, are not partitioned in the cell division process and must be formed de novo. However, this has an advantage in that more complex internal structures can be formed in budding cells than in cells that divide by binary fission, since the latter cells would have to partition these structures between the two daughter cells. Not coincidentally, many budding bacteria, particularly phototrophic and chemolithotrophic species, contain extensive internal membrane systems.

Budding Bacteria: *Hyphomicrobium*

Mastering Microbiology
Art Activity: Figure 15.53 Stages in the *Hyphomicrobium* cell cycle

Two well-studied budding bacteria are closely related *Alphaproteobacteria*: *Hyphomicrobium* (**Figure 15.50**), which is chemoorganotrophic, and *Rhodomicrobium*, which is phototrophic. These organisms release buds from the ends of long, thin hyphae. The hypha is a direct cellular extension and contains cell wall, cytoplasmic membrane, and ribosomes, and can contain DNA.

Figure 15.50 shows the life cycle of *Hyphomicrobium*. The mother cell, which is often attached by its base to a solid substrate, forms

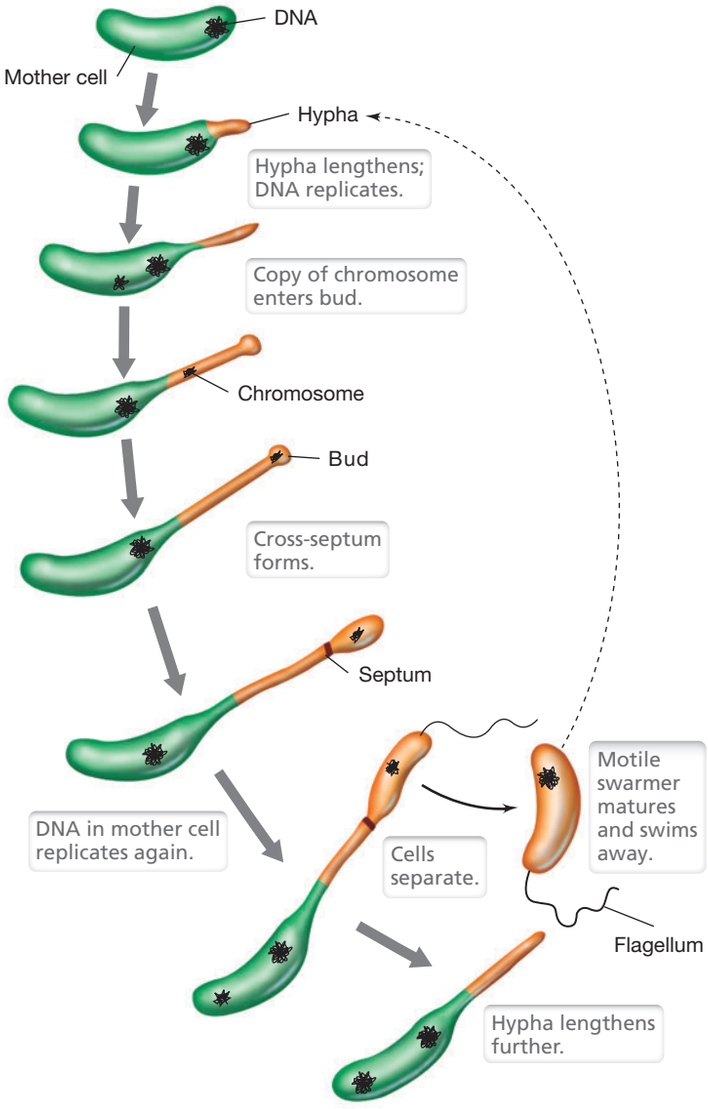
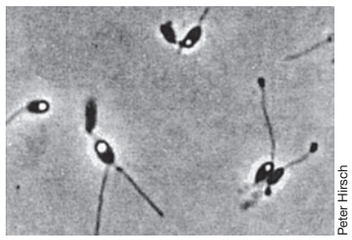


Figure 15.50 Stages in the *Hyphomicrobium* cell cycle. The single chromosome of *Hyphomicrobium* is circular.

a thin outgrowth that lengthens to become a hypha. At the end of the hypha, a bud forms. This bud enlarges, forms a flagellum, breaks loose from the mother cell, and swims away. Later, the daughter cell loses its flagellum and after a period of maturation forms a hypha and buds. More buds can also form at the hyphal tip of the mother cell, leading to arrays of cells connected by hyphae. In some cases, a bud begins to form directly from the mother cell without the intervening formation of a hypha, whereas in other cases a single cell forms hyphae from each end (**Figure 15.51**). Nucleoid replication events occur before the bud emerges, and then once a bud has formed, a copy of the chromosome moves down the hypha and into the bud. A cross-septum then forms, separating the still-developing bud from the hypha and mother cell (Figure 15.51).

Mastering Microbiology
Art Activity: Figure 15.56*a* Prosthecate bacteria

Physiologically, *Hyphomicrobium* is a methylotrophic bacterium (◀ Section 14.16 and Section 15.15), and it is widespread in freshwater, marine, and terrestrial habitats. Preferred carbon sources are



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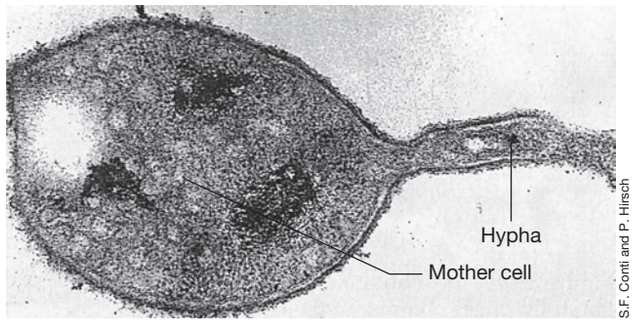


(b)

Figure 15.51 Morphology of *Hyphomicrobium*. *(a)* Phase-contrast micrograph of cells of *Hyphomicrobium*. Cells are about 0.7 μm wide. *(b)* Electron micrograph of a thin section of a single *Hyphomicrobium* cell. The hypha is about 0.2 μm wide.

methanol (CH_3OH), methylamine (CH_3NH_2), formaldehyde (CH_2O), and formate ($HCOO^-$). A fairly specific enrichment procedure for *Hyphomicrobium* is to use CH_3OH as an electron donor with nitrate (NO_3^-) as an electron acceptor in a dilute medium incubated under anoxic conditions. The only rapidly growing denitrifying bacterium known that uses CH_3OH as an electron donor is *Hyphomicrobium*, and so this procedure can select this organism out of a wide variety of environments.

Prosthecate and Stalked *Bacteria*

A variety of bacteria are able to produce cytoplasmic extrusions including *stalks* (**Figure 15.52**), *hyphae*, and *appendages* (**Table 15.3**). Extrusions of these kinds, which are smaller in diameter than the mature cell and contain cytoplasm and a cell wall, are collectively called **prosthecae** (**Figure 15.53**). Prosthecae allow organisms to attach to particulate matter, plant material, or other microorganisms in aquatic habitats. In addition, prosthecae can be used to increase the ratio of surface area to cell volume. Recall that the high surface-to-volume ratio of prokaryotic cells in general confers an increased ability to take up nutrients and expel wastes (◀ Section 1.3). The unusual morphology of appendaged bacteria (Figure 15.53) carries this theme to an extreme and may be an evolutionary adaptation to life in oligotrophic (nutrient-poor) waters where these organisms are most commonly found.

Prosthecae may also function to reduce cell sinking. Because these organisms are aquatic and their metabolism is typically aerobic, prosthecae may keep cells from sinking into anoxic zones in their aquatic environments where they would be unable to respire. Some prosthecate bacteria produce gas vesicles (◀ Section 2.7), which would also help prevent sinking.

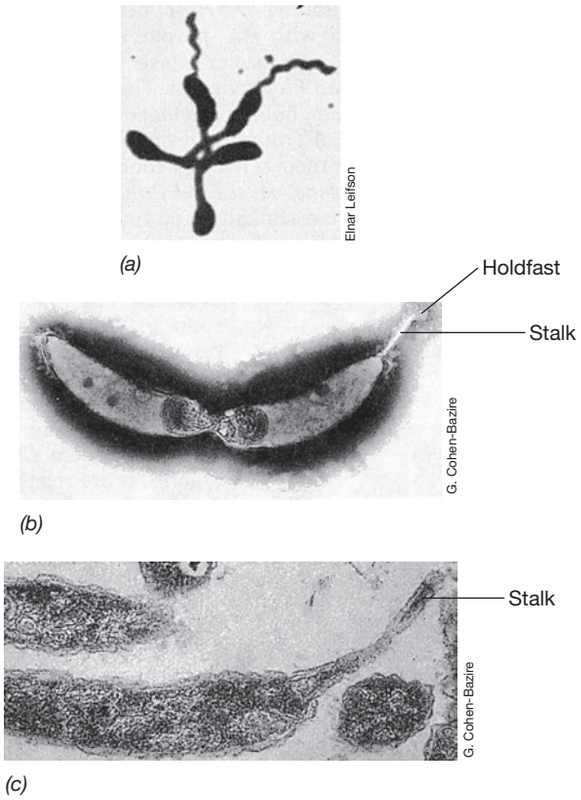


Figure 15.52 **Stalked bacteria.** *(a)* A *Caulobacter* rosette. A single cell is about 0.5 μm wide. The five cells are attached by their stalks, which are also prosthecae. Two of the cells have divided, and the daughter cells have formed flagella. *(b)* Negatively stained preparation of a *Caulobacter* cell in division. *(c)* A thin section of *Caulobacter* showing that cytoplasm is present in the stalk. Parts *b* and *c* are transmission electron micrographs.

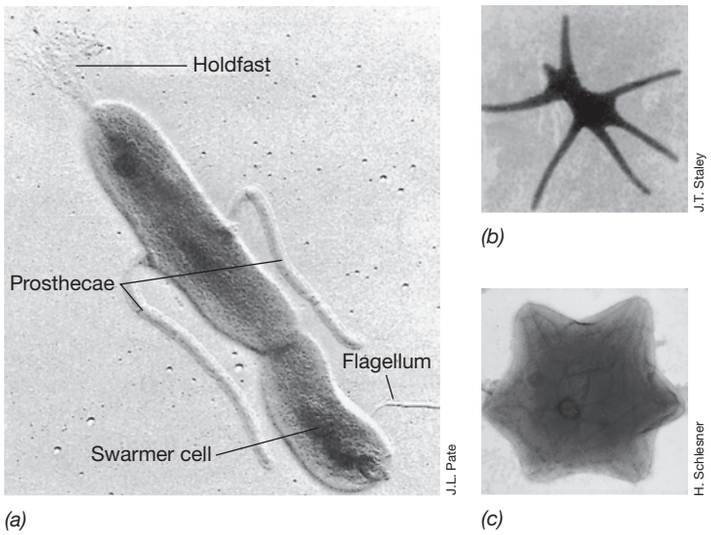


Figure 15.53 **Prosthecate bacteria.** *(a)* Electron micrograph of a shadow-cast preparation of *Asticcacaulis biprosthecum*, illustrating the location and arrangement of the prosthecae, the holdfast, and a swarmer cell. The swarmer cell breaks away from the mother cell and begins a new cell cycle. Cells are about 0.6 μm wide. *(b)* Negatively stained electron micrograph of a cell of *Ancalomicrobium adetum*. The prosthecae are bounded by the cell wall, contain cytoplasm, and are about 0.2 μm in diameter. *(c)* Electron micrograph of the star-shaped prosthecate bacterium *Stella*. Cells are about 0.8 μm in diameter.

Caulobacter

Two common stalked bacteria are *Caulobacter* (Figure 15.52) and *Gallionella* (Figure 15.36). The former is a chemoorganotroph that produces a cytoplasm-filled stalk, that is, a prostheca, while the latter is a chemolithotrophic iron-oxidizing bacterium whose stalk is composed of ferric hydroxide [$Fe(OH)_3$] (Section 15.14). *Caulobacter* cells are often seen on surfaces in aquatic environments with the stalks of several cells attached to form *rosettes* (Figure 15.52*a*). At the end of the stalk is a structure called a *holdfast* by which the stalk anchors the cell to a surface.

The *Caulobacter* cell division cycle (**Figure 15.54**; ◀ Section 8.8 and Figure 8.20) is unique because cells undergo unequal binary fission. A stalked cell of *Caulobacter* divides by elongation of the cell followed by binary fission, and a single flagellum forms at the pole opposite the stalk. The flagellated cell so formed, called a *swarmer*, separates from the nonflagellated mother cell and eventually attaches to a new surface, forming a new stalk at the flagellated pole; the flagellum is then lost. Stalk formation is a necessary precursor of cell division and is coordinated with DNA synthesis (Figure 15.54). The cell division cycle in *Caulobacter* is thus more complex than simple binary fission or budding division because the stalked and swarmer cells are structurally different and the growth cycle must include both forms.

UNIT 4

Mastering Microbiology
Art Activity: Figure 15.57 Growth of *Caulobacter*

TABLE 15.3 Characteristics of major genera of stalked, appendaged (prosthecate), and budding *Bacteria*

Characteristics	Genus	Phylogenetic group
Stalked bacteria		
Stalk an extension of the cytoplasm and involved in cell division	*Caulobacter*	*Alphaproteobacteria*
Stalk is an excretory product not containing cytoplasm (therefore it is not a prostheca)	*Gallionella*	*Betaproteobacteria*
Appendaged (prosthecate) bacteria		
Single or double prosthecae	*Asticcacaulis*	*Alphaproteobacteria*
Multiple prosthecae	*Prosthecomicrobium*	*Alphaproteobacteria*
Budding bacteria		
Phototrophic, produce hyphae	*Rhodomicrobium*	*Alphaproteobacteria*
Phototrophic, budding without hyphae	*Rhodopseudomonas*	*Alphaproteobacteria*
Chemoorganotrophic, buds on tips of slender hyphae	*Hyphomicrobium*	*Alphaproteobacteria*

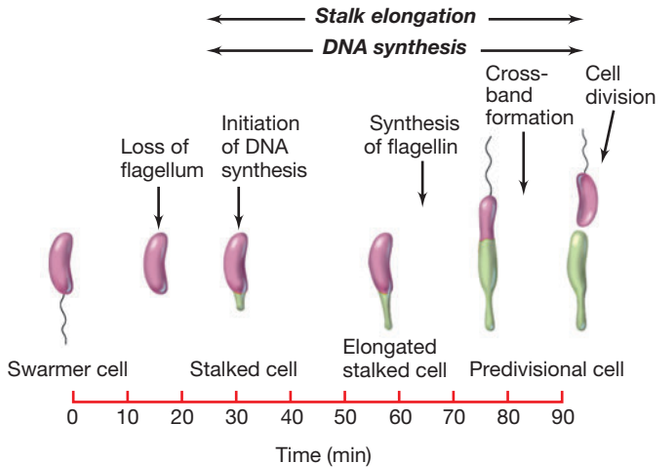


Figure 15.54 **Growth of *Caulobacter*.** Stages in the *Caulobacter* cell cycle, beginning with a swarmer cell. Compare with Figure 8.20.

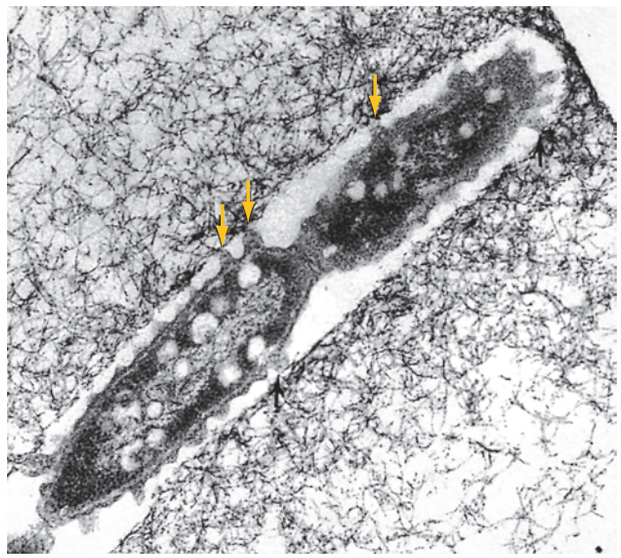


Figure 15.55 ***Leptothrix* and iron precipitation.** Transmission electron micrograph of a thin section of *Leptothrix* growing in a ferromanganese film in a swamp in Ithaca, New York. A single cell measures about 0.9 μm in diameter. Note the protuberances of the cell envelope that contact the sheath (arrows).

Check Your Understanding

- How does budding division differ from binary fission? How does binary fission differ from the division process in *Caulobacter*?
- What advantage might a prosthecate organism have in a very nutrient-poor environment?

UNIT 4

15.19 Sheathed *Bacteria*

KEY GENERA: *Sphaerotilus, Leptothrix*

Bacteria in many phyla form sheaths made of polysaccharide or protein that encase one or many cells. Sheaths often function to bind cells together into long multicellular filaments (Sections 15.3 and 15.12). *Sphaerotilus* and *Leptothrix* are filamentous bacteria that grow within a sheath and have a unique life cycle. Under favorable conditions, the cells grow vegetatively, leading to the formation of long, cell-packed sheaths. Flagellated swarmer cells form within the sheath under unfavorable growth conditions, and the swarmer cells break out and are dispersed to new environments, leaving behind the empty sheath.

Sphaerotilus and *Leptothrix* are common in freshwater habitats that are rich in organic matter, such as wastewaters and polluted streams. Because they are typically found in flowing waters, they are also abundant in trickling filters and activated sludge digesters in sewage treatment plants (▶ Section 22.6). In habitats in which reduced iron (Fe^{2+}) or manganese (Mn^{2+}) is present, the sheaths may become coated with ferric hydroxide [$Fe(OH)_3$] or manganese oxides from the oxidation of these metals.

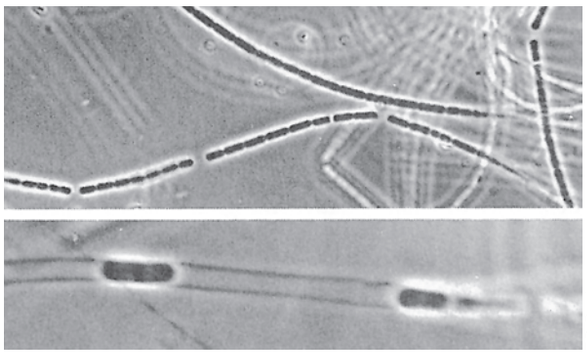
Leptothrix

The ability of *Sphaerotilus* and *Leptothrix* to precipitate iron oxides on their sheaths is well established, and when sheaths become iron encrusted, as occurs in iron-rich waters, they can frequently be seen microscopically (**Figure 15.55**). Iron precipitates form when ferrous iron (Fe^{2+}), chelated to organic materials such as humic or tannic acids, is oxidized. These chemoorganotrophic bacteria use the organic materials as a carbon or energy source and, when no longer chelated, the ferrous iron becomes oxidized and precipitates on the sheath. Iron oxidation is fortuitous and though these organisms are closely related to dissimilative iron-oxidizers (Section 15.14), the organism does not gain energy from iron oxidation. In a similar way, *Leptothrix* can also oxidize manganese.

Sphaerotilus

The *Sphaerotilus* filament is composed of a chain of rod-shaped cells enclosed in a closely fitting sheath. This thin, transparent structure is difficult to see when it is filled with cells, but when it is partially empty, the sheath can more easily be resolved (**Figure 15.56*a***). Individual cells are 1–2 × 3–8 μm in dimensions and stain gram-negatively. The cells within the sheath (Figure 15.56*b*) divide by binary fission, and the new cells synthesize new sheath material at the tips of the filaments. Eventually, motile swarmer cells are liberated from the sheaths (Figure 15.56*c*) and then migrate, attach to a solid surface, and begin to grow, with each swarmer being the forerunner of a new filament. The sheath, which is devoid of peptidoglycan, consists of protein and polysaccharide.

Sphaerotilus species are nutritionally versatile and use simple organic compounds as carbon and energy sources; one species can grow mixotrophically with thiosulfate as electron donor. Befitting its habitat in flowing waters, *Sphaerotilus* is an obligate aerobe. Large masses (blooms) of *Sphaerotilus* often occur in the fall of the year in streams and brooks when leaf litter causes a temporary increase in the organic content of the water. In addition, its filaments are the main component of a microbial complex that wastewater engineers call "sewage fungus," a filamentous slime found on the rocks in streams receiving sewage pollution. In activated sludge of sewage treatment plants (▶ Section 22.6), *Sphaerotilus* is often responsible for a condition called *bulking*, where the tangled masses of *Sphaerotilus* filaments so increase the bulk of the sludge that it remains suspended and does not settle as it should. This has a negative effect on the oxidation of organic matter and the recycling of inorganic nutrients and leads to treatment plant discharges with high nitrogen and carbon loads.



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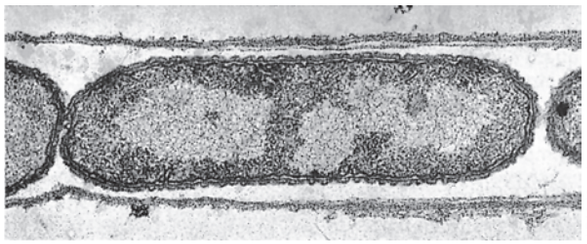

(b)

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Figure 15.56 ***Sphaerotilus natans***. A single cell is about 2 μm wide. *(a)* Phase-contrast photomicrographs of material collected from a polluted stream. Active growth stage (above) and swarmer cells leaving the sheath. *(b)* Electron micrograph of a thin section through a filament, clearly showing the sheath. *(c)* Electron micrograph of a negatively stained swarmer cell. Notice the polar flagellar tuft.

Check Your Understanding

- Describe how a sheathed bacterium such as *Sphaerotilus* grows.
- List two metals that are oxidized by sheathed bacteria.

15.20 Magnetic Microbes

KEY GENERA: *Magnetospirillum*

In a magnetic field, magnetic bacteria demonstrate a dramatic directed movement called *magnetotaxis*. Within these cells are structures called *magnetosomes*, which consist of chains of magnetic particles made of magnetite (Fe_3O_4) or greigite (Fe_3S_4) (◀ Section 2.7 and Figure 2.24). Magnetosomes are localized within invaginations of the cell membrane that are organized in a linear conformation by a protein scaffold. Magnetic bacteria orient along the north–south magnetic moment of a magnetic field, aligning parallel to the field lines in much the same manner as a compass needle. Magnetic bacteria are typically microaerophilic or anaerobic and are most often found near the oxic–anoxic interface in sediments or stratified lakes. The magnetosomes of aerobic species typically contain the mineral magnetite while those of anaerobes contain exclusively greigite.

Although the ecological role of bacterial magnets is unclear, the ability to orient in a magnetic field may be of selective advantage in maintaining these organisms in zones of low O_2 concentration. Generally, the concentration of O_2 decreases with depth through sediments or the water column of stratified lakes. Since Earth is spherical, its magnetic field lines have a strong vertical component in the Northern and Southern Hemispheres. Thus, bacteria that orient along these field lines can preferentially swim down and away from O_2. The magnetosome functions like a compass needle to "point" the bacterium in the right direction; rotation of the flagellum, by contrast, is controlled by a chemotactic response to O_2 (◀ Section 2.11).

Magnetic bacteria display one of two magnetic polarities depending on the orientation of magnetosomes within the cell. Cells in the Northern Hemisphere have the north-seeking pole of their magnetosomes forward with respect to their flagella and thus move in a northward direction (which in the Northern Hemisphere is downward). Cells in the Southern Hemisphere have the opposite polarity and move southward.

Most of the magnetic bacteria that have been described are species of *Alphaproteobacteria*, but species have also been observed in the *Gammaproteobacteria*, the *Deltaproteobacteria*, and the *Nitrospira* group. One of the best-characterized species is *Magnetospirillum magnetotacticum* (**Figure 15.57**), which is a chemoorganotrophic microaerophile that can also grow anaerobically by reducing NO_3^- or

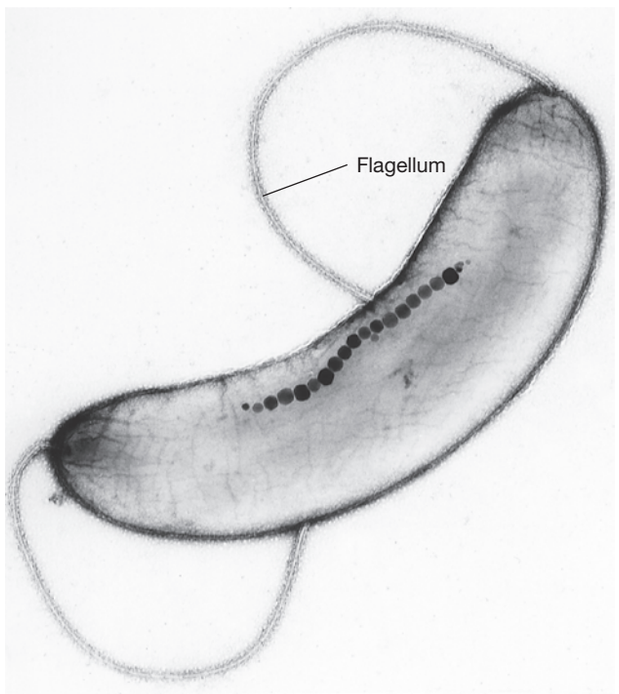

Figure 15.57 **A magnetotactic spirillum.** Electron micrograph of a single cell of *Magnetospirillum magnetotacticum*; a cell measures 0.3 × 2μm. The cell contains particles of magnetosomes made of Fe_3O_4 arranged in a chain.

N_2O. In contrast, the species *Desulfovibrio magneticus* is a sulfate reducer and an obligate anaerobe. In addition, magnetosomes have been observed in a few species of sulfur oxidizers and purple nonsulfur bacteria. Multicellular magnetotactic bacteria are also known. These are *Deltaproteobacteria* that form multicellular aggregates of 10–20 cells organized as a hollow sphere. While multicellular magnetotactic bacteria are obligate anaerobes, the basis of their metabolism has not yet been determined.

We transition now from viewing *Bacteria* through the lens of their ecological diversity to consider some other important phyla in a phylogenetic context in Chapter 16. We then conclude our coverage of prokaryotic microbes with Chapter 17 dedicated to the *Archaea*.

Check Your Understanding

- What benefit do magnetic bacteria accrue from having magnetosomes?
- Would you expect to find greigite or magnetite in the magnetosomes of *Desulfovibrio magneticus*?

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CHAPTER REVIEW

I • Ecological Diversity Among Microorganisms

15.1 Microbial diversity can be defined in several different ways. Metabolic diversity is defined in terms of the cellular processes that support growth. Ecological diversity is defined in terms of microbial interactions between organisms and their environments. Phylogenetic diversity is defined by evolutionary relationships between organisms.

Q What is convergent evolution and how is it different from horizontal gene transfer?

II • Ecological Diversity of Phototrophic *Bacteria*

15.2 Anoxygenic phototrophs, which do not produce oxygen, were the first phototrophic organisms to evolve. The evolution of photosynthesis has been affected strongly by patterns of horizontal gene transfer.

Q Which phyla of *Bacteria* contain anoxygenic phototrophs? Which phylum contains purple sulfur bacteria? Which phylum contains green sulfur bacteria?

15.3 *Cyanobacteria* is the only bacterial phylum that contains oxygenic phototrophs. All species of cyanobacteria can fix CO_2 and many can fix N_2, making these organisms important primary producers in many ecosystems.

Q Which morphological groups can *Cyanobacteria* be divided into?

15.4 Purple sulfur bacteria are anoxygenic phototrophic *Gammaproteobacteria*. Purple sulfur bacteria use H_2S and S^0 as electron donors and fix CO_2 by the Calvin cycle. These phototrophs have bacteriochlorophylls *a* or *b* and use a Q-type reaction center.

Q Compare and contrast the metabolism, morphology, and phylogeny of purple sulfur and purple nonsulfur bacteria.

15.5 Purple nonsulfur bacteria are anoxygenic phototrophic *Alpha-* and *Betaproteobacteria*. Purple nonsulfur bacteria are metabolically diverse, growing best as photoheterotrophs, and can also grow in darkness. These phototrophs have bacteriochlorophylls *a* or *b* and use type II photosystems with Q-type reaction centers. Aerobic anoxygenic phototrophs have type II photosystems but only possess bacteriochlorophyll *a*.

Q In what metabolic ways are aerobic anoxygenic phototrophs and purple nonsulfur bacteria similar? How do they differ?

15.6 Green sulfur bacteria are anoxygenic phototrophs of the phylum *Chlorobi*. Green sulfur bacteria use H_2S or S^0 as electron donors and fix CO_2 by the reverse citric acid cycle. These phototrophs contain bacteriochlorophylls *c*, *d*, or *e* localized in chlorosomes and bacteriochlorophyll *a* localized in their FeS-type photosynthetic reaction centers.

Q Compare and contrast the metabolism, morphology, and phylogeny of green sulfur and purple sulfur bacteria.

15.7 Green nonsulfur bacteria, also known as filamentous anoxygenic phototrophs, are anoxygenic phototrophs of the phylum *Chloroflexi* and grow best as photoheterotrophs. These phototrophs contain bacteriochlorophyll *c* in chlorosomes and bacteriochlorophyll *a* in their Q-type photosynthetic reaction centers.

Q What traits do green nonsulfur bacteria share with green sulfur bacteria and purple sulfur bacteria?

15.8 Heliobacteria are anoxygenic phototrophic *Firmicutes* that grow as photoheterotrophs or in darkness as chemotrophs. Heliobacteria produce bacteriochlorophyll *g* and have FeS-type reaction centers. *Chloracidobacterium thermophilum* is an anoxygenic phototrophic acidobacterium that grows photoheterotrophically, possesses bacteriochlorophyll *a* and *c* as well as chlorosomes, and has an FeS-type reaction center.

Q In what ways is *Chloracidobacterium thermophilum* similar to green sulfur bacteria, and in what ways is it different?

III • Diversity of *Bacteria* Defined by Metabolic Traits

15.9 Diazotrophs are bacteria that assimilate N_2 through activity of the enzyme nitrogenase. Diazotrophs are metabolically and phylogenetically diverse and employ various adaptations to protect nitrogenase from oxygen inactivation.

Q What are some ways that diazotrophs protect nitrogenase from O_2?

15.10 Nitrifying bacteria are aerobic chemolithotrophs that oxidize NH_3 to NO_2^- (genus prefix *Nitroso-*) or NO_2^- to NO_3^- (genus prefix *Nitro-*). Ammonia oxidizers are *Proteobacteria* or *Thaumarchaeota*, while nitrite oxidizers are *Proteobacteria* or *Nitrospirae*. Denitrifiers are metabolically and phylogenetically diverse facultative aerobes and chemoorganotrophs that reduce NO_3^- to the gaseous products NO, N_2O, and N_2.

Q Compare and contrast the nitrogen metabolism of nitrifiers with that of denitrifiers.

15.11 Dissimilative sulfate-reducers are obligate anaerobes that grow by reducing SO_4^{2-} with H_2 or simple organic compounds as electron donors. Most sulfate reducers are *Deltaproteobacteria*. Dissimilative sulfur-reducers are metabolically and phylogenetically diverse organisms that grow by reducing S^0 and other oxidized sulfur compounds (other than SO_4^{2-}) as electron acceptors.

Q With respect to sulfate-reducing bacteria and acetate as substrate, which genera contain "incomplete oxidizers" and which genera contain "complete oxidizers?" What are the physiological traits that can differentiate sulfur-reducing bacteria from sulfate-reducing bacteria?

15.12 Sulfur chemolithotrophs, most of which are species of *Proteobacteria*, oxidize H_2S and other reduced sulfur compounds as electron donors with O_2 or NO_3^- as electron acceptors and use either CO_2 or organic compounds as carbon sources. Sulfur chemolithotrophs use a variety of ecological strategies to conserve energy from H_2S and O_2, substances that otherwise react together spontaneously.

Q What are some ecological strategies that aerobic sulfide-oxidizers use to compete with the chemical oxidation of H_2S by atmospheric O_2?

15.13 Dissimilative iron-reducers reduce insoluble electron acceptors in anaerobic respirations. Most species can grow anaerobically by reducing ferric iron using H_2 or simple organic compounds as electron donor. The best-characterized genera include *Geobacter*, which contains exclusively obligate anaerobes, and *Shewanella*, which contains facultative aerobes.

Q In what ways are the dissimilative iron-reducing bacteria *Shewanella* and *Geobacter* similar, and in what ways are they different?

15.14 Dissimilative iron-oxidizers conserve energy from the aerobic oxidation of ferrous iron. These microbes use several ecological strategies to cope with the chemical instability of ferrous iron in oxic habitats at neutral pH. Iron oxidizers are found in four physiological groups: aerobic acidophiles, aerobic neutrophiles, anaerobic chemotrophs, and anaerobic phototrophs.

Q Which group of dissimilative iron-oxidizers is the least diverse, and in what way is this related to oxygen and pH?

15.15 Methylotrophs grow on organic compounds that lack carbon–carbon bonds. Some methylotrophs are also methanotrophs, organisms able to catabolize methane. Most methanotrophs are *Proteobacteria* that contain extensive internal membranes and incorporate carbon by either the serine or ribulose monophosphate pathways.

Q What are the differences between type I and type II methanotrophs?

IV • Morphologically and Ecologically Distinctive *Bacteria*

15.16 Bacterial predators such as *Bdellovibrio* and *Myxococcus* consume other microorganisms. Myxobacteria have a complex developmental cycle that involves the formation of fruiting bodies that contain myxospores.

Q Compare and contrast the life cycle of *Myxococcus* with that of *Bdellovibrio*.

15.17 The phylum *Spirochaetes* contains helically shaped bacteria that show a novel form of motility that allows them to "corkscrew" through viscous materials. These organisms are common in anoxic habitats and are the cause of many well-known human diseases, such as syphilis.

Q Contrast the motility of spirochetes with that of spirilla.

15.18 Prosthecate bacteria, such as *Hyphomicrobium*, *Caulobacter*, and *Gallionella*, are appendaged cells that form stalks or prosthecae used for attachment or nutrient absorption and are primarily aquatic. Some prosthecate bacteria, such as *Hyphomicrobium*, have a complex life cycle in which new cells form by budding from hyphae.

Q Contrast the life cycle of *Hyphomicrobium* with that of *Caulobacter*.

15.19 Sheathed bacteria are filamentous *Proteobacteria* in which individual cells form chains within an outer layer called the sheath. *Sphaerotilus* and *Leptothrix* are major genera of sheathed bacteria and can oxidize metals, such as Fe^{2+} and Mn^{2+}.

Q In what environment might you expect to find *Leptothrix*?

15.20 Magnetosomes are specialized magnetic structures present in magnetotactic bacteria. Magnetosomes orient cells along the magnetic field lines of Earth, and this allows cells to use their normal chemotactic response to move vertically in a directed fashion in sediments or stratified aquatic systems.

Q In what way does a magnetosome contribute to the fitness of microaerophilic bacteria in sediments?

APPLICATION QUESTIONS

1. Describe a key physiological feature of the following *Bacteria* that would differentiate each from the others: *Rhodobacter*, *Methylococcus*, *Azotobacter*, *Desulfovibrio*, and *Spirillum*.

2. Describe the metabolism for each of the following *Bacteria* and state whether the organism is an aerobe or an anaerobe: *Thiobacillus*, *Nitrosomonas*, *Methylomonas*, *Heliobacterium*, *Rhodospirillum*, *Myxococcus*, and *Gallionella*.

3. Using an example from each of the morphologically diverse groups of *Bacteria* (Sections 15.16–15.20), describe how you could distinguish them from each other using only microscopy. How do the habitats of your example organisms differ from each other? Could you find any of these organisms in or on the human body? Despite their ability to oxidize inorganic electron donors, why are *Sphaerotilus* and *Leptothrix* not considered chemolithotrophs?

CHAPTER GLOSSARY

Aerobic anoxygenic phototroph an organism that is an aerobic heterotroph that uses anoxygenic photosynthesis as a supplemental source of energy

Carboxysome a polyhedral cellular inclusion of crystalline ribulose bisphosphate carboxylase (RuBisCO), the key enzyme of the Calvin cycle

Chemolithotroph an organism able to oxidize inorganic compounds (such as H_2, Fe^{2+}, S^0, or NH_4^+) as energy sources (electron donors)

Chlorosome a cigar-shaped structure present in the periphery of cells of green sulfur and green nonsulfur bacteria and containing the antenna bacteriochlorophylls (*c*, *d*, or *e*)

Consortium a two- or more-membered association of bacteria, usually living in an intimate symbiotic fashion

Convergent evolution a circumstance where a trait or set of traits that are similar in form and/or function between two organisms are not inherited from a shared ancestor (that is, the traits are similar but not homologous)

Cyanobacteria prokaryotic oxygenic phototrophs containing chlorophyll *a* and in most cases phycobilins

Denitrifier an organism that carries out anaerobic respiration with NO_3^- or NO_2^-, reducing it to the gaseous products NO, N_2O, and N_2

Diazotroph an organism that can assimilate N_2 into biomass by activity of the enzyme nitrogenase

Dissimilative sulfate-reducer an anaerobic microorganism that conserves energy through the reduction of SO_4^{2-}

Dissimilative sulfur-oxidizer a microorganism that gains energy for growth through oxidation of reduced sulfur compounds

Dissimilative sulfur-reducer an anaerobic or facultatively aerobic microorganism that conserves energy through the reduction of S^0 but cannot reduce SO_4^{2-}

Ecological diversity a concept for understanding microbial diversity that defines diversity in terms of differences in the interactions between organisms and their environments.

Green nonsulfur bacteria anoxygenic phototrophs containing chlorosomes, Q-type photosynthetic reaction centers, bacteriochlorophylls *a* and *c* as light-harvesting chlorophylls, and typically growing best as photoheterotrophs

Green sulfur bacteria anoxygenic phototrophs containing chlorosomes, FeS-type photosynthetic reaction centers, bacteriochlorophylls *c*, *d*, or *e* as antenna bacteriochlorophylls, and typically growing with H_2S as an electron donor

Heliobacteria anoxygenic phototrophs containing bacteriochlorophyll *g* and FeS-type reaction centers

Horizontal gene transfer the unidirectional transfer of genes between cells through a process uncoupled from reproduction

Metabolic diversity a concept for understanding microbial diversity that defines diversity in terms of differences in the cellular processes that support microbial growth

Methanotroph an organism capable of oxidizing methane (CH_4) as an electron donor in energy metabolism

Methylotroph an organism capable of oxidizing organic compounds that do not contain carbon–carbon bonds; if able to oxidize CH_4, also a methanotroph

Mixotroph an organism that conserves energy from the oxidation of inorganic compounds but requires organic compounds as a carbon source

Nitrifier a chemolithotroph capable of carrying out the oxidation of NH_3 or NO_2^-

Phycobilins the pigments phycocyanin, allophycocyanin, and phycoerythrin that function as photosynthetic accessory pigments in cyanobacteria and are coupled to proteins to form phycobiliproteins

Phylogenetic diversity a concept for understanding microbial diversity that defines diversity in terms of differences in the evolutionary relationships between organisms

Prochlorophyte a bacterial oxygenic phototroph that contains chlorophylls *a* and *b* but lacks phycobilins

Prosthecae extrusions of cytoplasm, often forming distinct appendages, bounded by the cell wall

Purple nonsulfur bacteria a group of phototrophic bacteria that contain bacteriochlorophyll *a* or *b* and Q-type reaction centers, and that grow best as photoheterotrophs

Purple sulfur bacteria a group of phototrophic bacteria that contain bacteriochlorophylls *a* or *b*, have Q-type reaction centers, and can oxidize H_2S as photosynthetic electron donor

Spirilla (singular, spirillum) spiral-shaped cells

Spirochete a slender, tightly coiled, gram-negative bacterium of the phylum *Spirochaetes* characterized by possession of endoflagella used for motility

Phylogenetic Diversity of *Bacteria* 16

I *Proteobacteria* 556

II *Firmicutes, Tenericutes,* and *Actinobacteria* 567

III *Bacteroidetes* 578

IV *Chlamydiae, Planctomycetes,* and *Verrucomicrobia* 580

V Hyperthermophilic *Bacteria* 584

VI Other *Bacteria* 586

MICROBIOLOGYNOW

Bacterial Diversity and Human Health

Animals, particularly those that eat plants, have gut microbes that convert food into nutrients for the host. These animals lack enzymes for digesting the lignin and cellulose of plant cell walls and have evolved specialized organs that house microbes to perform these tasks. In humans, this organ is the colon, also known as the large intestine.

The average human colon contains hundreds of microbial species, and up to a hundred trillion microbial cells, many of which belong to the order *Clostridiales* within the phylum *Firmicutes*. The *Clostridiales* are a highly diverse group of gram-positive anaerobes that employ a fermentative metabolism. *Clostridiales* are crucial to gut function, and changes in their abundance and diversity can have important consequences for human health.

Christensenella minuta (see scanning electron micrograph), a member of the *Clostridiales,* was first discovered in human feces. *C. minuta* is a strictly anaerobic, nonsporulating and nonmotile fermentative bacterium that ferments sugars to short-chain fatty acids, H_2, and CO_2. *C. minuta* often co-occurs with methane-producing *Archaea* and may grow syntrophically with these H_2-consuming organisms. Microbiome research suggests that *C. minuta* and other species of the family *Christensenellaceae* are indicators of a healthy gut. These bacteria are depleted in obese individuals as well as those suffering from metabolic syndrome, inflammatory bowel disease, Crohn's disease, and ulcerative colitis. In addition, the introduction of live cells of *C. minuta* into mice reduces their body fat levels compared with control mice given heat-killed cells. Moreover, the relative abundance of *Christensenellaceae* in the gut is genetically determined in humans, with up to 40% of variation in their relative abundance attributable to host genetics.

These results show that our genes influence our microbial relationships and that our gut microbes can influence our health. A full understanding of our gut health therefore depends upon our understanding of gut bacterial diversity.

Source: Goodrich, J.K., et al. 2014. Human genetics shape the gut microbiome. *Cell 159*: 789.

In the previous chapters we examined the *metabolic and ecological* diversity of microorganisms. In this and the next two chapters, we shift our focus to the *phylogenetic* diversity of microorganisms. We considered the difference between metabolic, ecological, and phylogenetic diversity in Section 15.1. In this chapter, we examine the major lineages of *Bacteria* (**Figure 16.1*a***) and focus on the *Archaea* and microbial *Eukarya* in Chapters 17 and 18, respectively.

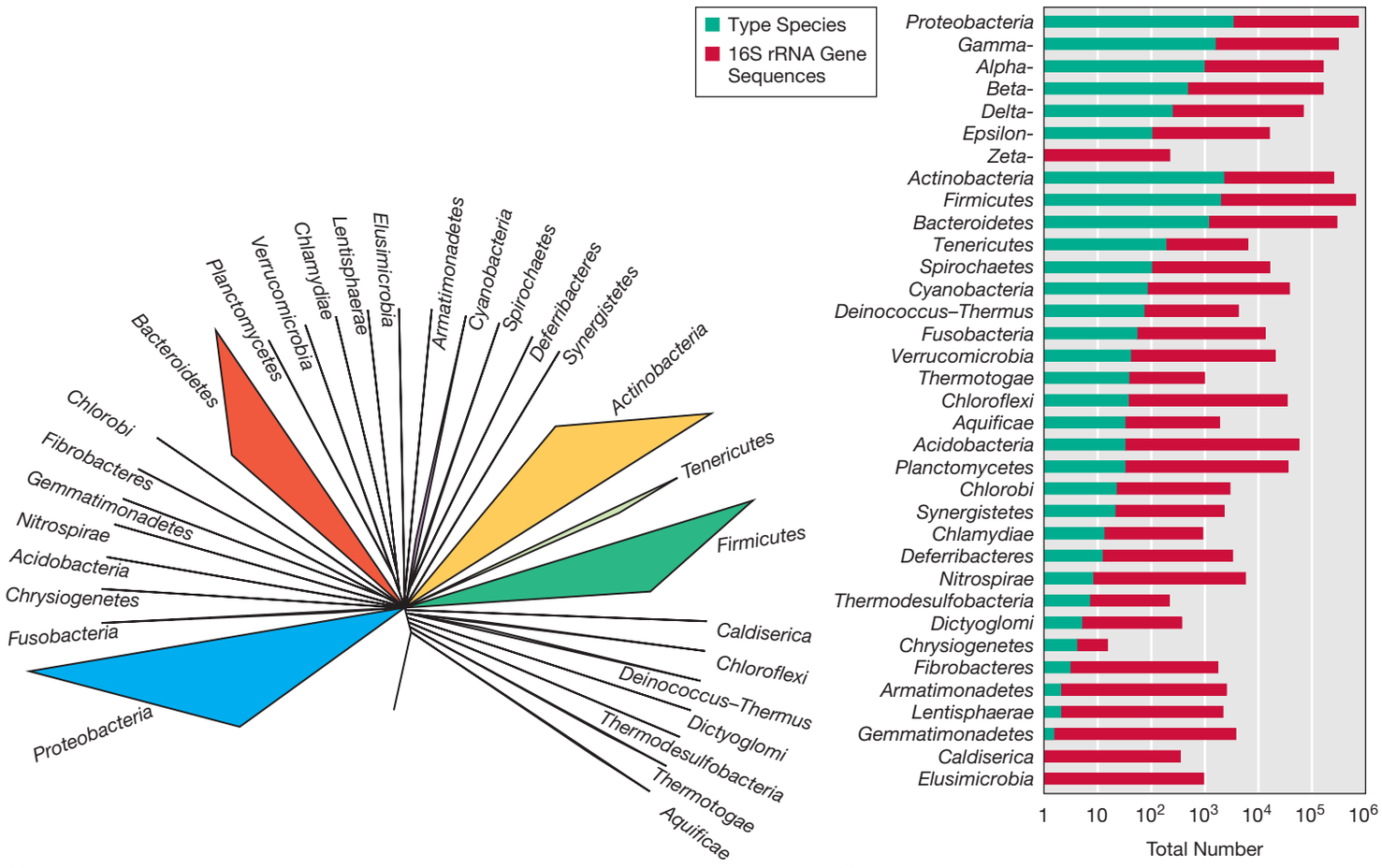
Including phyla of *Bacteria* known only from 16S ribosomal RNA (rRNA) gene sequences retrieved from the environment (▶ Sections 19.6 and 19.8), over 80 phyla can be distinguished. However, only about 30 of these contain species that have been characterized in laboratory culture (Figure 16.1*b*). Remarkably, more than 90% of cultivated genera and species of *Bacteria* originate in only *four* phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Figure 16.1*b*).

With more than 12,000 species of bacteria described, we obviously cannot consider them all. Therefore, using phylogenetic trees to focus our discussion, we will explore some of the best-known species from a broad diversity of phyla. In this chapter, we will consider species from 20 bacterial phyla, focusing on those with the largest numbers of characterized species. We begin our tour of the *Bacteria* with the phylum *Proteobacteria*, a hotbed of cultured species in this domain.

I • *Proteobacteria*

The *Proteobacteria* are divided into six classes, the *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Epsilon-*, and *Zetaproteobacteria*. *Proteobacteria* is the largest and most metabolically diverse phylum of *Bacteria* and leads in numbers of cultivated and well-characterized species.

The *Proteobacteria* are by far the largest and most metabolically diverse phylum of *Bacteria* (**Figure 16.2**). More than a third of characterized species of *Bacteria* originate within this group



(a) **Major phyla of *Bacteria***

(b) **Cultured representatives versus phylotypes**

Figure 16.1 Some major phyla of *Bacteria* based on 16S ribosomal RNA gene sequence comparisons. *(a)* Depicted are the major phyla of *Bacteria* that have cultivated species. The area of each wedge is roughly proportional to the number of described cultivated species in each group. Analyses of 16S rRNA gene sequences from natural environments suggest there are more than 80 bacterial phyla. *(b)* Numbers of cultured and characterized species (green bars) and known 16S rRNA gene sequences (phylotypes, red bars) for each of the 29 major bacterial phyla that have at least one type species in pure culture. Also shown are related data for the different classes of *Proteobacteria*. Differences between the size of the red and green bars indicate the degree to which members of each group are common in natural environments but difficult to cultivate in isolation. Note that the abscissa is a log scale.

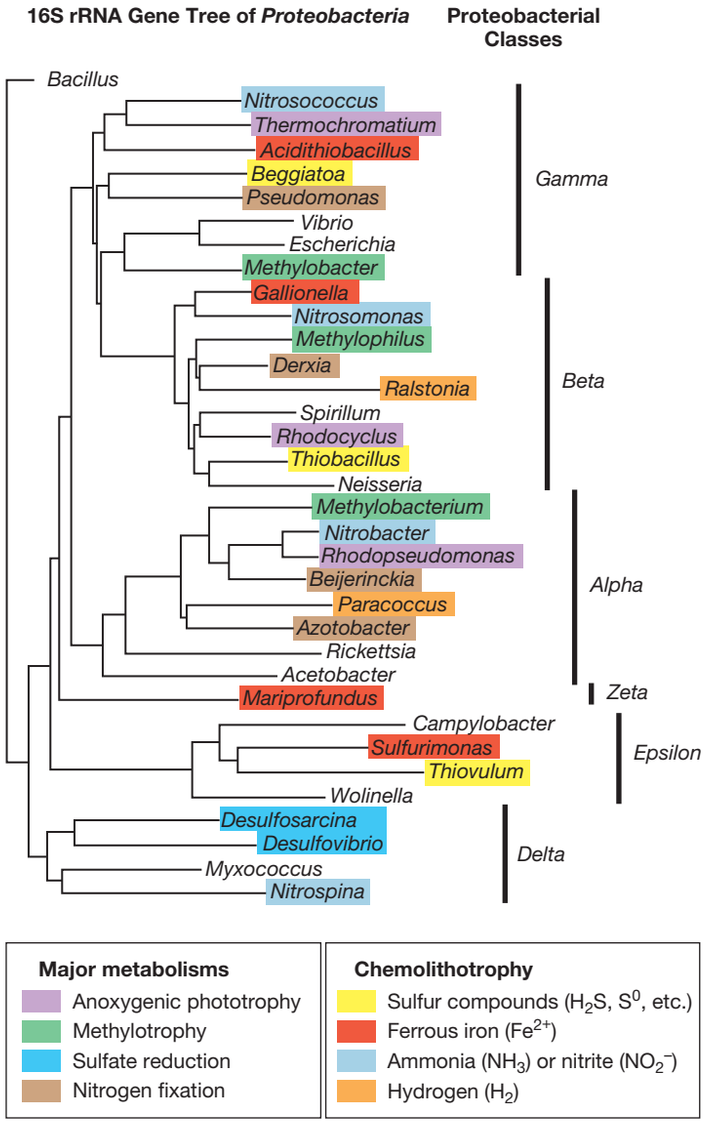


Figure 16.2 Phylogenetic tree and metabolic links of some key genera of *Proteobacteria*. Phylogeny of representative genera of *Proteobacteria* as revealed by analysis of 16S rRNA gene sequences. Note how identical metabolisms are often distributed in phylogenetically distinct genera, suggesting that horizontal gene flow has been extensive in the *Proteobacteria*. Some organisms listed may have multiple properties; for example, some sulfur chemolithotrophs are also iron or hydrogen chemolithotrophs, and several of the organisms listed can fix nitrogen. Phylogenetic analyses were performed and the phylogenetic tree constructed by Marie Asao, Ohio State University.

(Figure 16.1*b*), and *Proteobacteria* constitute the majority of known bacteria of medical, industrial, and agricultural significance.

As a group, the *Proteobacteria* are all gram-negative bacteria. They show an exceptionally wide diversity of energy-generating mechanisms, with chemolithotrophic, chemoorganotrophic, and phototrophic species (Figure 16.2). We have already encountered diverse representatives of this group in Chapters 14 and 15, where we considered the tremendous metabolic and ecological diversity of the *Proteobacteria*. Indeed, only a few types of metabolism do not occur within the *Proteobacteria*, including methanogenesis, which is only in *Archaea* (▶ Section 17.2); oxygenic phototrophy, which is only in *Cyanobacteria* (◀ Section 15.3); anammox, which is only in *Planctomyces* (◀ Section 14.10); the complete oxidation of ammonia all the way to nitrate, a process called *comammox* (▶ Section 21.3), which is only found in *Nitrospirae* (Section 16.21); and anaerobic methanotrophy (◀ Section 14.16). The *Proteobacteria* are equally diverse in terms of their relationship to O_2, with anaerobic, microaerophilic, and facultatively aerobic species known. Morphologically, they also exhibit a wide range of cell shapes, including straight and curved rods, cocci, spirilla, and filamentous, budding, and appendaged forms (◀ Section 1.3 and Figure 1.8).

As we proceed through this chapter, we will need to recall the descending hierarchy of microbial systematics: domain (*Bacteria* or *Archaea*) → phylum → class → order → family → genus → species (◀ Section 13.12 and Table 13.1). We will use these terms frequently in this chapter. Based on 16S rRNA gene sequences, the phylum *Proteobacteria* can be divided into six classes: *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Epsilon-*, and *Zetaproteobacteria*. Each class contains many genera with the exception of the *Zetaproteobacteria*, which is composed of a single characterized species, the marine iron-oxidizing bacterium *Mariprofundus ferrooxydans* (◀ Section 15.14).

Despite the phylogenetic breadth of the *Proteobacteria*, species in different classes often have similar metabolisms. For example, phototrophy and methylotrophy occur in three different classes of *Proteobacteria*, and nitrifying bacteria span four classes of *Proteobacteria* (◀ Figure 15.1). This suggests that horizontal gene flow (Chapter 9 and ◀ Section 13.9) has played a major role in shaping the metabolic diversity of the *Proteobacteria*. The sharing of metabolic traits in the different classes of *Proteobacteria* is also a good reminder that phenotype and phylogeny often provide different views of prokaryotic diversity (◀ Section 15.1).

16.1 *Alphaproteobacteria*

With about one thousand described species, the *Alphaproteobacteria* are the second largest class of *Proteobacteria* (Figure 16.1*b*). The *Alphaproteobacteria* contain extensive functional diversity (Figure 16.2, ◀ Figure 15.1), and many genera in this group have already been considered in Chapters 14 and 15. Most species are obligate aerobes or facultative aerobes and many are **oligotrophic**, preferring to grow in environments that have low nutrient concentrations. There are 10 well-characterized orders within the *Alphaproteobacteria*, but the vast majority of species fall within the *Rhizobiales*, *Rickettsiales*, *Rhodobacterales*, *Rhodospirillales*, *Caulobacterales*, and *Sphingomonadales* (**Figure 16.3** and **Table 16.1**).

Rhizobiales

KEY GENERA: *Bartonella, Methylobacterium, Pelagibacter, Rhizobium, Agrobacterium*

The *Rhizobiales* (Figure 16.3) are the largest and most metabolically diverse order of *Alphaproteobacteria* and contain phototrophs (e.g., *Rhodopseudomonas*), chemolithotrophs (e.g., *Nitrobacter*), symbionts (e.g., rhizobia), free-living nitrogen-fixing bacteria (e.g., *Beijerinckia*), a few pathogens of plants and animals, and diverse chemoorganotrophs. The group gets its name from the *rhizobia*, a *polyphyletic* collection of genera that form root nodules and fix nitrogen in symbiotic association with leguminous plants (▶ Section 23.4).

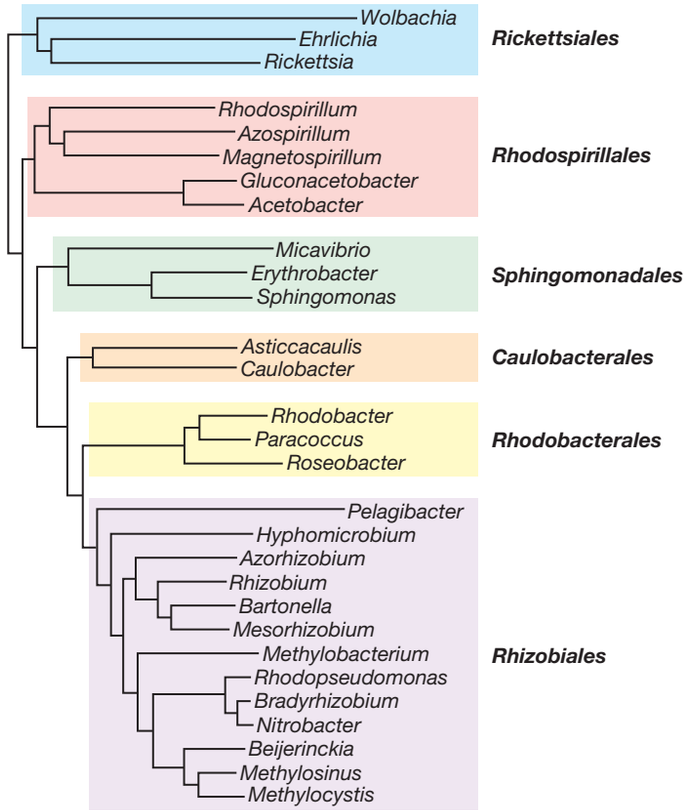


Figure 16.3 Major orders of *Proteobacteria* in the class *Alphaproteobacteria*. The phylogenetic tree was constructed using 16S rRNA gene sequences from representative genera of *Alphaproteobacteria*. Order names are shown in bold.

TABLE 16.1 Notable genera of *Alphaproteobacteria*

Order	Genus	Notable characteristics
Caulobacterales	*Caulobacter*	Asymmetric cell division and formation of prosthecae
Rickettsiales	*Rickettsia*	Obligate intracellular parasites of humans, transmitted by arthropods
	Wolbachia	Obligate intracellular parasites of arthropods, affect their reproduction, lack peptidoglycan
Rhizobiales	*Bartonella*	Obligate intracellular parasites, transmitted by arthropods
	Bradyrhizobium	Form root nodules with soybean and other legumes
	Brucella	Facultative intracellular parasites of animals, zoonotic pathogen
	Hyphomicrobium	Stalked cells, metabolically versatile
	Mesorhizobium	Form root nodules with bird's-foot trefoil and other legumes
	Methylobacterium	Methylotroph found on plants and in soil
	Nitrobacter	Nitrifying bacterium that oxidizes NO_2^- to NO_3^-
	Pelagibacter	Oligotrophic chemoorganotroph; high abundance in ocean surface
	Rhodopseudomonas	Metabolically versatile purple nonsulfur bacterium
Rhodobacterales	*Paracoccus*	Species used as a model for studying denitrification
	Rhodobacter	Metabolically versatile purple nonsulfur bacteria
	Roseobacter	Aerobic anoxygenic phototroph
Rhodospirillales	*Acetobacter*	Used industrially for producing acetic acid
	Azospirillum	Obligately aerobic diazotroph
	Gluconobacter	Used industrially for producing acetic acid
	Magnetospirillum	Magnetotactic bacterium
Sphingomonadales	*Sphingomonas*	Aerobic degradation of aromatic organics, biodegradation
	Zymomonas	Ferments sugars into ethanol, potential for biofuel production

Among the *Rhizobiales* are nine genera that contain rhizobia: *Bradyrhizobium*, *Ochrobactrum*, *Azorhizobium*, *Devosia*, *Methylobacterium*, *Mesorhizobium*, *Phyllobacterium*, *Sinorhizobium*, and *Rhizobium*. These are typically chemoorganotrophs and obligate aerobes, and the genes that convey the ability to form root nodules have been distributed among these genera by horizontal gene transfer. Indeed, nodulation genes are found on large plasmids that can be transferred between cells (▶ Section 23.4). Each rhizobial genus has a distinct range of plant hosts that can be colonized (▶ Table 23.1). Rhizobia can be isolated by crushing nodules and spreading their contents on nutrient-rich solid media; colonies typically produce copious amounts of exopolysaccharide slime (**Figure 16.4**).

The organism *Agrobacterium tumefaciens* (also called *Rhizobium radiobacter*) is closely related to root nodule *Rhizobium* species but is a plant pathogen that causes crown gall disease (▶ Section 23.6). *A. tumefaciens* is unable to form root nodules, and the genes that encode gall formation are also found on a plasmid. These pathogenicity genes, however, are unrelated to the plasmid-borne genes that mediate nodule formation in symbiotic rhizobia.

The genus *Methylobacterium* is one of the largest in the *Rhizobiales*. These species are often called "pink-pigmented facultative methylotrophs" because of the pink color of their colonies (◀ Section 15.15) and their good growth on methanol. Species are commonly found on the surface of plants and in soils and freshwater systems. These organisms are also commonly encountered in toilets and baths where their growth on shower curtains, caulk, and in toilet bowls results in the formation of pink-pigmented biofilms. Species of *Methylobacterium* are readily isolated by pressing the surface of a plant leaf onto an agar Petri plate containing methanol as the sole source of carbon.

Odile Berge

Figure 16.4 Colonies of *Rhizobium mongolense*. Colonies of rhizobia often produce copious exopolysaccharide slime. These colonies of *Rhizobium mongolense* were grown on a medium low in nitrogen with sucrose as carbon source.

Bartonella is another notable genus of *Rhizobiales*. These organisms, once classified with the *Rickettsiales*, are intracellular pathogens of humans. Species of *Bartonella* can cause a variety of diseases in humans and other vertebrate animals. *Bartonella quintana* is the causative agent of *trench fever*, a disease that got its name because it decimated soldiers in the brutal trench warfare practiced in World War I. Other species of *Bartonella* can cause bartonellosis, cat scratch disease, and a variety of inflammatory diseases. These diseases are mediated by arthropod vectors including fleas, lice, and sand flies (Chapter 32). Species of *Bartonella* are fastidious and difficult to cultivate, and isolation is most commonly achieved using blood agar. When growing in tissue culture, cells of *Bartonella* grow on the outside surface of the eukaryotic host cells rather than within the cytoplasm or the nucleus.

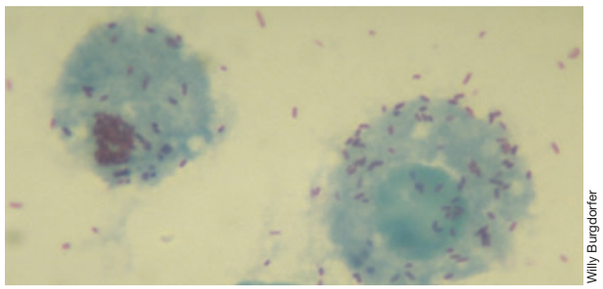
Finally, the genus *Pelagibacter* also belongs to the *Rhizobiales*. *Pelagibacter ubique* is an oligotroph and an obligately aerobic chemoorganotroph that inhabits the photic zone of Earth's oceans. This organism can make up 25% of the bacterial cells found at the ocean's surface, and its numbers can reach 50% of cells in temperate waters in the summer; as a consequence, *Pelagibacter ubique* is likely the most abundant bacterial species on Earth (▶ Section 20.12).

Rickettsiales

KEY GENERA: *Rickettsia, Wolbachia*

Rickettsiales (Figure 16.3) are all obligate intracellular parasites or mutualists of animals. Species in this order have not yet been cultivated in the absence of host cells (**Figure 16.5**) and must be grown in chicken eggs or in host cell tissue culture. Typically, *Rickettsiales* are closely associated with arthropods. Those genera that cause disease, such as *Rickettsia* and *Ehrlichia*, are transmitted by arthropod bites; other genera such as *Wolbachia* are obligate parasites or mutualists of insects and other arthropods.

Species of the genus *Rickettsia* are the causative agents of several human diseases, including typhus (*Rickettsia prowazekii*) and spotted fever rickettsiosis, commonly called Rocky Mountain spotted fever (*Rickettsia rickettsii*)(▶ Section 32.3). These organisms are closely associated with arthropod vectors and can be transmitted by ticks, fleas, lice, and mites. Most rickettsias are metabolically specialized, able to oxidize only the amino acids glutamate or glutamine and unable to oxidize glucose or organic acids. Rickettsias are unable to synthesize certain metabolites and must instead obtain them from host cells. Rickettsias do not survive long outside their hosts, and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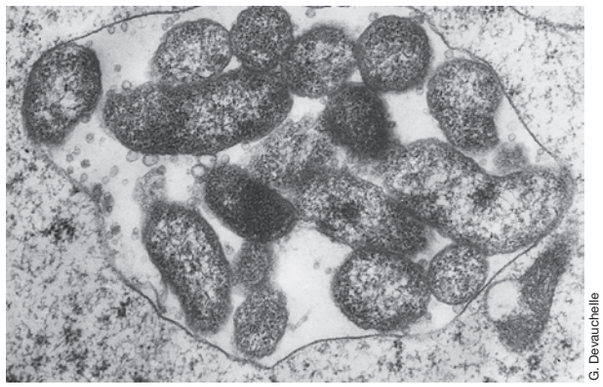
(a)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(b)

Figure 16.5 Rickettsias growing within host cells. *(a) Rickettsia rickettsii* in tissue culture. Cells are about 0.3 μm in diameter. *(b)* Electron micrograph of cells of *Rickettsiella popilliae* within a blood cell of its host, the beetle *Melolontha melolontha*. The bacteria grow inside a vacuole within the host cell.

this may explain why they must be transmitted from animal to animal by arthropod vectors.

Electron micrographs of thin sections of rickettsial cells show a typical prokaryotic morphology including a cell wall (Figure 16.5*b*). The penetration of a host cell by a rickettsial cell is an active process, requiring both host and parasite to be viable and metabolically active. Once inside the host cell, the bacteria multiply primarily in the cytoplasm and continue replicating until the host cell is loaded with parasites (Figure 16.5; ▶ Figures 32.6 and 32.7). The host cell then bursts and liberates the bacterial cells.

The genus *Wolbachia* contains intracellular parasites found within many arthropods and some nematodes (**Figure 16.6**). Species of *Wolbachia* infect an enormous diversity of insect species, and 10–70% of individual insects in a susceptible species carry *Wolbachia*. *Wolbachia* species can have any of several effects on their insect hosts. These include inducing parthenogenesis (development of unfertilized eggs), the killing of males, and feminization (the conversion of male insects into females). *Wolbachia*, because of their ability to alter host reproduction, have a major impact on the evolution of their insect hosts (▶ Section 23.7).

Wolbachia pipientis is the best-studied species in the genus. Cells of *W. pipientis* colonize the insect egg (Figure 16.6), where they multiply in vacuoles of host cells surrounded by a membrane of host origin. *Wolbachia* lack a cell wall and the ability to make peptidoglycan. Peptidoglycan can trigger host immune responses (Chapter 26), and the loss of genes for peptidoglycan synthesis helps this obligate

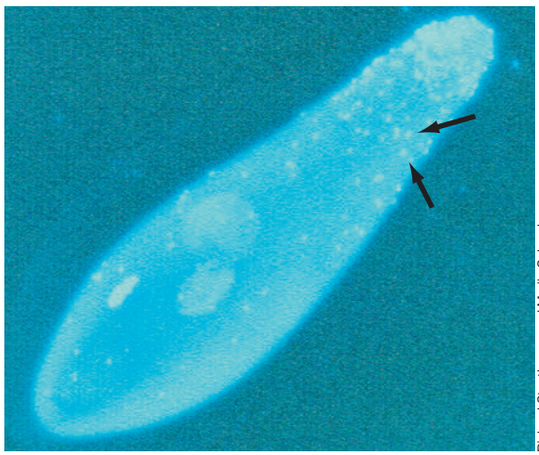


Figure 16.6 ***Wolbachia.*** Photomicrograph of a DAPI-stained egg of the parasitoid wasp *Trichogramma kaykai* infected with *Wolbachia pipientis*, which induces parthenogenesis. The *W. pipientis* cells are primarily located in the narrow end of the egg (arrows).

intracellular organism escape host immune detection. Cells of *W. pipientis* are passed from an infected female to her offspring through infected eggs. *Wolbachia*-induced parthenogenesis occurs in a number of species of wasps. In the normal reproductive cycle of these insects, eggs that are unfertilized (and haploid) develop into males, while eggs that are fertilized (and diploid) develop into females. However, *Wolbachia* infection triggers a doubling of chromosome number in haploid eggs, and so an infected mother can only lay female eggs that contain her own DNA. Predictably, if female insects are fed antibiotics that kill *Wolbachia*, parthenogenesis ceases.

Other Groups of *Alphaproteobacteria*

KEY GENERA: *Rhodobacter, Acetobacter, Caulobacter, Sphingomonas*

The orders *Rhodobacterales* and *Rhodospirillales* (Figure 16.3) contain metabolically diverse organisms that have been discussed previously, including purple nonsulfur bacteria (*Rhodobacter* and *Rhodospirillum*, ◀ Section 15.5), aerobic anoxygenic phototrophs (*Roseobacter*, ◀ Section 15.5), nitrogen-fixing bacteria (*Azospirillum*, ◀ Section 15.9), denitrifiers (*Paracoccus*, ◀ Section 15.10), and magnetotactic bacteria (*Magnetospirillum*, ◀ Section 15.20), among others.

The *Caulobacterales* are typically oligotrophic and strictly aerobic chemoorganotrophs. Species typically form prosthecae or stalks (◀ Section 15.18), and many species display asymmetric forms of cell division. The characteristic genus is *Caulobacter*, which has a characteristic life cycle that we have discussed previously (◀ Sections 8.8 and 15.18).

The *Sphingomonadales* include diverse aerobic and facultatively aerobic chemoorganotrophs as well as species of aerobic anoxygenic phototrophs (*Erythrobacter*) and a few obligate anaerobes. The characteristic genus is *Sphingomonas*, which consists of obligately aerobic and nutritionally versatile species. Sphingomonads are widespread in aquatic and terrestrial environments and are notable for their ability to metabolize a wide range of organic compounds including many aromatic compounds that are common environmental contaminants (toluene, nonylphenol, dibenzo-*p*-dioxin, naphthalene, and anthracene, among others). Consequently, sphingomonads have been widely studied as potential agents of bioremediation (▶ Sections 22.4 and 22.5). These organisms are typically easy to cultivate and grow well on a variety of complex culture media.

Check Your Understanding

- What are some ways in which *Wolbachia* species can affect insects? What evolutionary pressures have likely caused *Wolbachia* to lose the ability to make peptidoglycan?
- *Agrobacterium tumefaciens* has been reclassified into the genus *Rhizobium*. What mechanism underlies the distribution of genes for virulence or symbiosis among the species of this genus?

16.2 *Betaproteobacteria*

With about 500 described species, the *Betaproteobacteria* are the third largest class of *Proteobacteria* (Figure 16.1). The *Betaproteobacteria* contain an immense amount of functional diversity (Figure 16.2 and ◀ Figure 15.1), and many species in this group have already been considered in Chapter 15. A total of six orders of *Betaproteobacteria* have many characterized species: *Burkholderiales*, *Hydrogenophilales*, *Methylophilales*, *Neisseriales*, *Nitrosomonadales*, and *Rhodocyclales* (**Figure 16.7**), and we focus on these here.

Burkholderiales

KEY GENUS: *Burkholderia*

The *Burkholderiales* contain species with a wide range of metabolic and ecological characteristics. Species include strictly aerobic, facultatively aerobic, and obligately anaerobic chemoorganotrophs, anoxygenic

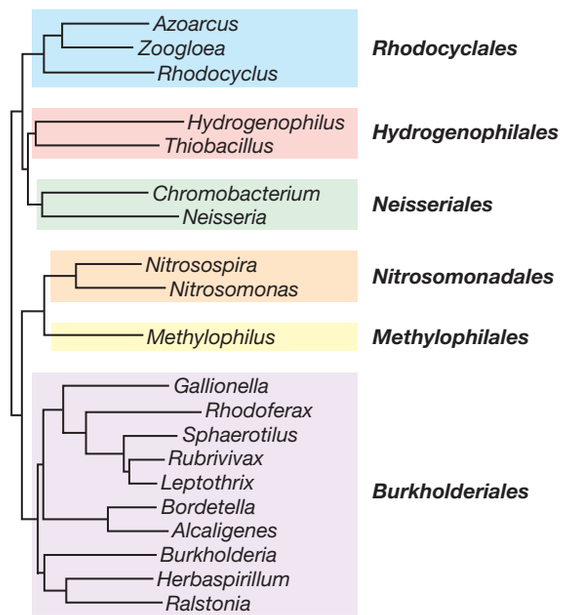


Figure 16.7 **Major orders of *Proteobacteria* in the class *Betaproteobacteria*.** The phylogenetic tree was constructed using 16S rRNA gene sequences from representative genera of *Betaproteobacteria*. Order names are shown in bold.

phototrophs, obligate and facultative chemolithotrophs, free-living nitrogen fixers, and pathogens of plants, animals, and humans.

Burkholderia is the type genus for the *Burkholderiales*. The genus *Burkholderia* includes diverse species of chemoorganotrophs with strictly respiratory metabolism. All species can grow aerobically, some also grow anaerobically with nitrate as the electron acceptor, and many strains are able to fix N_2. The metabolic versatility of *Burkholderia* species with respect to organic compounds, and aromatic compounds in particular, has led to interest in their use in bioremediation (▶ Section 22.4). Certain strains of *Burkholderia* have also been shown to promote plant growth. However, many species are potentially pathogenic for plants or animals. One of the best known of the pathogenic species is *Burkholderia cepacia* (**Figure 16.8**).

B. cepacia is primarily a soil bacterium but also an opportunistic pathogen. Often found in the rhizosphere of plants, *B. cepacia* can produce both antifungal and anti-nematodal compounds, and thus its ability to colonize plant roots can provide disease protection and promote plant growth. However, *B. cepacia* is also known as a plant pathogen in certain circumstances, and it is a major cause of soft rot in onions. *B. cepacia* has also emerged as an opportunistic hospital-acquired infection in humans, as it is a hardy organism that is difficult to eradicate from the clinical setting. *B. cepacia* can form secondary lung infections in patients who are immunocompromised or have pneumonia or cystic fibrosis. The ability of *B. cepacia* to form biofilms in the lung and its natural resistance to many antibiotics has made this organism, along with *Pseudomonas aeruginosa*, particularly dangerous for patients with cystic fibrosis (◀ Section 8.10 and ▶ Section 20.4).

Rhodocyclales

KEY GENERA: *Rhodocyclus, Zoogloea*

Like the *Burkholderiales*, the order *Rhodocyclales* contains species with diverse metabolic and ecological characteristics. The type genus for the *Rhodocyclales* is *Rhodocyclus*, a purple nonsulfur bacterium (◀ Section 15.5). Like most purple nonsulfur bacteria, *Rhodocyclus* species grow best as photoheterotrophs but most can also grow as photoautotrophs with H_2 as electron acceptor. Species can also grow by respiration in darkness, but they are typically found in illuminated anoxic environments where organic matter is present.

Zoogloea is another important genus of the *Rhodocyclales*. *Zoogloea* species are aerobic chemoorganotrophs that are distinctive for producing a thick gelatinous capsule that binds cells together into a complex matrix with branching, fingerlike projections. This gelatinous matrix can cause *flocculation*, the formation of macroscopic particles that settle out of solution. *Zoogloea ramigera* is of particular importance in aerobic wastewater treatment (▶ Section 22.6), where it degrades much of the organic carbon in the waste stream and promotes flocculation and settling, crucial steps in water purification.

Neisseriales

KEY GENERA: *Chromobacterium, Neisseria*

The order *Neisseriales* contains at least 29 genera of diverse chemoorganotrophs. The best-characterized species are in the genera *Neisseria* and *Chromobacterium*. Species of *Neisseria* are commonly isolated from animals, and some of them are pathogenic. *Neisseria* species are always cocci (**Figure 16.9*a***). Some *Neisseria* are free-living saprophytes and reside in the oral cavity and other moist areas on the animal body. Others are serious pathogens, such as *Neisseria meningitidis*, which can cause a potentially fatal inflammation of the membranes lining the brain (meningitis, ▶ Section 31.5). We discuss the clinical microbiology of *Neisseria gonorrhoeae*—the causative agent of the sexually transmitted disease gonorrhea—in Section 31.5 and the pathogenesis of gonorrhea itself in Section 31.13.

Chromobacterium is a close phylogenetic relative of *Neisseria* but is rod-shaped in morphology. The best-known *Chromobacterium* species is *C. violaceum*, a purple-pigmented organism (Figure 16.9*b*) found in soil and water and occasionally in pus-forming wounds of humans and other animals. *C. violaceum* and a few other chromobacteria produce the purple pigment *violacein* (Figure 16.9*b*), a water-insoluble pigment with both antimicrobial and antioxidant properties. *Chromobacterium* is a facultative aerobe, growing fermentatively on sugars and aerobically on various carbon sources.

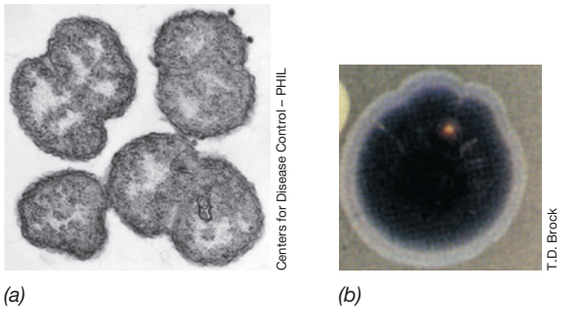
Hydrogenophilales, *Methylophilales,* and *Nitrosomonadales*

KEY GENERA: *Hydrogenophilus, Thiobacillus, Methylophilus, Nitrosomonas*

These three orders contain organisms that have fairly specialized metabolic capabilities including chemolithotrophs and methylotrophs; most species are obligate aerobes and many are autotrophic. *Hydrogenophilus thermoluteolus* is an obligate aerobe that can grow as a chemolithotroph using H_2 as an electron donor for respiration

James Shapiro

Figure 16.8 Colonies of *Burkholderia*. Photograph of colonies of *Burkholderia cepacia* on an agar plate.



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(a) *(b)*

Figure 16.9 *Neisseria* and *Chromobacterium*. *(a)* Transmission electron micrograph of cells of *Neisseria gonorrhoeae* showing the typical diplococcus cell arrangements. *(b)* A large colony of *Chromobacterium violaceum*.

(◀ Section 3.11) and the Calvin cycle to fix CO_2. This species is a facultative chemolithotroph but can also grow as a chemoorganotroph on simple carbon sources. *Thiobacillus* is another important genus of *Hydrogenophilales.* Species of *Thiobacillus* can be chemoorganotrophs or chemolithotrophs. Chemolithotrophic species of *Thiobacillus* are sulfur bacteria (◀ Sections 14.7 and 15.12) that oxidize reduced sulfur compounds as electron donors and grow by aerobic respiration or denitrification (◀ Sections 14.11 and 15.10). Species of *Thiobacillus* can also fix CO_2 using the Calvin cycle and are commonly found in soils, sulfur springs, marine habitats, and other locales where reduced sulfur compounds are available.

The *Methylophilales* and *Nitrosomonadales* contain metabolically specialized organisms. *Methylophilus* species are obligate and facultative methylotrophs (◀ Section 14.16) that grow on methanol and other C_1 compounds, but not on CH_4. Facultative species can grow as chemoorganotrophs through aerobic respiration of simple sugars. The order *Nitrosomonadales* contains obligately chemolithotrophic ammonia-oxidizing bacteria, the key genera being *Nitrosomonas* and *Nitrosospira* (◀ Section 15.10).

Check Your Understanding

- List three species of *Betaproteobacteria* that are known to be human pathogens.
- List three genera of *Betaproteobacteria* that contain chemolithotrophic species.

16.3 *Gammaproteobacteria: Enterobacteriales*

KEY GENERA: *Enterobacter, Escherichia, Klebsiella, Proteus, Salmonella, Serratia, Shigella*

The *Gammaproteobacteria* are the largest and most diverse class of *Proteobacteria*, containing nearly half of all characterized species in the phylum. The class contains more than 1500 characterized species and at least 15 well-characterized orders (**Figure 16.10**, Figure 16.1*b*). Its species have diverse metabolic and ecological characteristics (Figure 16.2 and ◀ Figure 15.1) and include many well-known human pathogens. Species can be phototrophic (including the purple sulfur bacteria, ◀ Section 15.4), chemoorganotrophic, or chemolithotrophic, and can have either respiratory or fermentative metabolisms. Members of this group often grow rapidly in laboratory media and can be isolated from a wide diversity of habitats. In this section, we consider the *Enterobacteriales*, one of the largest and best-known orders within the *Gammaproteobacteria*.

The *Enterobacteriales*, commonly called the **enteric bacteria**, comprise a relatively homogeneous phylogenetic group within the *Gammaproteobacteria* and consist of facultatively aerobic, gram-negative, nonsporulating rods that are either nonmotile or motile by peritrichous flagella (**Figure 16.11**). The *oxidase test* and the *catalase test* are common assays used to characterize bacteria (▶ Section 29.3 and Figure 29.6), and these tests can be used to discriminate enteric bacteria from many other *Gammaproteobacteria*. The oxidase test is an assay for the presence of cytochrome *c* oxidase, an enzyme present in many respiring bacteria. The catalase test assays for the enzyme catalase, which detoxifies hydrogen peroxide and is commonly found in bacteria able to grow in the presence of oxygen (◀ Section 4.16 and Figure 4.32). Enteric bacteria are oxidase-negative and catalase-positive. They also produce acid from glucose and reduce nitrate but only to nitrite. Enteric bacteria have relatively simple nutritional requirements and ferment sugars to a variety of end products.

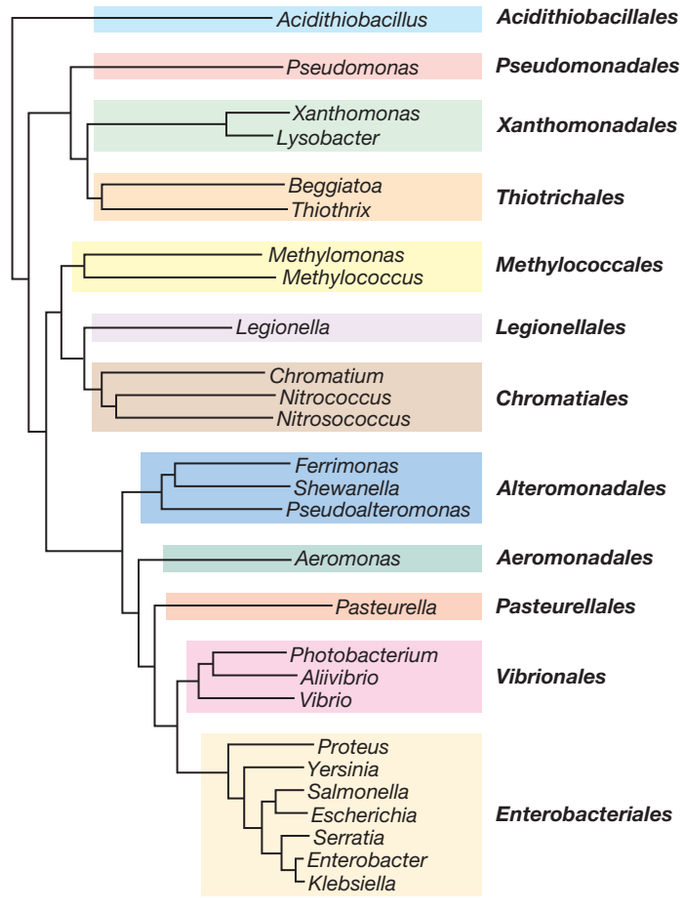


Figure 16.10 Major orders of *Proteobacteria* in the class *Gammaproteobacteria*. The phylogenetic tree was constructed using 16S rRNA gene sequences from representative genera of *Gammaproteobacteria*. Order names are shown in bold.

Among the enteric bacteria are many species pathogenic to humans, other animals, or plants, as well as other species of industrial importance. *Escherichia coli*, the best known of all organisms, is the classic enteric bacterium. Because of the medical importance of many enteric bacteria, an extremely large number have been characterized, and numerous genera and species have been defined, largely for ease in identification purposes in clinical microbiology. However, because enteric bacteria are genetically very closely related, their positive identification often presents considerable difficulty. In clinical laboratories, identification is typically based on the combined analysis of a large number of diagnostic tests carried out using miniaturized rapid diagnostic media kits along with immunological and genomic analyses to identify signature proteins or genes of particular species (Chapter 29).

Fermentation Patterns in Enteric Bacteria

One major taxonomic characteristic separating the various genera of enteric bacteria is the type and proportion of fermentation products generated from the fermentation of glucose. Two broad patterns are recognized, the *mixed-acid fermentation* and the *2,3-butanediol fermentation* (**Figure 16.12**).

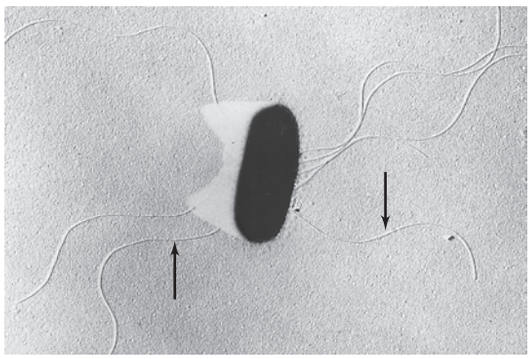
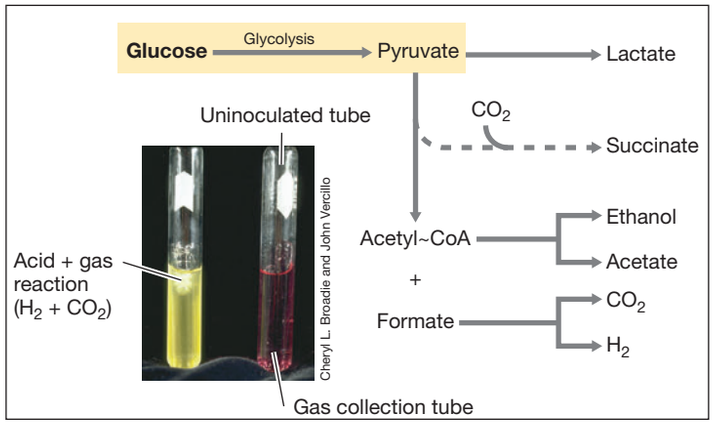
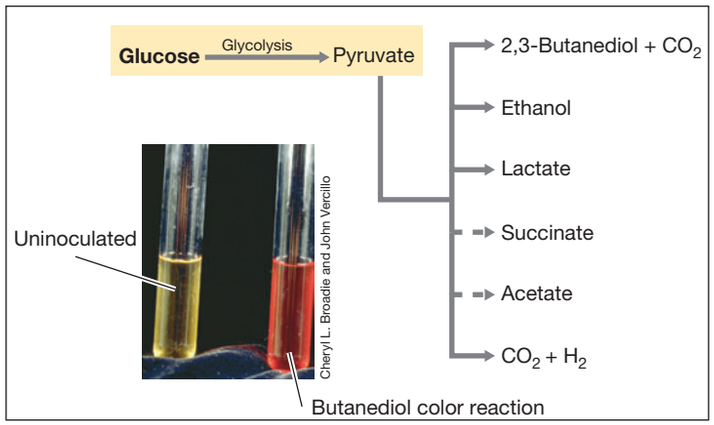


Figure 16.11 A butanediol-producing enteric bacterium. Electron micrograph of a shadow-cast preparation of a cell of the butanediol-producing bacterium *Erwinia carotovora*. The cell is about 0.8 μm wide. Note the peritrichously arranged flagella (arrows), typical of enteric bacteria.



(a) **Mixed-acid fermentation** (for example, *Escherichia coli*)



(b) **Butanediol fermentation** (for example, *Enterobacter aerogenes*)

Figure 16.12 Enteric fermentations. Distinction between *(a)* mixed-acid and *(b)* butanediol fermentation in enteric bacteria (see also Figure 14.45). The solid arrows indicate reactions leading to major products. Dashed arrows indicate minor products. *(a)* The photo shows the production of acid (yellow) and gas (in the inverted Durham tube) in a culture of *Escherichia coli* carrying out a mixed-acid fermentation (purple tube was uninoculated). *(b)* The photo shows the pink-red color in the Voges–Proskauer (VP) test, which indicates butanediol production, following growth of *Enterobacter aerogenes*. The left (yellow) tube was not inoculated. Note that the mixed-acid fermentation produces less CO_2 but more acid products from glucose than does the butanediol fermentation.

In the mixed-acid fermentation, three acids are formed in significant amounts: acetic, lactic, and succinic. Ethanol, CO_2, and H_2 are also formed, but not butanediol. In the butanediol fermentation, smaller amounts of acids are formed, and butanediol, ethanol, CO_2, and H_2 are the main products (◀ Figure 14.45). As a result of mixed-acid fermentation, equal amounts of CO_2 and H_2 are produced, whereas in the butanediol fermentation, considerably more CO_2 than H_2 is produced. This is because mixed-acid fermenters produce CO_2 only from formic acid by means of the enzyme formate hydrogenlyase:

$$HCOOH \rightarrow H_2 + CO_2$$

This reaction results in equal amounts of CO_2 and H_2. The butanediol fermenters also produce CO_2 and H_2 from formic acid, but they produce two additional molecules of CO_2 during the formation of each molecule of butanediol (Figure 16.12*b*). Butanediol fermentation is characteristic of *Enterobacter*, *Klebsiella*, *Erwinia*, and *Serratia*, whereas mixed-acid fermentation is observed in *Escherichia*, *Salmonella*, *Shigella*, *Citrobacter*, *Proteus*, and *Yersinia*.

Mixed-Acid Fermenters: *Escherichia, Salmonella, Shigella,* and *Proteus*

Species of *Escherichia* are almost universal inhabitants of the intestinal tract of humans and other warm-blooded animals, although they are by no means the dominant organisms in this habitat. *Escherichia* may play a nutritional role in the intestinal tract by synthesizing vitamins, particularly vitamin K. As a facultative aerobe, this organism probably also helps consume O_2, thus rendering the large intestine anoxic. Wild-type *Escherichia* strains rarely show any growth-factor requirements and are able to grow on a wide variety of carbon and energy sources such as sugars, amino acids, and organic acids.

Some strains of *Escherichia* are pathogenic and have been implicated in diarrheal diseases, especially in infants; diarrheal diseases are a major public health problem in developing countries (▶ Sections 33.1, 33.2, 33.7, and 33.11). *Escherichia* is also a major cause of urinary tract infections in women. Enteropathogenic *E. coli* strains are becoming more frequently implicated in gastrointestinal infections and generalized fevers. Some strains, such as enterohemorrhagic *E. coli*, an important representative of which is strain O157:H7, can cause sporadic outbreaks of severe foodborne disease. Infection occurs primarily through consumption of contaminated foods, such as raw or undercooked ground beef, unpasteurized milk, or contaminated water. In a small percentage of cases, *E. coli* O157:H7 causes a life-threatening complication related to its production of a potent enterotoxin.

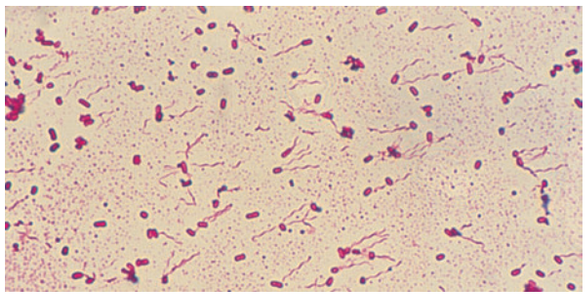
Salmonella and *Escherichia* are quite closely related. However, in contrast to *Escherichia*, species of *Salmonella* are almost always pathogenic, either to humans or to other warm-blooded animals (*Salmonella* is also found in the intestines of cold-blooded animals, such as turtles and lizards). In humans the most common diseases caused by salmonellas are typhoid fever and gastroenteritis (▶ Sections 33.5 and 33.10). The shigellas are also genetically very closely related to *Escherichia*. Genomic analyses strongly suggest that *Shigella* and *Escherichia* have exchanged a significant number of genes by horizontal gene flow. In contrast to most *Escherichia*, however, species of *Shigella* are typically pathogenic to humans, causing a rather severe gastroenteritis called *bacillary dysentery*. *Shigella dysenteriae*, transmitted by food- and waterborne routes, is a good example of this. The bacterium, which contains endotoxin,

invades intestinal epithelial cells, where it excretes a neurotoxin that causes acute gastrointestinal distress.

The genus *Proteus* typically contains highly motile cells (**Figure 16.13**) that produce the enzyme *urease*. Unlike *Salmonella* and *Shigella*, *Proteus* shows only a distant relationship to *E. coli*. *Proteus* is a frequent cause of urinary tract infections in humans and probably benefits in this regard from its ready ability to degrade urea by urease. Because of the rapid motility of *Proteus* cells, colonies growing on agar plates often exhibit a characteristic *swarming* phenotype (Figure 16.13*b*). Cells at the edge of the growing colony are more rapidly motile than those in the center of the colony. The former move a short distance away from the colony in a mass and then undergo a reduction in motility, settle down, and divide, forming a new population of motile cells that again swarm. As a result, the mature colony appears as a series of concentric rings, with higher concentrations of cells alternating with lower concentrations (Figure 16.13*b*).

Butanediol Fermenters: *Enterobacter*, *Klebsiella*, and *Serratia*

The butanediol fermenters are genetically more closely related to each other than to the mixed-acid fermenters, a finding that is in agreement with the observed physiological differences (Figure 16.12).



(a)

(b)

Figure 16.13 **Swarming in *Proteus*.** *(a)* Cells of *Proteus mirabilis* stained with a flagella stain; the peritrichous flagella of each cell form into a bundle to rotate in synchrony. *(b)* Photo of a swarming colony of *Proteus vulgaris*. Note the concentric rings.

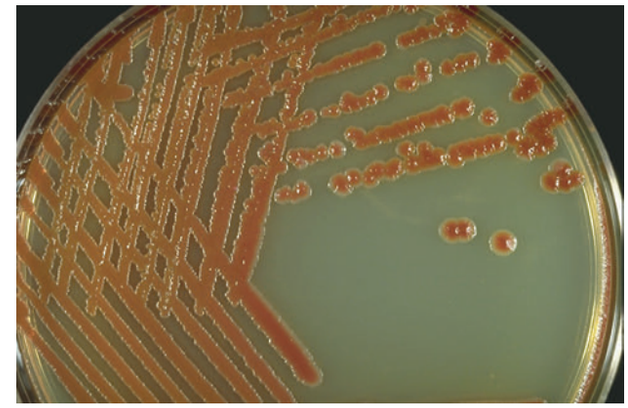


Figure 16.14 **Colonies of *Serratia marcescens*.** The orange-red pigmentation is due to the pyrrole-containing pigment prodigiosin.

Enterobacter aerogenes is a common species in water and sewage as well as the intestinal tract of warm-blooded animals and is an occasional cause of urinary tract infections. One species of *Klebsiella*, *K. pneumoniae*, occasionally causes pneumonia in humans, but klebsiellas are most commonly found in soil and water. Most *Klebsiella* strains also fix nitrogen (◀ Sections 3.12 and 15.9), a property not characteristic of other enteric bacteria.

The genus *Serratia* forms a series of red pyrrole-containing pigments called *prodigiosins* (**Figure 16.14**). Prodigiosin is produced in stationary phase as a secondary metabolite and is of interest because it contains the pyrrole ring also found in the pigments for energy transfer: porphyrins, chlorophylls, bacteriochlorophylls, and phycobilins (◀ Sections 14.3–14.5). However, it is unclear if prodigiosin plays any role in energy transfer, and its exact function is unknown. Species of *Serratia* can be isolated from water and soil as well as from the gut of various insects and vertebrates and occasionally from the intestines of humans. *Serratia marcescens* is also a human pathogen that can cause infections in many body sites. It has been implicated in infections caused by some invasive medical procedures and is an occasional contaminant in intravenous fluids.

Check Your Understanding

- What is a mixed-acid fermentation, and how does it differ from a butanediol fermentation?
- What characteristics would you use to distinguish between *Escherichia coli* and *Klebsiella pneumoniae*?

16.4 *Gammaproteobacteria*: *Pseudomonadales* and *Vibrionales*

KEY GENERA: *Aliivibrio, Pseudomonas, Vibrio*

The phylogenetic and metabolic diversity of the *Gammaproteobacteria* is remarkable (Figure 16.2 and ◀ Figure 15.1), making it difficult to select any particular species as characteristic of the class. We focus here on the *Pseudomonadales* and *Vibrionales*, since these groups (along with the *Enterobacteriales*) represent some of the most commonly encountered orders of *Gammaproteobacteria* (Figure 16.10).

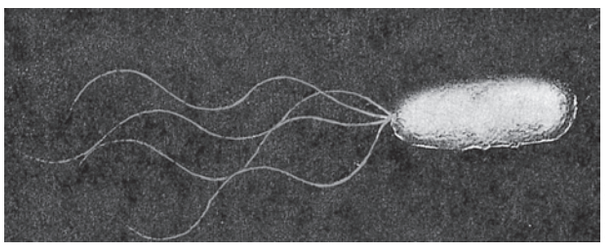
Pseudomonadales

The *Pseudomonadales* contain exclusively chemoorganotrophs that carry out respiratory metabolisms. All species can grow as aerobes and are typically oxidase- and catalase-positive, but some are also capable of anaerobic respiration with nitrate as the electron acceptor. Most species are able to use a wide diversity of organic compounds as sources of carbon and energy for growth. These organisms are ubiquitous in soil and aquatic systems, and many species cause diseases of plants and animals, including humans. The term **pseudomonad** is often used to describe any gram-negative, polarly flagellated, aerobic rod that is able to use diverse carbon sources. Pseudomonads can be found in several different groups of *Proteobacteria*, but here we consider only those organisms in the order *Pseudomonadales*. The type genus for this order is *Pseudomonas*.

Several species of *Pseudomonas* are pathogenic. Among these, *Pseudomonas aeruginosa* (**Figure 16.15**) is frequently associated with infections of the urinary and respiratory tracts in humans. *P. aeruginosa* is not an obligate pathogen. Instead, the organism is an opportunist, initiating infections in individuals with weakened immune systems. *P. aeruginosa* is a model organism used to study the development of microbial biofilms (◀ Sections 4.9 and 8.10). Due to its ability to readily colonize surfaces, *P. aeruginosa* is a common cause of hospital-acquired (nosocomial) infections from catheterizations, tracheostomies, lumbar punctures, and intravenous infusions. *P. aeruginosa* is also a common pathogen in patients receiving treatment for severe burns or other traumatic skin damage; it often infects patients undergoing prolonged treatment with immunosuppressive agents; and it often colonizes the lungs of people with cystic fibrosis. In addition to localized infections, *P. aeruginosa* can also cause systemic infections, usually in individuals who have experienced extensive skin damage.

P. aeruginosa is naturally resistant to many widely used antibiotics, so treatment of infections is often difficult. Resistance is typically due to a resistance transfer plasmid (R plasmid)(◀ Section 6.2 and ▶ Section 28.7), which is a plasmid whose genes encode proteins that detoxify various antibiotics or pump them out of the cell. Resistance to antibiotics is further enhanced by biofilm growth (◀ Section 8.10). Polymyxin, an antibiotic not ordinarily used in human therapy because of its toxicity, is effective against *P. aeruginosa* and is used in critical medical situations.

Certain species of *Pseudomonas*, such as *Pseudomonas syringae*, are well-known plant pathogens (phytopathogens). Phytopathogens frequently inhabit nonhost plants (in which disease symptoms are inapparent) and from there become transmitted to host plants and initiate infection. Disease symptoms vary considerably, depending on the particular phytopathogen and host plant. The pathogen releases plant toxins, lytic enzymes, plant growth factors, and other substances that destroy or distort plant tissue, releasing nutrients for use by the bacterium. In many cases the disease symptoms help identify the phytopathogen. Thus, *Pseudomonas syringae* is typically isolated from leaves showing chlorotic (yellowing) lesions, whereas *Pseudomonas marginalis*, a "soft-rot" pathogen, infects stems and shoots but rarely leaves.



Arthur Kelman

Figure 16.15 Cell morphology of pseudomonads. Shadow-cast transmission electron micrograph of a *Pseudomonas* cell. The cell measures about 1 μm in diameter.

Vibrionales

The *Vibrionales* contain facultatively aerobic rods and curved rods that employ a fermentative metabolism. One key difference between the *Vibrio* group and enteric bacteria is that *Vibrio* are oxidase-positive whereas enteric bacteria are oxidase-negative. Although *Pseudomonas* species are also oxidase-positive, they are not fermentative and so are clearly distinct from *Vibrio* species. The best-known genera in this group are *Vibrio*, *Aliivibrio*, and *Photobacterium*, which contain several species that are bioluminescent (▶ Section 23.10).

Most vibrios and related bacteria are aquatic, being found in marine, brackish, or freshwater habitats. *Vibrio cholerae* is the cause of the disease cholera in humans (▶ Sections 30.8 and 33.3); the organism does not normally cause disease in other hosts. Cholera is one of the most common human infectious diseases in developing countries and is transmitted almost exclusively via water.

Vibrio parahaemolyticus inhabits the marine environment and is a major cause of gastroenteritis in Japan, where raw fish is widely consumed; the organism has also been implicated in outbreaks of gastroenteritis in other parts of the world, including the United States. *V. parahaemolyticus* can be isolated from seawater itself or from shellfish and crustaceans, and its primary habitat is probably marine animals, with humans being an accidental host.

Check Your Understanding

- What species of *Pseudomonas* is a common cause of lung infection in cystic fibrosis patients? What properties of this species contribute to its pathogenesis?
- What major characteristic could be used to differentiate strains of *Pseudomonas* from those of *Vibrio*?

16.5 *Deltaproteobacteria* and *Epsilonproteobacteria*

These classes of *Proteobacteria* contain fewer species and less functional diversity than we have encountered in the *Alpha-*, *Beta-*, and *Gammaproteobacteria* (Figure 16.2 and ◀ Figure 15.1). The *Deltaproteobacteria* are primarily sulfate- and sulfur-reducing bacteria (◀ Sections 14.12, 15.11), dissimilative iron-reducers (◀ Section 15.13), and bacterial predators (◀ Section 15.16). *Epsilonproteobacteria*, by contrast, contain many species that oxidize the H_2S produced by the sulfate and sulfur reducers (◀ Sections 14.7, 15.12). The final class of *Proteobacteria*, the *Zetaproteobacteria*, contains only one characterized species (the iron oxidizer *Mariprofundus ferrooxydans*)and was considered earlier (◀ Section 15.14).

Deltaproteobacteria

KEY GENERA: *Bdellovibrio, Myxococcus, Desulfovibrio, Geobacter, Syntrophobacter*

Eight orders have been well characterized within the *Deltaproteobacteria* (**Figure 16.16**). The *Myxococcales* and *Bdellovibrionales* contain notable genera of bacterial predators (◀ Section 15.16). In contrast, the *Desulfuromonadales* contains diverse species of metal- and sulfur-reducing genera such as *Geobacter* (◀ Sections 14.13, 15.13). Indeed, like the *Desulfuromonadales*, many genera from the *Deltaproteobacteria* are associated with the reduction of sulfur compounds.

The largest and most common order containing sulfate reducers is the *Desulfovibrionales*. These organisms are readily cultivated from marine sediments and nutrient-rich anoxic environments that contain sulfate. Species of *Desulfovibrionales* are typically incomplete oxidizers (◀ Section 15.11). All use sulfate as the terminal electron acceptor, and all require small organic compounds such as lactate as a source of carbon and energy for growth. Species within the orders *Desulfobacterales* and *Desulfarculales* also typically reduce sulfate; however, in contrast to the *Desulfovibrionales*, these species can be complete or incomplete acetate oxidizers (◀ Section 15.11). In addition to sulfate, some species in these three orders can also reduce sulfite, thiosulfate, or nitrate, and some are capable of certain fermentations.

The final order containing sulfate reducers is the *Syntrophobacterales*. Some but not all species of the *Syntrophobacterales* are able to reduce sulfate. In nature, however, species of *Syntrophobacterales* primarily interact with H_2-consuming bacteria in a metabolic partnership called *syntrophy* (◀ Section 14.22). For example, syntrophic species such as *Syntrophobacter wolinii* oxidize propionate, producing acetate, CO_2, and H_2. However, such growth is only possible when a H_2-consuming partner is present. If sulfate is present, *S. wolinii* can grow as a sulfate reducer without the need for a partner. *S. wolinii* can also grow without a partner organism by fermenting pyruvate, fumarate, or malate.

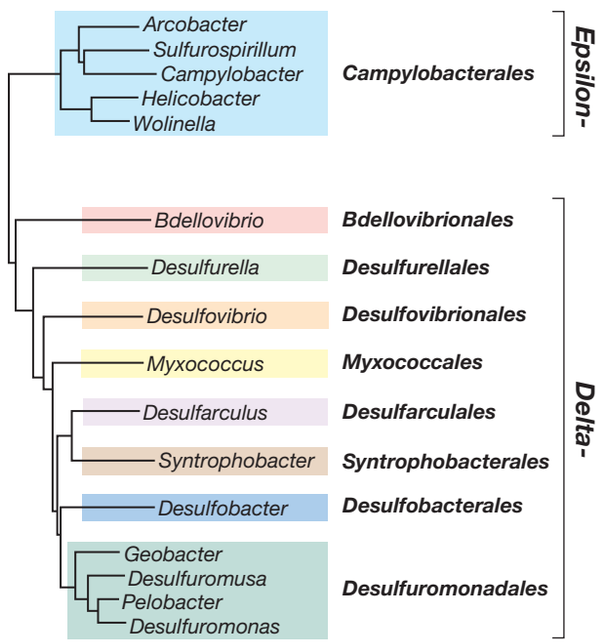


Figure 16.16 Major orders of *Proteobacteria* in the classes *Deltaproteobacteria* and *Epsilonproteobacteria*. The phylogenetic tree was constructed using 16S rRNA gene sequences from representative genera in the *Delta-* and *Epsilonproteobacteria*. Order names are shown in bold.

Epsilonproteobacteria

KEY GENERA: *Campylobacter, Helicobacter*

The *Epsilonproteobacteria* (Figure 16.16) were initially defined by pathogenic species of the genera *Campylobacter* and *Helicobacter*. While these pathogenic species remain the best characterized in the phylum, environmental studies of marine and terrestrial microbial habitats have shown that a diversity of *Epsilonproteobacteria* exist in nature. Nearly all of these environmental *Epsilonproteobacteria* metabolize sulfur compounds in one way or another, and chemolithotrophs and autotrophs are common. We will see that many *Epsilonproteobacteria* are associated with hosts as either pathogens or symbionts.

Campylobacter and *Helicobacter*

These two genera of *Epsilonproteobacteria* share a number of characteristics. *Campylobacter* and *Helicobacter* species are gram-negative, oxidase- and catalase-positive, motile spirilla, and most species are pathogenic to humans or other animals. These organisms are also microaerophilic (◀ Section 4.16) and must therefore be cultured from clinical specimens at low (3–15%) O_2 and high (3–10%) CO_2.

Campylobacter species, over a dozen of which have been described, cause acute gastroenteritis that typically results in a bloody diarrhea. Pathogenesis is due to several factors, including an enterotoxin that is related to cholera toxin. *Helicobacter pylori*, also a pathogen, causes both chronic and acute gastritis, leading to the formation of peptic ulcers.We consider these diseases, including their modes of transmission and clinical symptoms, in more detail in Section 31.10.

Sulfur-Metabolizing *Epsilonproteobacteria*

Through environmental sequencing studies (▶ Sections 19.6 and 19.8) and ongoing cultivation efforts, species of *Epsilonproteobacteria* are now recognized as ubiquitous in marine and terrestrial environments. These microbes are particularly abundant where sulfur-cycling activities are ongoing, particularly in deep-sea hydrothermal vent habitats and marine sediments where sulfide-rich and oxygenated waters mix (▶ Sections 20.15 and 20.16). Thermophilic species are common in this class of bacteria, contributing to their prevalence at hydrothermal systems (▶ Section 20.16). Chemolithotrophy and autotrophy (by the reverse TCA cycle, ◀ Section 14.2) are also widespread among *Epsilonproteobacteria*. Many species are able to grow aerobically or anaerobically (using oxidized nitrogen or sulfur compounds as electron acceptor) while using inorganic electron donors such as reduced sulfur compounds or H_2. *Epsilonproteobacteria* are especially abundant attached to surfaces at the oxic–anoxic interfaces in sulfur-rich environments and play major roles in the oxidation of sulfur compounds in nature.

The ability of *Epsilonproteobacteria* to fix CO_2 is of particular importance to many animals that live in sulfur-rich environments. For example, *Epsilonproteobacteria* can account for up to 85% of the microbial biomass growing on hydrothermal vent chimneys (▶ Section 20.16 and Figure 20.44). In addition,

Epsilonproteobacteria grow as ectosymbionts and endosymbionts of many animals such as oligochaete and polychaete worms, snails, and even shrimp. These symbionts can play two roles for the host, both providing a source of nutrition and helping to detoxify H_2S that would otherwise be deleterious to the hosts (▶ Section 23.11). Further exploration of the phylogeny, metabolic activities, and ecological roles of *Epsilonproteobacteria* will likely uncover exciting new aspects of prokaryotic diversity.

Check Your Understanding

- What four metabolic traits are most common in species of *Deltaproteobacteria*?
- What are some characteristic metabolic traits you would expect to find in *Epsilonproteobacteria* living at a hydrothermal vent?

II • *Firmicutes, Tenericutes,* and *Actinobacteria*

The *Firmicutes* and *Actinobacteria* contain gram-positive *Bacteria* and include many well-characterized bacteria. The *Tenericutes* include species such as *Mycoplasma* that have lost the ability to make peptidoglycan and a cell wall of any kind.

We continue our tour of phylogenetic bacterial diversity with the gram-positive bacteria of the phyla *Actinobacteria* and *Firmicutes*, and the closely related phylum *Tenericutes* (**Figure 16.17**). These three phyla contain nearly half of all characterized species of *Bacteria* (Figure 16.1*b*).

The *Actinobacteria* include the actinomycetes, a huge group of primarily filamentous soil bacteria. One distinguishing feature of the *Actinobacteria* is that their genomic DNA typically has a high frequency of GC base pairs, and as a result they are also called the **high G + C gram-positive bacteria**. The *Tenericutes* include cells that lack a cell wall, and the *Firmicutes* include the endospore-forming bacteria, lactic acid bacteria, and several other groups. In contrast to the *Actinobacteria*, the genomes of *Firmicutes* generally have a low GC content, and as a result, they are also called the **low G + C gram-positive bacteria**.

We begin by examining *Firmicutes* that do not form endospores.

16.6 *Firmicutes: Lactobacillales*

KEY GENERA: *Lactobacillus, Streptococcus*

The order *Lactobacillales* contains the **lactic acid bacteria**, fermentative organisms that produce lactic acid as a major end product of metabolism. These organisms are used widely in food production and preservation (◀ Section 1.6). Lactic acid bacteria are nonsporulating, oxidase- and catalase-negative rods or cocci that show an exclusively fermentative metabolism. All lactic acid bacteria produce lactic acid as a major or sole fermentation product (◀ Sections 3.7, 14.18). Members of this group lack porphyrins and cytochromes; thus they do not carry out oxidative phosphorylation and obtain energy only by substrate-level phosphorylation. However, unlike many anaerobes, most lactic acid bacteria are not sensitive to oxygen (O_2) and can grow in its presence; thus they are called *aerotolerant anaerobes* (◀ Section 4.16).

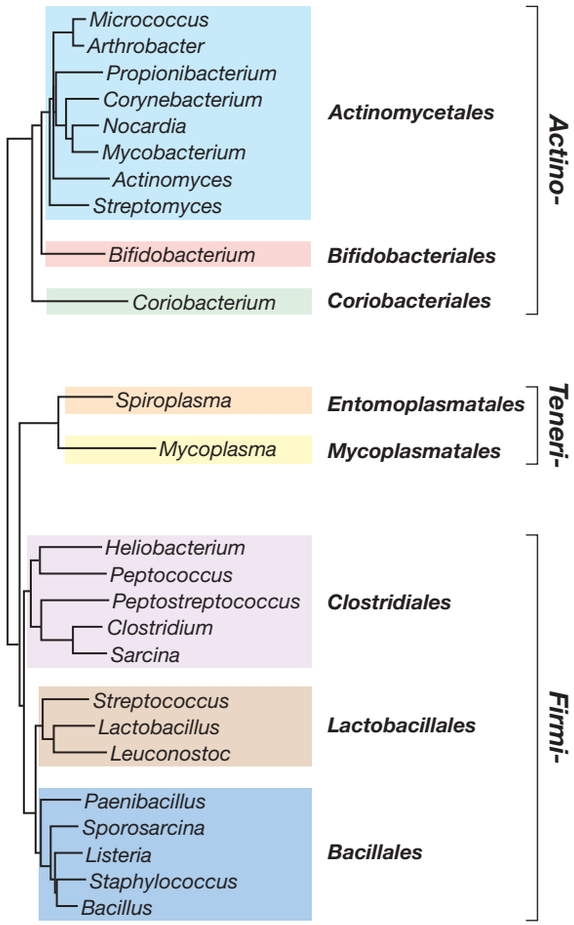


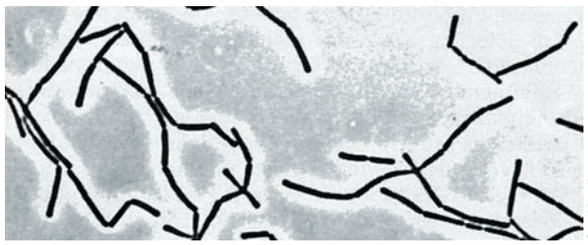
Figure 16.17 Major orders of gram-positive bacteria and relatives. The phylogenetic tree was constructed from 16S rRNA gene sequences of representative genera of *Actinobacteria*, *Firmicutes*, and *Tenericutes*. Order names are shown in bold.

Most lactic acid bacteria obtain energy only from the metabolism of sugars and therefore are usually restricted to habitats in which sugars are present. They typically have limited biosynthetic abilities, and their complex nutritional requirements include needs for amino acids, vitamins, purines, and pyrimidines (for example, ◀ Table 4.2 for *Leuconostoc mesenteroides*). One important difference between subgroups of the lactic acid bacteria lies in the pattern of products formed from the fermentation of sugars. One group, called **homofermentative**, produces a single fermentation product, *lactic acid*. The other group, called **heterofermentative**, produces other products, mainly ethanol and CO_2, as well as lactate (◀ Section 14.18 and Figure 14.44).

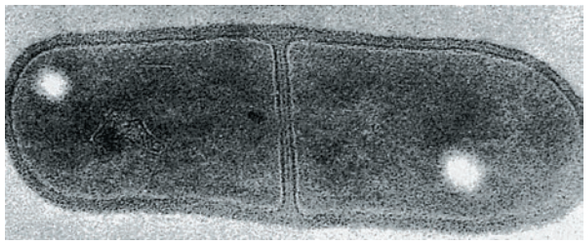
Mastering Microbiology
Art Activity: Figure 16.17 Major orders of gram-positive bacteria and relatives

Lactobacillus

Lactobacilli are typically rod-shaped and grow in chains, varying from long and slender to short, bent rods (**Figure 16.18**), and most are homofermentative. Lactobacilli are common in dairy products, and some strains are used in the preparation of fermented milk products. For instance, *Lactobacillus acidophilus* (Figure 16.18*a*) is used in the production of acidophilus milk; *Lactobacillus delbrueckii* (Figure 16.18*c*) is used in the preparation of yogurt; and other species are used in the production of sauerkraut, silage, and pickles (▶ Section 33.6).



(a)



(b)

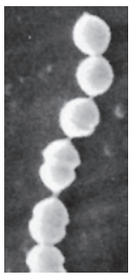
(c)

Figure 16.18 ***Lactobacillus*** **species.** *(a) Lactobacillus acidophilus,* phase-contrast. Cells are about 0.75 μm wide. *(b) Lactobacillus brevis,* transmission electron micrograph. Cells measure about 0.8 × 2 μm. *(c) Lactobacillus delbrueckii,* scanning electron micrograph. Cells are about 0.7 μm in diameter.

Lactobacilli are typically more resistant to acidic conditions than are other lactic acid bacteria and are able to grow well at pH values as low as 4. Because of this, they can be selectively enriched from dairy products and fermenting plant material on acidic carbohydrate-containing media. The acid resistance of the lactobacilli enables them to continue growing during natural lactic fermentations, even when the pH value has dropped too low for other lactic acid bacteria to grow. The lactobacilli are therefore typically responsible for the final stages of most lactic acid fermentations. They are rarely, if ever, pathogenic.

Streptococcus and Other Cocci

The genera *Lactococcus* and *Streptococcus* (**Figure 16.19**) contain homofermentative species of coccoid-shaped lactic acid bacteria with quite distinct habitats and activities that are of considerable practical importance to humans. Some species are pathogenic to humans and animals (▶ Section 31.2). *Streptococcus* species (Figure 16.19*a*) have a characteristic cell morphology of cocci in chains or tetrads and so are readily resolved from the rod-shaped lactobacilli. As producers of lactic acid, other streptococci play important roles in the production of buttermilk, silage, and other fermented products (▶ Section 33.6), and certain species play a major role in the formation of dental caries (▶ Sections 24.3 and 25.1).



(a)

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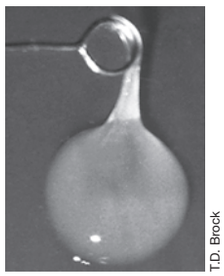


(c)

Figure 16.19 **Gram-positive cocci.** *(a) Streptococcus* sp., scanning electron micrograph. *(b) Lactococcus lactis,* phase-contrast micrograph. Cells in both photos are 0.5–1 μm in diameter. *(c)* Colony of *Leuconostoc mesenteroides* showing the extensive dextran slime produced by cells grown on sucrose.

There are several other genera of homofermentative cocci. The genus *Lactococcus* (Figure 16.19*b*) contains those streptococci of dairy significance, whereas the genus *Enterococcus* includes streptococci that are primarily of fecal origin and can be human pathogens. Species of the genera *Peptococcus* and *Peptostreptococcus* are obligate anaerobes that ferment proteins rather than sugars.

Streptococci have been divided into two groups of related species: the *pyogenes subgroup*, characterized by *Streptococcus pyogenes*, the cause of strep throat (▶ Section 31.2), and the *viridans subgroup*, characterized by *Streptococcus mutans*, the major cause of dental caries (▶ Sections 24.3 and 25.1). Hemolysis on blood agar is of considerable importance in the subdivision of the genus into species. For example, species that produce the virulence factors streptolysin O or S form colonies surrounded by a large zone of complete red blood cell hemolysis when plated on blood agar, a condition called *β-hemolysis* (▶ Figures 25.17*a*, 31.4*b*, and 31.8). β-hemolysis is diagnostic for streptococci in the pyogenes subgroup. In contrast, streptococci in the viridans subgroup cause incomplete hemolysis on blood agar, a condition that leads to greening of the agar under colonies. Streptococci are also divided into immunological groups (designated by the letters A, B, C, F, G), based on the presence of specific carbohydrate antigens (antigens are substances that elicit an immune response). Those β-hemolytic streptococci found in humans usually contain the group A antigen, whereas enterococci contain the group D antigen.

Heterofermentative lactococci reside in the genus *Leuconostoc*. Strains of *Leuconostoc* also produce the flavoring ingredients diacetyl and acetoin from the catabolism of citrate; they have been used as starter cultures in dairy fermentations. Some strains of *Leuconostoc* produce large amounts of glucose or fructose polysaccharide slimes, especially when cultured on sucrose as the carbon and energy source (Figure 16.19*c*), and some of these polymers have found medical use as plasma extenders in blood transfusions.

Check Your Understanding

- How do heterofermentative and homofermentative bacteria differ physiologically?
- How can *Streptococcus pyogenes* be distinguished from *Streptococcus mutans*?

16.7 *Firmicutes:* Nonsporulating *Bacillales* and *Clostridiales*

KEY GENERA: *Listeria, Staphylococcus, Sarcina*

Firmicutes that form endospores reside in the orders *Bacillales* and *Clostridiales*. However, numerous *Bacillales* and *Clostridiales* are unable to form endospores, and we consider some of these here.

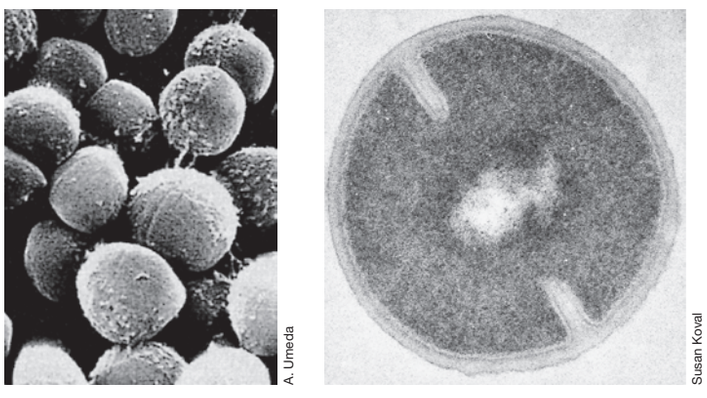
Listeria

The order *Bacillales* typically contains aerobic and facultatively aerobic chemoorganotrophs. Members of this group are widespread and particularly common in soils. For example, *Listeria* is found widely in soils and is an opportunistic pathogen and a common cause of foodborne illness. *Listeria* are gram-positive, catalase-positive, rod-shaped, facultatively aerobic chemoorganotrophs. Although several species of *Listeria* are known, the species *Listeria monocytogenes* is most noteworthy because it causes a major foodborne illness, *listeriosis* (▶ Section 33.13). The organism is transmitted in contaminated, usually ready-to-eat foods such as cheese and sausages and can cause anything from a mild illness to a fatal form of meningitis. Species of *Listeria* often grow well at low temperatures, allowing growth in refrigerated foods.

Staphylococcus

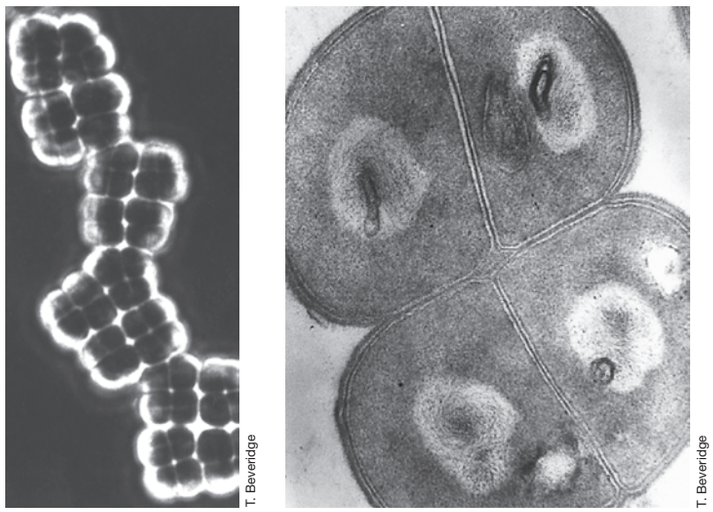
Staphylococcus (**Figure 16.20**) is a facultative aerobe that shows a typical respiratory metabolism but can also grow fermentatively. Cells typically grow in clusters and produce acid from glucose both aerobically and anaerobically. *Staphylococcus* species are catalase-positive, and this permits their distinction from *Streptococcus* and some other genera of lactic acid bacteria. Staphylococci are relatively resistant to reduced water potential and tolerate drying and high salt (NaCl) fairly well. Their ability to grow in media containing salt provides a selective means for isolation. For example, if an appropriate inoculum such as a skin swab, dry soil, or room dust is spread on a rich-medium agar plate containing 7.5% NaCl and the plate is incubated aerobically, gram-positive cocci often form the predominant colonies. Many species are pigmented, and this provides an additional aid in selecting gram-positive cocci.

Staphylococci are common commensals and parasites of humans and animals, and they occasionally cause serious infections. In humans, there are two major species, *Staphylococcus epidermidis*, a nonpigmented, nonpathogenic organism usually found on the skin or mucous membranes, and *Staphylococcus aureus* (Figure 16.20), a yellow-pigmented species that is most commonly associated with pathological conditions including boils, pimples, pneumonia, osteomyelitis, meningitis, and arthritis. Some *S. aureus* strains are resistant to multiple antibiotics (so-called *MRSA* strains) and are fierce pathogens that can cause extensive tissue damage (▶ Figure 31.9). We discuss the pathogenesis of MRSA and other strains of *S. aureus* and staphylococcal diseases in Sections 24.5, 29.2, and 31.9.



(a) (b)

Figure 16.20 ***Staphylococcus.*** *(a)* Scanning electron micrograph of typical *Staphylococcus aureus* cells, showing the irregular arrangement of the cell clusters. Individual cells are about 0.8 μm in diameter. *(b)* Transmission electron micrograph of a dividing cell of *S. aureus*. Note the thick gram-positive cell wall.



(a) (b)

Figure 16.21 ***Sarcina.*** *(a)* Phase-contrast photomicrograph of cells of a typical gram-positive coccus *Sarcina*. A single cell is about 2 μm in diameter. *(b)* Electron micrograph of a thin section from *Sarcina ventriculi*. The outermost layer of the cell consists of cellulose.

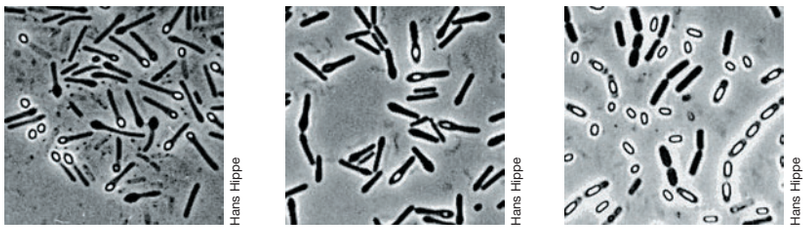
Sarcina

The genus *Sarcina* contains obligate anaerobes that are catalase-negative within the order *Clostridiales*. *Sarcina* species divide in three perpendicular planes to yield packets of eight or more cells and are notable for this morphology (**Figure 16.21**). *Sarcina* are also extremely acid-tolerant, being able to ferment sugars and grow in environments at a pH as low as 2. Cells of one species, *Sarcina ventriculi*, contain a thick, fibrous layer of cellulose surrounding the cell wall (Figure 16.21*b*). The cellulose layers of adjacent cells become attached, and this functions as a cementing material to hold together packets of *S. ventriculi* cells.

Sarcina species can be isolated from soil, mud, feces, and stomach contents. Because of its extreme acid tolerance, *S. ventriculi* is one of only a few bacteria that can inhabit and grow in the stomach of humans and other monogastric animals. Rapid growth of *S. ventriculi* is observed in the stomach of humans suffering from certain gastrointestinal disorders, such as pyloric ulcerations. These pathological conditions retard the flow of food to the intestine and often require surgery to correct.

Check Your Understanding

- How could species of *Staphylococcus* be differentiated from *Streptococcus*?
- What characteristics differentiate *Sarcina* from *Staphylococcus*?



(a) (b) (c)

Figure 16.22 ***Clostridium*** **species and endospore location.** *(a) Clostridium cadaveris*, terminal endospores. Cells are about 0.9 μm wide. *(b) Clostridium sporogenes,* subterminal endospores. Cells are about 1 μm wide. *(c) Clostridium bifermentans,* central endospores. Cells are about 1.2 μm wide. All are phase-contrast micrographs.

16.8 *Firmicutes*: Sporulating *Bacillales* and *Clostridiales*

KEY GENERA: ***Bacillus, Clostridium, Sporosarcina***

All endospore-forming bacteria are gram-positive species of *Bacillales* or *Clostridiales*. The ability to form endospores evolved only once in a common ancestor of the *Bacillales*, *Clostridiales*, and *Lactobacillales* (Figure 16.17). However, many *Bacillales* and *Clostridiales* and the entire order *Lactobacillales* are unable to form endospores. The capacity to make endospores requires many genes (◀ Sections 2.8 and 8.6) and has not been acquired by horizontal gene transfer. It thus appears that the phylogenetic distribution of endospores has seen many cases where the capacity to form endospores has been lost during the course of evolution.

Endospore-forming bacteria are distinguished on the basis of cell morphology, shape and cellular position of the endospore (**Figure 16.22**), relationship to O_2, and energy metabolism. The two genera about which most is known are *Bacillus*, species of which are aerobic or facultatively aerobic, and *Clostridium*, which contains species that are obligately anaerobic and fermentative. All endospore-forming bacteria are ecologically related because they are found in nature primarily in soil. Even those species that are pathogenic to humans or other animals are primarily saprophytic soil organisms and infect animals only incidentally. Indeed, the ability to produce endospores should be advantageous for a soil microorganism because soil is a highly variable environment in terms of nutrient levels, temperature, and water activity.

Endospore-forming bacteria can be selectively isolated from soil, food, dust, and other materials by heating the sample to 80°C for 10 min, a treatment that effectively kills vegetative cells while any endospores present remain viable. Streaking such heat-treated samples on plates of the appropriate medium and incubating either aerobically or anaerobically selectively yields species of *Bacillus* or *Clostridium*, respectively.

Bacillus and *Paenibacillus*

Species of *Bacillus* and *Paenibacillus* grow well on defined media containing any of a number of carbon sources. Many bacilli produce extracellular hydrolytic enzymes that break down complex polymers such as polysaccharides, nucleic acids, and lipids, permitting the organisms to use these products as carbon sources and electron donors. Many bacilli produce antibiotics, including bacitracin, polymyxin, tyrocidine, gramicidin, and circulin. In most cases the antibiotics are released when the culture enters the stationary phase of growth and is committed to sporulation.

Several bacilli, most notably *Paenibacillus popilliae* and *Bacillus thuringiensis*, produce toxic insecticidal proteins. *P. popilliae* causes a fatal condition called milky disease in Japanese beetle larvae and larvae of closely related beetles of the family *Scarabaeidae*. *B. thuringiensis* causes a fatal disease of many different groups of insects. Both of these insect pathogens form a crystalline protein during sporulation called the *parasporal body*, which is deposited within the sporangium but outside the endospore proper (**Figure 16.23**). In *B. thuringiensis*, the parasporal body is a protoxin that is converted to a toxin in the insect gut. The toxin binds to specific receptors in the intestinal epithelial cells of certain insects and induces pore formation that causes leakage of the host cell cytoplasm followed by lysis. Diverse strains of *B. thuringiensis* can make different types of toxin that have specificity for different groups of insects. Endospore preparations derived from *B. thuringiensis* and *P. popilliae* are commercially available as biological insecticides.

The *cry* genes that encode crystal proteins have been isolated from several *B. thuringiensis* strains. The genes for the *B. thuringiensis* crystal protein (known commercially as "Bt toxin") have been introduced into genetically modified crops (e.g., maize, soybeans, and cotton) to render the plants resistant to insects. These genetically modified "Bt crops" are used widely around the world.

Clostridium

Clostridia lack a respiratory chain, unlike *Bacillus* species, and so they obtain ATP by substrate-level phosphorylation. Many anaerobic energy-yielding mechanisms are known in the clostridia (◀ Section 14.19). Indeed, the separation of the genus *Clostridium* into subgroups is based primarily on these properties and on the fermentable substrate used. A number of clostridia are *saccharolytic* and ferment sugars, producing butyric acid as a major end product. Some of these also produce acetone and butanol, such as *Clostridium pasteurianum*, which is also a vigorous nitrogen-fixing bacterium (◀ Sections 3.12 and 15.9).

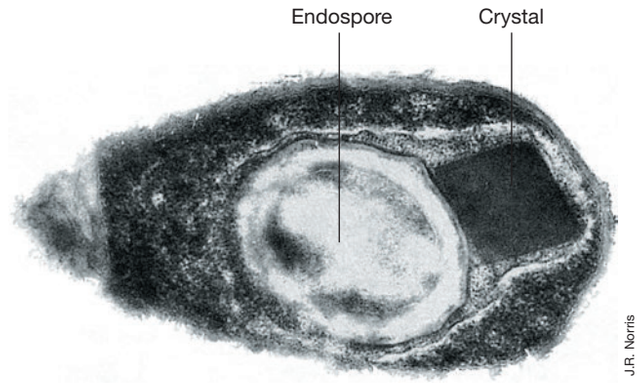


Figure 16.23 **The toxic parasporal crystal in the insect pathogen** ***Bacillus thuringiensis.*** Electron micrograph of a thin section of a sporulating cell. The crystalline protein (Bt toxin) is toxic to certain insects by causing lysis of their intestinal cells.

One group of clostridia including the species *C. thermocellum*, *C. cellulolyticum*, and *C. cellulovorans* ferments cellulose with the formation of acids and alcohols. These species are likely the major organisms decomposing cellulose in anoxic environments such as the rumen and sediments. Cellulolytic clostridia possess *cellulosomes*, complex multienzyme structures found on the outer surface of the cell wall. The cellulosome binds insoluble cellulose and degrades it into soluble products that are transported into the cytoplasm and metabolized by the cell. This cellulosome mechanism is common to bacteria that are able to degrade cellulose anaerobically.

Another group of clostridia are *proteolytic* and conserve energy from the fermentation of amino acids. Some species ferment individual amino acids, but others ferment only amino acid pairs. The products of amino acid fermentation are typically acetate, butyrate, CO_2, and H_2. The coupled catabolism of an amino acid pair is called a *Stickland reaction*; for example, *Clostridium sporogenes* ferments glycine plus alanine. In the Stickland reaction, one amino acid functions as the electron donor and is oxidized, whereas the other is the electron acceptor and is reduced (◀ Figure 14.47). Many of the products of amino acid fermentation by clostridia are foul-smelling substances, and the odor that results from putrefaction is mainly the result of clostridial action. In addition to butyric acid, other odoriferous compounds produced are isobutyric acid, isovaleric acid, caproic acid, hydrogen sulfide, methyl mercaptan (from sulfur amino acids), cadaverine (from lysine), putrescine (from ornithine), and ammonia.

The main habitat of clostridia is the soil, where they live primarily in anoxic "pockets" made free of O_2 by the respiratory activities of facultative or obligately aerobic bacteria. In addition, a number of clostridia inhabit the anoxic environment of the mammalian intestinal tract. Several clostridia are capable of causing severe diseases in humans, as will be discussed in Sections 24.9, 30.7, 32.9 and 33.9. For example, botulism is caused by *Clostridium botulinum*, tetanus by *Clostridium tetani*, and gas gangrene by *Clostridium perfringens* and a number of other clostridia, both sugar and amino acid fermenters. These pathogenic clostridia seem in no way unusual metabolically but are distinct in that they produce specific toxins or, in those causing gas gangrene, a group of toxins. *C. perfringens* and related species can also cause gastroenteritis in humans and domestic animals (▶ Section 33.9), and botulism outbreaks are not uncommon in birds such as ducks and a variety of other animals.

Sporosarcina

The genus *Sporosarcina* (**Figure 16.24**) is unusual among endospore formers because cells are cocci instead of rods. *Sporosarcina* consists of strictly aerobic spherical to oval cells that divide in two or three perpendicular planes to form tetrads or packets of eight or more cells. The major species is *Sporosarcina ureae*. This bacterium can be enriched from soil by plating dilutions of a pasteurized soil sample on alkaline nutrient agar supplemented with 8% urea and incubating in air. Most soil bacteria are strongly inhibited by as little as 2% urea. However, *S. ureae* tolerates this, catabolizing urea to CO_2 and ammonia (NH_3), which dramatically raises the pH. *S. ureae* is remarkably alkaline-tolerant and can be grown in media up to pH 10, and this feature can be used to advantage in its enrichment from soil.

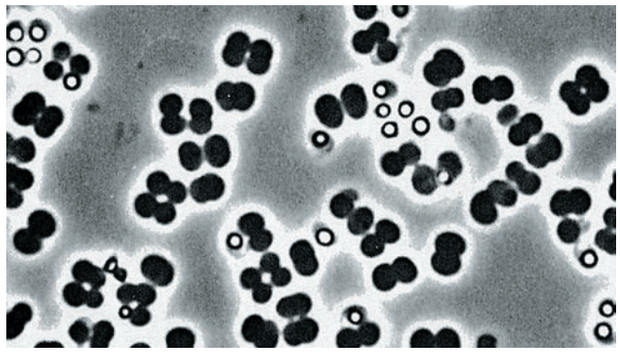


Figure 16.24 ***Sporosarcina ureae.*** Phase-contrast micrograph. A single cell is about 2 μm wide. Note bright refractile endospores. Most cell packets contain eight cells.

Check Your Understanding

- What is the major physiological distinction between *Bacillus* and *Clostridium* species?
- What is the crystalline protein made by *Bacillus thuringiensis* and what is its significance to agriculture?

16.9 *Tenericutes:* The Mycoplasmas

KEY GENERA: *Mycoplasma, Spiroplasma*

The *Tenericutes*, which contain the single class *Mollicutes*, are bacteria that lack cell walls (*mollis* is Latin for "soft") and are some of the smallest organisms known. This group is often called the *mycoplasmas* because *Mycoplasma*, a notable genus containing human pathogens, is the best-characterized genus in the phylum.

Although they do not stain gram-positively (because they lack cell walls), mycoplasmas are phylogenetically related to the *Firmicutes*. Mycoplasmas typically live within animal and plant hosts and this may eliminate the need for a gram-positive cell wall. These organisms also have small genomes (ranging in size from 0.6 to 2.2 megabase pairs [Mbp]), a characteristic common in obligate symbionts and intracellular pathogens (◀ Section 10.3 and ▶ Section 23.7).

Properties of Mycoplasmas

The absence of cell walls in mycoplasmas has been confirmed by electron microscopy and chemical analyses, which show that peptidoglycan is absent. Mycoplasmas resemble protoplasts (bacteria treated to remove their cell walls), but they are more resistant to osmotic lysis and are able to survive conditions under which protoplasts lyse. This ability to resist osmotic lysis is at least partially determined by the presence of sterols, which make the cytoplasmic membrane of mycoplasmas more stable than that of other bacteria. Indeed, some mycoplasmas require sterols in their growth media, and this sterol requirement can aid in the classification of mycoplasmas.

In addition to sterols, certain mycoplasmas contain compounds called *lipoglycans*. Lipoglycans are long-chain heteropolysaccharides covalently linked to membrane lipids and embedded in the cytoplasmic membrane of many mycoplasmas. Lipoglycans in some ways resemble the lipopolysaccharides in the outer membrane of gram-negative bacteria, except that they lack the lipid A backbone (◀ Section 2.4). Lipoglycans function to help stabilize the

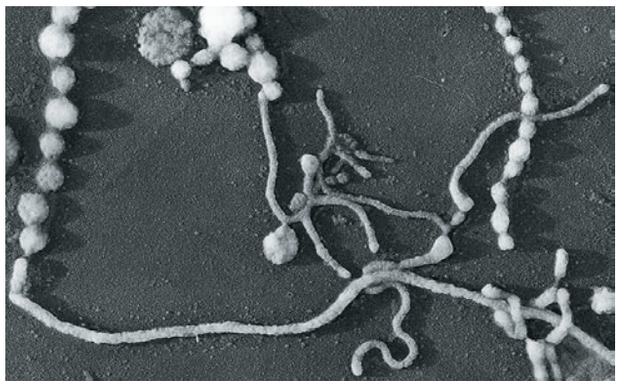


Figure 16.25 ***Mycoplasma mycoides.*** Metal-shadowed transmission electron micrograph. Note the coccoid and hyphae-like elements. The average diameter of cells in chains is about 0.5 μm.

cytoplasmic membrane and have also been identified as facilitating attachment of mycoplasmas to cell surface receptors of animal cells.

Growth of Mycoplasmas

Mycoplasmas can be grown in the laboratory and are small and pleomorphic cells. A single culture may exhibit small coccoid elements; larger, swollen forms; and filamentous forms, often highly branched (**Figure 16.25**). The small coccoid elements (0.2–0.3 μm in size) are among the smallest of free-living cells. The mode of growth of mycoplasmas differs in liquid and agar cultures. On agar the organisms tend to grow so that they become embedded in the medium. These colonies show a characteristic "fried-egg" appearance consisting of a dense central core that penetrates downward into the agar, surrounded by a circular spreading area that is lighter in color (**Figure 16.26**). As would be expected of cells lacking cell walls, growth of *Mollicutes* is not inhibited by antibiotics that inhibit cell wall synthesis. However, mycoplasmas are as sensitive as most *Bacteria* to antibiotics whose targets are other than the cell wall.

Media for the culture of mycoplasmas are typically quite complex. For many species, growth is poor or absent even in complex yeast extract–peptone–beef heart infusion media. Fresh serum or ascitic fluid (peritoneal fluid) is needed as well to provide unsaturated fatty acids and sterols. Some mycoplasmas can be cultivated on relatively simple culture media, however, and even defined media have been developed for some species. Most mycoplasmas use carbohydrates as carbon and energy sources and require vitamins, amino acids, purines, and pyrimidines as growth factors.

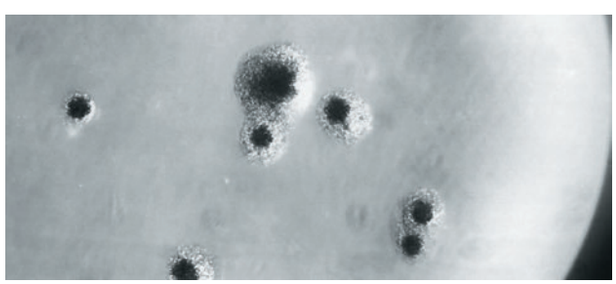


Figure 16.26 **Colonies of a *Mycoplasma* species on agar.** Note the typical "fried-egg" appearance. The colonies are about 0.5 mm in diameter.

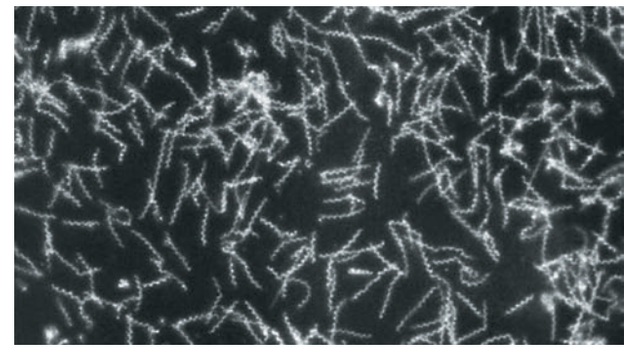


Figure 16.27 **"Sex-ratio" spiroplasma from the hemolymph of the fly *Drosophila pseudoobscura*.** Dark-field micrograph. Female flies infected with the sex-ratio spiroplasma bear only female progeny. Cells are about 0.15 μm in diameter.

Spiroplasma

The genus *Spiroplasma* consists of helical or spiral-shaped *Mollicutes*. Amazingly, although they lack a cell wall and flagella, spiroplasmas are motile by means of a rotary (screw) motion or a slow undulation. Intracellular fibrils that are thought to play a role in motility have been demonstrated. The organism has been isolated from ticks, the hemolymph (**Figure 16.27**) and gut of insects, vascular plant fluids and insects that feed on these fluids, and the surfaces of flowers and other plant parts. For example, *Spiroplasma citri* has been isolated from the leaves of citrus plants, where it causes a disease called citrus stubborn disease, and from corn plants suffering from corn stunt disease. A number of other mycoplasma-like organisms have been detected in diseased plants by electron microscopy, which indicates that a large group of plant-associated *Mollicutes* may exist. Some species of *Spiroplasma* are known that cause insect diseases, such as honeybee spiroplasmosis and lethargy disease of the beetle *Melolontha*.

Check Your Understanding

- Why do mycoplasmas need to have stronger cytoplasmic membranes than other bacteria?
- Motile spiroplasmas cannot contain a normal bacterial flagellum; why?

16.10 *Actinobacteria:* Coryneform and Propionic Acid Bacteria

KEY GENERA: ***Arthrobacter, Corynebacterium, Propionibacterium***

The other major group of gram-positive bacteria is the *Actinobacteria*, which form their own phylum within the *Bacteria*. The *Actinobacteria* contain rod-shaped to filamentous and primarily aerobic bacteria that are common inhabitants of soil and plant materials. For the most part they are harmless commensals, species of *Mycobacterium* (for example, *Mycobacterium tuberculosis*) being notable exceptions. Some are of great economic value in either the production of antibiotics or certain fermented dairy products. While there are nine orders of *Actinobacteria*, the vast majority of species belong to the order *Actinomycetales* (Figure 16.17). We consider here the coryneform bacteria, species of *Actinomycetales* that have an unusual method of cell division, and the propionic acid bacteria, important agents in the ripening of Swiss cheese.

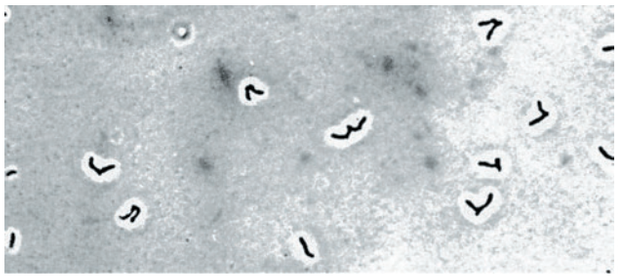


Figure 16.28 Snapping division in *Arthrobacter*. Phase-contrast micrograph of characteristic V-shaped cell groups in *Arthrobacter crystallopoietes* resulting from snapping division. Cells are about 0.9 μm in diameter.

Coryneform Bacteria

Coryneform bacteria are gram-positive, aerobic, nonmotile, rod-shaped organisms that form irregular-shaped, club-shaped, or V-shaped cell arrangements during growth. V-shaped cells arise as a result of an abrupt movement that occurs just after cell division, a process called *snapping division* (**Figure 16.28**). Snapping division occurs because the cell wall consists of two layers; only the inner layer participates in cross-wall formation, and so after the cross-wall is formed, the two daughter cells remain attached by the outer layer of the cell wall. Localized rupture of this outer layer on only one side of the cell results in a bending of the two cells away from the ruptured side (**Figure 16.29**) and thus development of V-shaped forms.

The main genera of coryneform bacteria are *Corynebacterium* and *Arthrobacter*. The genus *Corynebacterium* consists of an extremely diverse group of bacteria, including animal and plant pathogens and saprophytes. Some species, such as *Corynebacterium diphtheriae*, are pathogenic (diphtheria, ▶ Section 31.3). The genus *Arthrobacter*, consisting primarily of soil organisms, is distinguished from *Corynebacterium* on the basis of a developmental cycle during which it changes morphology between rod and coccus (**Figure 16.30**). *Corynebacterium* cells frequently have a swollen end, so they have a club-shaped appearance, whereas *Arthrobacter* species are less commonly club-shaped.

Along with the *Acidobacteria* (Section 16.21), species of *Arthrobacter* are among the most common of all soil bacteria. They are remarkably resistant to desiccation and starvation, despite the fact that they do not form spores or other resting cells. Arthrobacters are a heterogeneous group that have considerable nutritional versatility, and strains have been isolated that decompose herbicides, caffeine, nicotine, phenols, and other unusual organic compounds.

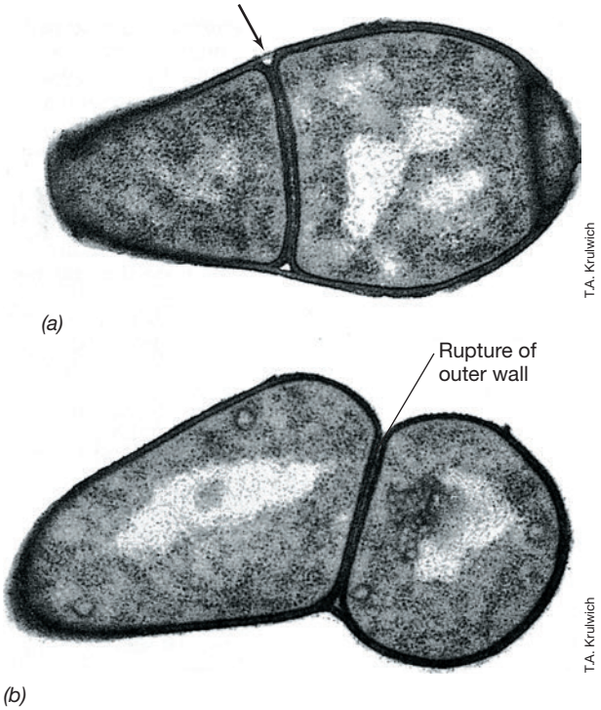


Figure 16.29 Cell division in *Arthrobacter*. Transmission electron micrograph of cell division in *Arthrobacter crystallopoietes*, illustrating how snapping division and V-shaped cell groups arise. *(a)* Before rupture of the outer cell wall layer (arrow). *(b)* After rupture of the outer layer on one side. Cells are 0.9–1 μm in diameter.

UNIT 4

Propionic Acid Bacteria

The **propionic acid bacteria** (genus *Propionibacterium*) were first discovered in Swiss (Emmentaler) cheese, where their fermentative production of CO_2 produces the characteristic holes. In addition, the propionic acid they produce is at least partly responsible for the unique flavor of the cheese. The bacteria in this group are gram-positive anaerobes that ferment lactic acid, carbohydrates, and polyhydroxy alcohols, producing primarily propionic acid, acetic acid, and CO_2 (◀ Section 14.20).

The fermentation of lactate is of interest because lactate itself is a fermentation product of many bacteria (Section 16.6). The starter culture in Swiss cheese manufacture consists of a mixture of homofermentative streptococci and lactobacilli, plus propionic acid bacteria. The homofermentative organisms carry out the initial fermentation of lactose to lactic acid during formation of the curd

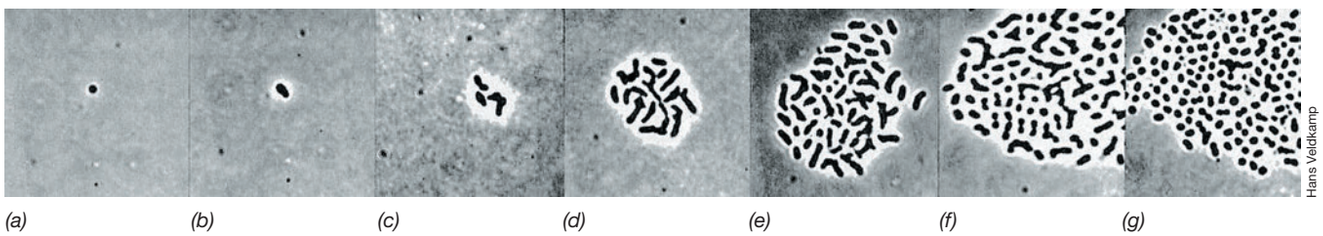


Figure 16.30 Stages in the life cycle of *Arthrobacter globiformis* as observed in slide culture. *(a)* Single coccoid element; *(b–e)* conversion to rod and growth of a microcolony consisting predominantly of rods; *(f–g)* conversion of rods to coccoid forms. Cells are about 0.9 μm in diameter.

(protein and fat). After the curd has been drained, the propionic acid bacteria develop rapidly. The eyes (or holes) characteristic of Swiss cheese are formed by the accumulation of CO_2, the gas diffusing through the curd and gathering at weak points. The propionic acid bacteria are thus able to obtain energy anaerobically from a product that other bacteria have produced by fermentation. This metabolic strategy is called a *secondary fermentation*.

Propionate is also formed in the fermentation of succinate by the bacterium *Propionigenium*. This organism is phylogenetically and ecologically unrelated to *Propionibacterium*, but energetic aspects of its fermentation are of considerable interest.We discussed the mechanism of the *Propionigenium* fermentation in Section 14.20.

Check Your Understanding

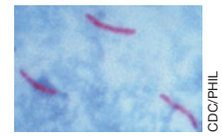
- What is snapping division and what organism exhibits it?
- What organism is involved in the production of Swiss cheese, and what products does it make that help to flavor the cheese and make the holes?

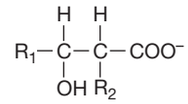
16.11 *Actinobacteria: Mycobacterium*

KEY GENUS: *Mycobacterium*

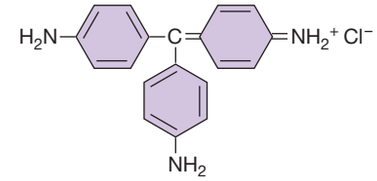
Mycobacteria are common in soils and most are harmless, but the genus *Mycobacterium* contains several notable human pathogens, chief among them *Mycobacterium tuberculosis*, the cause of tuberculosis (▶ Section 31.4). Species are rod-shaped bacteria that at some stage of their growth cycle possess the distinctive staining property called **acid-fastness**. This property is due to the presence of unique lipids called *mycolic acids*, found only in species of the genus *Mycobacterium*, on the surface of the mycobacterial cell. Mycolic acids are a group of complex branched-chain hydroxylated lipids (**Figure 16.31*a***) covalently bound to peptidoglycan in the cell wall; the complex gives the cell surface a waxy, hydrophobic consistency.

Because of their waxy surface, mycobacteria do not stain well with Gram stain. A mixture of the red dye basic fuchsin and phenol is used in the acid-fast (Ziehl–Neelsen) stain. The stain is driven into the cells by slow heating, and the role of the phenol is to enhance penetration of the fuchsin into the lipids. After washing in distilled water, the preparation is decolorized with acid alcohol and counterstained with methylene blue. Cells of acid-fast organisms stain red (Figure 16.31 inset), whereas the background and non-acid-fast organisms appear blue (▶ Figure 31.15*a*).





(a) **Mycolic acid; R_1 and R_2 are long-chain aliphatic hydrocarbons**

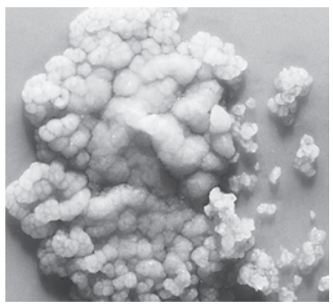


(b) **Basic fuchsin**

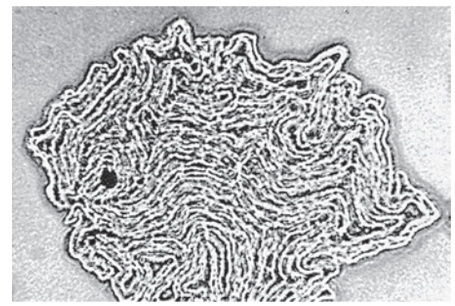
Figure 16.31 Acid-fast staining. Structure of *(a)* mycolic acid and *(b)* basic fuchsin, the dye used in the acid-fast stain. The fuchsin dye combines with mycolic acids in the cell wall via ionic bonds between COO^- and NH_2^+. Inset: Acid-fast stain of cells (red) of *Mycobacterium tuberculosis* present in a sputum sample from a tuberculosis patient.

Mycobacteria are somewhat pleomorphic and may undergo branching or even filamentous growth. However, in contrast to the filaments of the actinomycetes (Section 16.12), the filaments of the mycobacteria do not form a true mycelium. Mycobacteria can be separated into two major groups: slow-growing species (e.g., *M. tuberculosis, M. avium, M. bovis*, and *M. gordonae*) and fast-growing species (e.g., *M. smegmatis, M. phlei, M. chelonae, M. parafortuitum*). *Mycobacterium tuberculosis* is a typical slow grower, and visible colonies are produced from dilute inoculum only after days to weeks of incubation. When growing on solid media, mycobacteria form tight, compact, often wrinkled colonies (**Figure 16.32**). This colony morphology is probably due to the high lipid content and hydrophobic nature of the cell surface, which facilitates cells sticking together.

For the most part, mycobacteria have relatively simple nutritional requirements. Most species can grow aerobically in a simple mineral-salts medium with ammonium as the nitrogen source and glycerol or acetate as the sole carbon source and electron donor. Growth of *M. tuberculosis* is more difficult and is stimulated by lipids and fatty acids. The virulence of *M. tuberculosis* cultures has been correlated with the formation of long, cordlike structures (Figure 16.32*b*) that



(a)



(b)

(c)

Figure 16.32 Characteristic colony morphology of mycobacteria. *(a) Mycobacterium tuberculosis,* showing the compact, wrinkled appearance of the colony. The colony is about 7 mm in diameter. *(b)* A colony of virulent *M. tuberculosis* at an early stage, showing the characteristic cordlike growth. Individual cells are about 0.5 μm in diameter. (See also the historic drawings of *M. tuberculosis* cells made by Robert Koch, Figure 1.35). *(c)* Colonies of *Mycobacterium avium* from a strain of this organism isolated as an opportunistic pathogen from an AIDS patient.

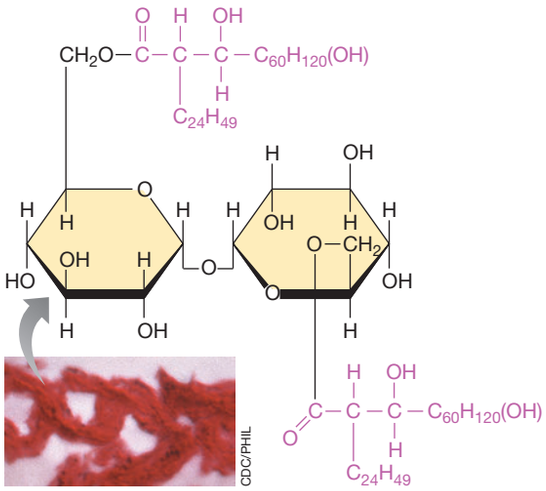


Figure 16.33 Structure of cord factor, a mycobacterial glycolipid: 6,6′-di-*O*-mycolyl trehalose. The two identical long-chain dialcohol groups are shown in purple. Inset: Photomicrograph of acid-fast stained cells of *Mycobacterium tuberculosis* (Figure 16.31) that have formed cords.

form as a result of side-to-side aggregation and intertwining of long chains of bacteria. Growth in cords reflects the presence of a characteristic glycolipid, the *cord factor*, on the cell surface (**Figure 16.33**).The pathogenesis of tuberculosis, along with the related mycobacterial disease leprosy, is discussed in Section 31.00.

Some mycobacteria produce yellow carotenoid pigments (Figure 16.32*c*), and pigmentation can aid in identification. Mycobacteria can either be nonpigmented (e.g., *M. tuberculosis*, *M. bovis*, *M. smegmatis*, *M. chelonae*); or can form pigment only when cultured in light, a property called *photochromogenesis* (e.g., *M. parafortuitum*); or can form pigment even when cultured in the dark, a property called *scotochromogenesis* (e.g., *M. gordonae*, *M. phlei*). Photochromogenesis is triggered by the blue region of the visible spectrum and is characterized by the photoinduction of one of the early enzymes in carotenoid biosynthesis. As with other carotenoid-containing bacteria, it is likely that carotenoids protect mycobacteria against oxidative damage from singlet oxygen (◀ Section 4.16).

Check Your Understanding

- What is mycolic acid, and what properties does this substance confer on mycobacteria?

16.12 Filamentous *Actinobacteria*: *Streptomyces* and Relatives

KEY GENERA: *Streptomyces, Actinomyces, Nocardia*

The **actinomycetes** are a large group of phylogenetically related, filamentous and aerobic gram-positive *Bacteria* common in soils. Many actinomycetes have a characteristic developmental cycle that culminates in the production of desiccation-resistant spores. Filaments elongate from their ends and form branching *hyphae*. Hyphal growth results in a network of filaments called a *mycelium* (**Figure 16.34**), analogous to that formed by filamentous fungi (▶ Section 18.9). When nutrients are depleted, the mycelium forms aerial hyphae that differentiate into spores that allow for survival and dispersal. We focus here on the genus *Streptomyces*, the most important genus in this group.

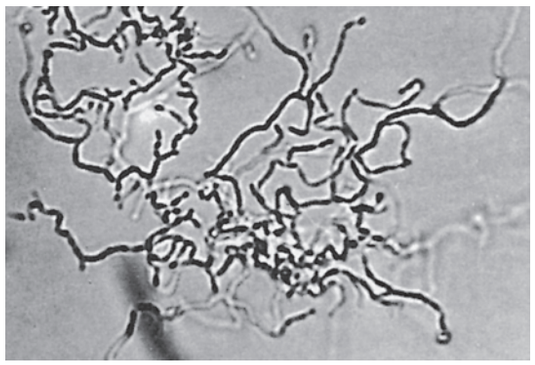
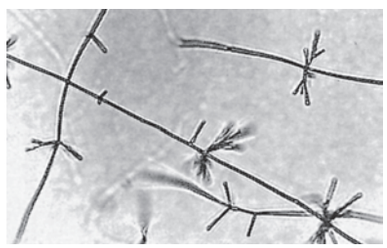


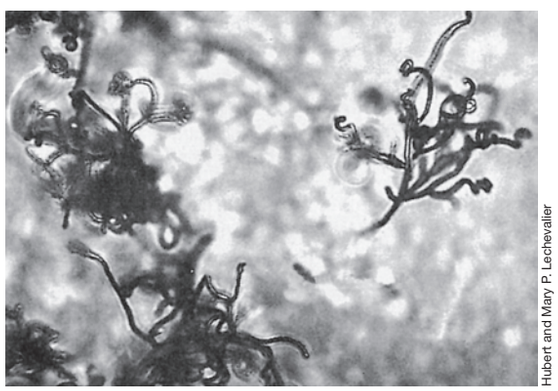
Figure 16.34 *Nocardia*. A young colony of an actinomycete of the genus *Nocardia*, showing typical filamentous cellular structure (mycelium). Each filament is about 0.8–1 μm in diameter.

Streptomyces

Over 600 species of *Streptomyces* are recognized. *Streptomyces* filaments are typically 0.5–1.0 μm in diameter and of indefinite length, and often lack cross-walls in the vegetative phase. *Streptomyces* grow at the tips of the filaments and may branch often. Thus, the vegetative phase consists of a complex, tightly woven matrix, resulting in a compact, convoluted mycelium and subsequent colony. As the colony ages, characteristic aerial filaments called *sporophores* are formed, which project above the surface of the colony and give rise to spores (**Figure 16.35**).



(a)



(b)

Figure 16.35 Spore-bearing structures of actinomycetes. Phase-contrast micrographs. Compare these photos with the art in Figure 16.37. *(a) Streptomyces,* a monoverticillate type. *(b) Streptomyces,* a closed spiral type. Filaments are about 0.8 μm wide in both types.

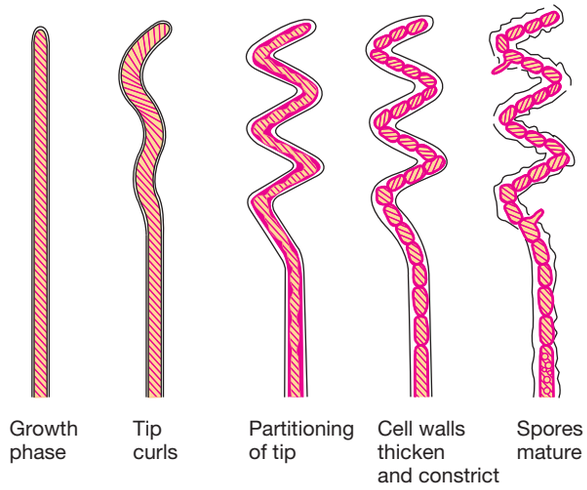


Figure 16.36 Spore formation in *Streptomyces*. Diagram of stages in the conversion of an aerial hypha (sporophore) into spores (conidia).

Streptomyces spores, called *conidia*, are quite distinct from the endospores of *Bacillus* and *Clostridium*. Unlike the elaborate cellular differentiation that leads to the formation of an endospore, conidia are produced by the formation of cross-walls in the multinucleate sporophores followed by separation of the individual cells directly into spores (**Figure 16.36**). Differences in the shape and arrangement of aerial filaments and spore-bearing structures of various species are among the fundamental features used in classifying the *Streptomyces* species (**Figure 16.37**). The conidia and sporophores are often pigmented and contribute a characteristic color to the mature colony (**Figure 16.38**). The dusty appearance of the mature colony, its compact nature, and its color make detection of *Streptomyces* colonies on agar plates relatively easy (Figure 16.38*b*).

UNIT 4

Mastering Microbiology
Art Activity: Figure 16.36 Spore formation in *Streptomyces*

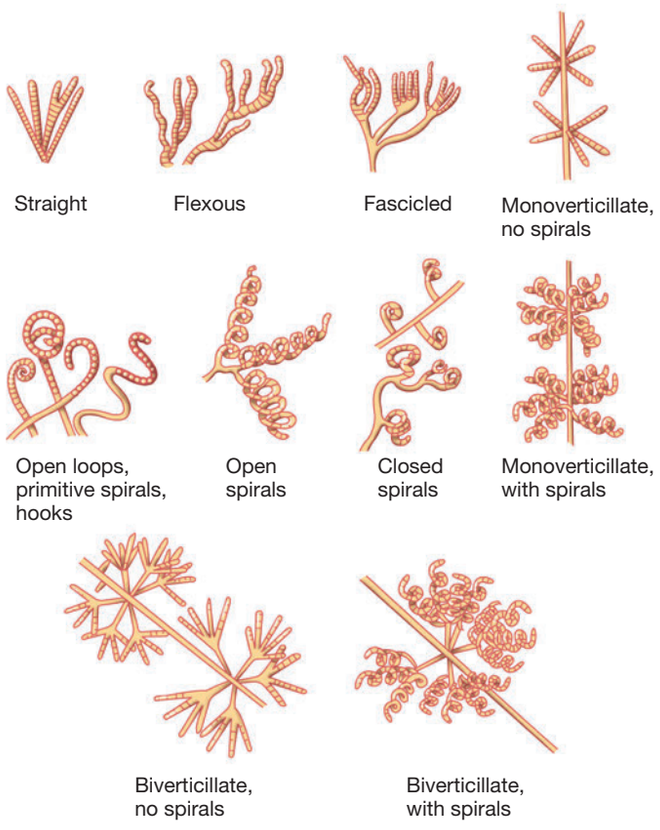


Figure 16.37 Morphologies of spore-bearing structures in the streptomycetes. A given species of *Streptomyces* produces only one morphological type of spore-bearing structure. The term "verticillate" means "whorls."

Ecology and Isolation of *Streptomyces*

Although a few streptomycetes are aquatic, they are primarily soil organisms. In fact, the characteristic earthy odor of soil is caused by the production by streptomycetes of a series of complex metabolites all called *geosmin*. Alkaline to neutral soils are more favorable for the development of *Streptomyces* than are acid soils. Moreover, higher numbers of *Streptomyces* are found in well-drained soils (such as sandy loams or soils covering limestone), where conditions are more likely to be aerobic, than in waterlogged soils, which quickly become anoxic.

Isolation of *Streptomyces* from soil is relatively easy: A suspension of soil in sterile water is diluted and spread on selective agar medium, and the plates are incubated aerobically at 25°C (Figure 16.38). Media selective for *Streptomyces* contain mineral salts plus polymeric substances such as starch or casein as organic nutrients. Streptomycetes typically produce extracellular hydrolytic enzymes that permit utilization of polysaccharides (starch, cellulose, and hemicellulose), proteins, and fats, and some strains

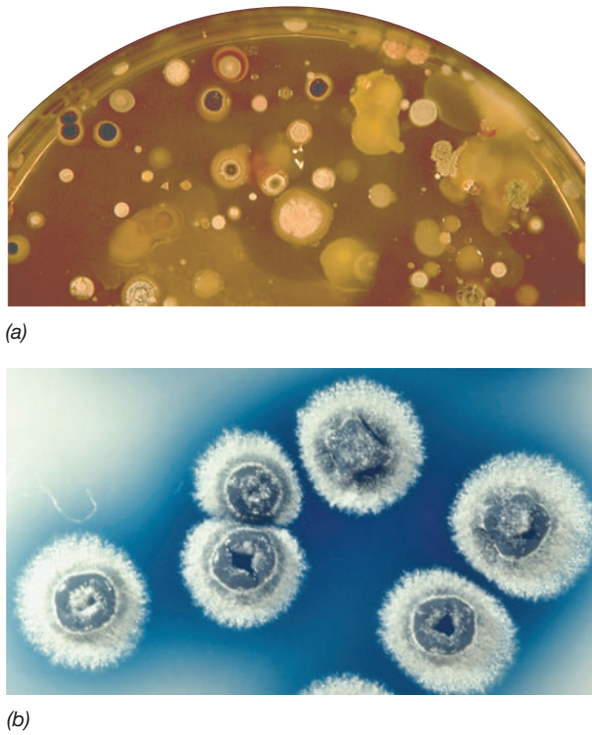


Figure 16.38 Streptomycetes. *(a)* Colonies of *Streptomyces* and other soil bacteria derived from spreading a soil dilution on a casein–starch agar plate. The *Streptomyces* colonies are of various colors (several black *Streptomyces* colonies are near the top of the plate) but can easily be identified by their opaque, rough, nonspreading morphology. *(b)* Close-up photo of colonies of *Streptomyces coelicolor*.

TABLE 16.2 Some common antibiotics synthesized by species of *Streptomyces* and related *Actinobacteria*

Chemical class	Common name	Produced by	Active against[a]
Aminoglycosides	Streptomycin	*S. griseus*[b]	Most gram-negative *Bacteria*
	Spectinomycin	*Streptomyces* spp.	*Mycobacterium tuberculosis*, penicillinase-producing *Neisseria gonorrhoeae*
	Neomycin	*S. fradiae*	Broad spectrum, usually used in topical applications because of toxicity
Tetracyclines	Tetracycline	*S. aureofaciens*	Broad spectrum, gram-positive and gram-negative *Bacteria*, rickettsias and chlamydias, *Mycoplasma*
	Chlortetracycline	*S. aureofaciens*	As for tetracycline
Macrolides	Erythromycin	*Saccharopolyspora erythraea*	Most gram-positive *Bacteria*, frequently used in place of penicillin; *Legionella*
	Clindamycin	*S. lincolnensis*	Effective against obligate anaerobes, especially *Bacteroides fragilis*, the major cause of anaerobic peritoneal infections
Polyenes	Nystatin	*S. noursei*	Fungi, especially *Candida* (a yeast) infections
	Amphotericin B	*S. nodosus*	Fungi
None	Chloramphenicol	*S. venezuelae*	Broad spectrum; drug of choice for typhoid fever

[a]Most antibiotics are effective against several different *Bacteria*. The entries in this column refer to the common clinical application of a given antibiotic. The structures and mode of action of many of these antibiotics are discussed in ▶ Sections 28.5–28.7.
[b]All species names beginning with an "*S.*" are species of *Streptomyces*.

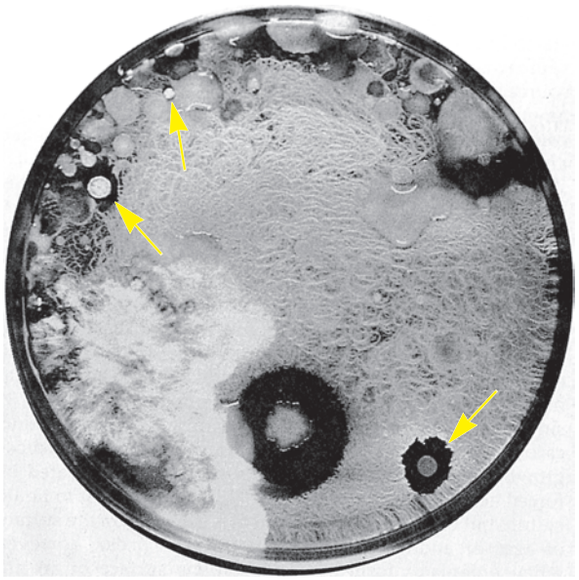
can use hydrocarbons, lignin, tannin, and other polymers. After incubation for 5–7 days in air, the plates are examined for the presence of the characteristic *Streptomyces* colonies (Figure 16.38), and spores from colonies can be restreaked to isolate pure cultures.

Antibiotics of *Streptomyces*

Perhaps the most striking physiological property of the streptomycetes is the extent to which they produce *antibiotics* (**Table 16.2**). Evidence for antibiotic production is often seen on the agar plates used in their initial isolation: Adjacent colonies of other bacteria show zones of inhibition (**Figure 16.39*a***).

About 50% of all *Streptomyces* isolated have been found to be antibiotic producers. Over 500 distinct antibiotics are produced by streptomycetes and many more are suspected; most of these have been identified chemically. Some species produce more than one antibiotic, and often the several antibiotics produced by one organism are chemically unrelated. Although an antibiotic-producing organism is resistant to its own antibiotics, it usually remains sensitive to antibiotics produced by other streptomycetes. Many genes are required to encode the enzymes for antibiotic synthesis, and because of this, the genomes of *Streptomyces* species are typically quite large (8 Mbp and larger; compare with genome sizes in Table 10.1). More than 60 streptomycete antibiotics have been used in human and veterinary medicine, and some of the most commonly used are listed in Table 16.2.

Surprisingly, despite the extensive research done on antibiotic-producing streptomycetes by the antibiotic industry and the fact that *Streptomyces* antibiotics are a multibillion-dollar-a-year industry, the



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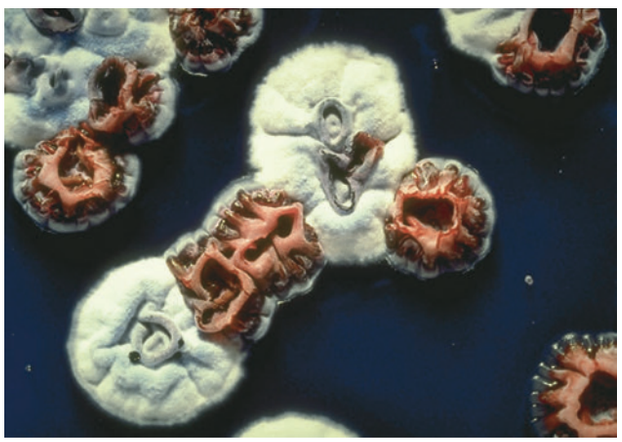
(a)



David A. Hopwood

(b)

Figure 16.39 Antibiotics from *Streptomyces*. *(a)* Antibiotic action of soil microorganisms on a crowded agar plate. The smaller colonies surrounded by inhibition zones (arrows) are streptomycetes; the larger, spreading colonies are *Bacillus* species, some of which are also producing antibiotics. *(b)* The red-colored antibiotic undecylprodigiosin is being excreted by colonies of *S. coelicolor*.

ecology of *Streptomyces* remains poorly understood. The interactions of these organisms with other bacteria and the ecological rationale for antibiotic production remains an important topic about which we know very little. One hypothesis for why *Streptomyces* species produce antibiotics is that antibiotic production, which is linked to sporulation (a process itself triggered by nutrient depletion), might be a mechanism to inhibit the growth of other organisms competing with *Streptomyces* cells for limiting nutrients. This would allow the *Streptomyces* to complete the sporulation process and form a dormant structure that would increase their chances of survival.

Check Your Understanding

- Contrast spores and sporulation in *Streptomyces* and *Bacillus* species.
- Why might antibiotic production be of advantage to streptomycetes?

III • *Bacteroidetes*

The *Bacteroidetes* include aerobes such as *Cytophaga*, which are common in natural habitats, and obligate anaerobes such as *Bacteroides*, which are common in the guts of many animals. Many of these species are motile by gliding.

The phylum *Bacteroidetes* contains more than 1000 characterized species spread across four primary orders: *Bacteroidales*, *Cytophagales*, *Flavobacteriales*, and *Sphingobacteriales* (Figure 16.40). The *Bacteroidetes* are gram-negative, nonsporulating rods; species are typically saccharolytic and can be aerobic or fermentative, including obligate aerobes, facultative aerobes, and obligate anaerobes. Gliding motility (◀ Section 2.10) is widespread in the phylum, though many species are nonmotile and a few are motile by flagella. The genus *Bacteroides* has been particularly well studied as these organisms are a major component of the microbial community in the human gut. But as we will see in Chapter 20, as a group, *Bacteroidetes* are found almost everywhere in nature and are diverse and common inhabitants of most terrestrial and aquatic environments.

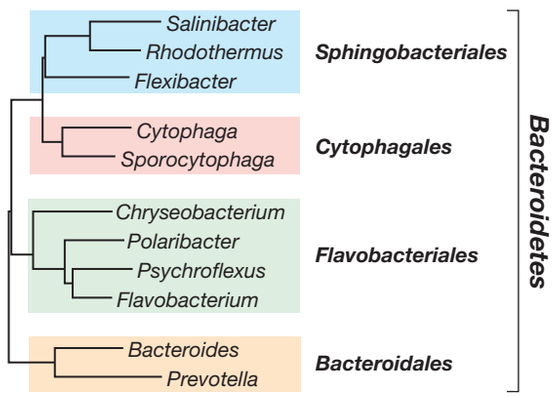


Figure 16.40 Major orders of *Bacteroidetes*. The phylogenetic tree was constructed from 16S rRNA gene sequences of representative genera of *Bacteroidetes*. Order names are shown in bold.

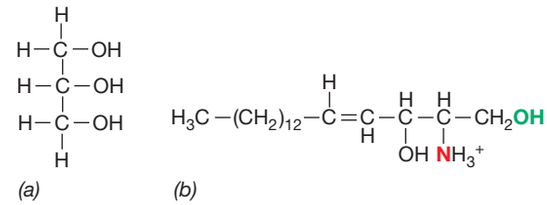


Figure 16.41 Sphingolipids. Comparison of *(a)* glycerol with *(b)* sphingosine. In sphingolipids, characteristic of *Bacteroides* species, sphingosine is the esterifying alcohol; a fatty acid is bonded by peptide linkage through the N atom (shown in red), and the terminal —OH group (shown in green) can be part of any of a number of compounds including phosphatidylcholine (sphingomyelin) or various sugars (cerebrosides and gangliosides).

16.13 *Bacteroidales*

KEY GENUS: *Bacteroides*

The order *Bacteroidales* primarily contains obligately anaerobic fermentative species. The type genus is *Bacteroides*, which contains species that are saccharolytic, fermenting sugars or proteins (depending on the species) to acetate and succinate as major fermentation products. *Bacteroides* are normally commensals, found in the intestinal tract of humans and other animals. In fact, *Bacteroides* species are the numerically dominant bacteria in the human large intestine, where measurements have shown that about 10^{11} prokaryotic cells are present per gram of wet feces (▶ Section 24.2). However, species of *Bacteroides* can occasionally be pathogens and are the most important anaerobic bacteria associated with human infections such as *bacteremia* (bacteria in the blood).

Bacteroides thetaiotaomicron is one of the most prominent species of *Bacteroides* found in the lumen of the large intestine. *B. thetaiotaomicron* specializes in the degradation of complex polysaccharides. A majority of its genome is devoted to making enzymes that degrade polysaccharides. The diversity and number of genes for carbohydrate metabolism found in its genome far exceeds those found in any other bacterial species. *B. thetaiotaomicron* produces many enzymes that are not encoded by the human genome and thus it vastly increases the diversity of plant polymers that can be degraded in the human digestive tract.

Species of *Bacteroides* are unusual in that they are one of the few groups of bacteria to synthesize a special type of lipid called *sphingolipid* (Figure 16.41), a collection of lipids characterized by the long-chain amino alcohol sphingosine in place of glycerol in the lipid backbone. Sphingolipids such as sphingomyelin, cerebrosides, and gangliosides are common in mammalian tissues, especially in the brain and other nervous tissues, but are rare in most bacteria. The production of sphingolipids can be found in a number of other genera in the phylum *Bacteroidetes* including *Flectobacillus*, *Prevotella*, *Porphyromonas*, and *Sphingobacterium*.

Check Your Understanding

- What is the role of *Bacteroides thetaiotaomicron* in the human gut?

16.14 *Cytophagales, Flavobacteriales,* and *Sphingobacteriales*

KEY GENERA: ***Cytophaga, Flavobacterium, Flexibacter***

Cytophagales

The order *Cytophagales* (Figure 16.40) contains almost exclusively obligate aerobes, though some species have limited fermentative capabilities. Cells are typically long, slender, gram-negative rods, often containing pointed ends, and move by gliding (**Figure 16.42**). Cytophagas specialize in the degradation of complex polysaccharides. They are widespread in toxic soils and freshwaters, where they probably account for much of the bacterial cellulose digestion. Cellulose decomposers can easily be isolated by placing small crumbs of soil on pieces of cellulose filter paper laid on the surface of mineral salts agar. The bacteria attach to and digest the cellulose fibers, forming spreading colonies (Figure 16.42*b*).

Cellulose degradation by cytophagas can proceed by two different mechanisms. The typical mechanism is the free cellulase mechanism in which cells secrete extracellular enzymes called *exoenzymes* that degrade insoluble cellulose outside of the cell. A complex mixture of enzymes is secreted including *processive endocellulases*, which cleave *internal* β-1,4 glycosidic bonds, and *processive exocellulases*, which cleave *terminal* β-1,4 glucosidic bonds, releasing cellobiose. These exoenzymes degrade insoluble cellulose into soluble polysaccharides and disaccharides that can be readily assimilated by cells. *Cytophaga hutchinsonii* does not produce processive cellulases, and its degradation of cellulose likely requires physical contact of cellulose fibers with cellulase enzymes located on the outer surface of its cell wall.

The genus *Cytophaga* contains species that can degrade not only cellulose (Figure 16.42*c*) but also agar (Figure 16.42*a*) and chitin. In pure culture *Cytophaga* can be grown on agar containing embedded cellulose fibers (Figure 16.42*b*). The related genus *Sporocytophaga* is similar to *Cytophaga* in morphology and physiology, but the cells form resting spherical structures called *microcysts* (Figure 16.42*d*), similar to those produced by some fruiting myxobacteria (◀ Section 15.16).

Several species of *Cytophaga* are fish pathogens and can cause serious problems in the cultivated fish industry. Two of the most important diseases are *columnaris disease*, caused by *Cytophaga columnaris*, and *cold-water disease*, caused by *Cytophaga psychrophila*. Both diseases preferentially affect stressed fish, such as those living in waters receiving pollutant discharges or living in high-density confinement situations such as fish hatcheries and aquaculture facilities. Infected fish show tissue destruction, frequently around the gills, probably from proteolytic activities of the *Cytophaga* pathogen.

Flavobacteriales and *Sphingobacteriales*

Flavobacteriales and *Sphingobacteriales* (Figure 16.40) typically contain aerobic and facultatively aerobic chemoorganotrophs. Like most *Bacteroidetes*, these organisms are gram-negative rods, and are saccharolytic with many species motile by gliding. Species are found widely in soils and in aquatic habitats, where they typically degrade complex polysaccharides.

Flavobacteriales can be particularly abundant in marine waters including aquatic systems in polar environments. *Flavobacterium*

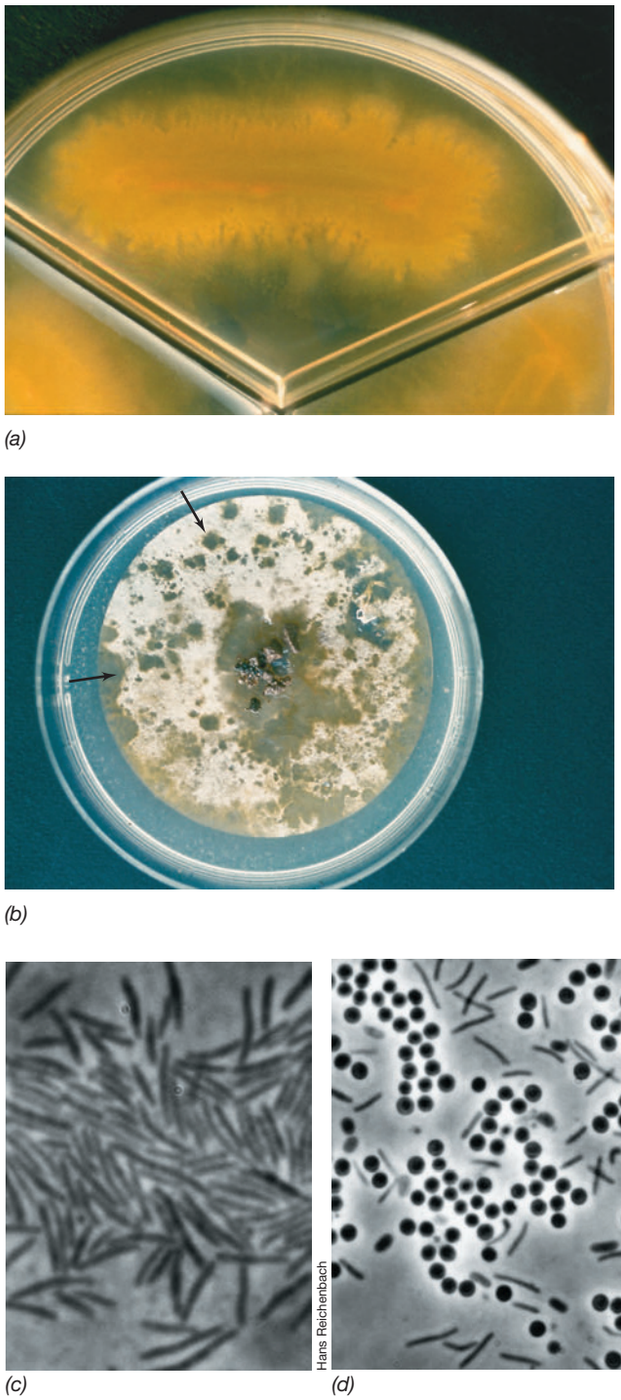


Figure 16.42 ***Cytophaga* and *Sporocytophaga*.** *(a)* Streak of an agarolytic marine *Cytophaga* hydrolyzing agar in a Petri dish. *(b)* Colonies of *Sporocytophaga* growing on cellulose. Note the clearing zones (arrows) where the cellulose has been degraded. *(c)* Phase-contrast micrograph of cells of *Cytophaga hutchinsonii* grown on cellulose filter paper (cells are about 1.5 μm in diameter). *(d)* Phase-contrast micrograph of the rod-shaped cells and spherical microcysts of *Sporocytophaga myxococcoides* (cells are about 0.5 μm and microcysts about 1.5 μm in diameter). Although *Sporocytophaga* microcysts are only slightly more heat-tolerant than vegetative cells, they are extremely resistant to desiccation and thus help the organism survive dry periods in soil. The genera *Cytophaga* and *Sporocytophaga* form a major clade within the phylum *Bacteriodetes* (Figure 16.40).

species are primarily found in aquatic habitats, both freshwater and marine, as well as in foods and food-processing plants. Most species are obligate aerobes, though some species are able to reduce nitrate in an anaerobic respiration. Flavobacteria frequently produce yellow pigments and are generally saccharophilic; most can also degrade starch and proteins. Flavobacteria are rarely pathogenic; however, one species, *Flavobacterium meningosepticum*, has been implicated in cases of infant meningitis, and several fish pathogens are also known.

Some *Flavobacteriales* are psychrophilic or psychrotolerant (◀ Section 4.12). These include, in particular, the genera *Polaribacter* and *Psychroflexus*, organisms commonly isolated from cold environments, especially permanently cold environments such as polar waters and sea ice. Many related genera are also capable of good growth below 20°C and can thus be agents of food spoilage. None are pathogenic.

Sphingobacteriales are phenotypically similar to many *Flavobacteriales*. In terms of physiology, species of *Sphingobacteriales* are generally able to degrade a greater breadth of complex polysaccharides than are *Flavobacteriales*, and in this regard they resemble species of *Cytophagales*. The genus *Flexibacter* is typical of many genera of *Sphingobacteriales*. Species of *Flexibacter* differ from those of *Cytophaga* in that they usually require complex media for good growth and are unable to degrade cellulose. Cells of some *Flexibacter* species also undergo changes in cell morphology from long, gliding, threadlike filaments lacking cross-walls to short, nonmotile rods. Many flexibacteria are pigmented due to carotenoids located in their cytoplasmic membrane, or from related pigments called *flexirubins*, located in the cell's outer membrane. *Flexibacter* species are common in soil and freshwaters where they degrade polysaccharides, and none have been identified as pathogens.

Check Your Understanding

- Describe a method for isolating *Cytophaga* species from nature.
- What characteristics are shared between the genera *Cytophaga* and *Bacteroides*, and in what ways do they differ?

IV • *Chlamydiae, Planctomycetes,* and *Verrucomicrobia*

***Chlamydiae* and *Planctomycetes* lack the key cell-division protein FtsZ and produce unusual cellular structures. *Chlamydiae* include intracellular parasites while *Planctomycetes* and *Verrucomicrobia* are widespread in soils and aquatic systems.**

The phyla *Chlamydiae*, *Planctomycetes*, and *Verrucomicrobia* share an ancestor and are more closely related to each other than to other bacterial phyla (**Figure 16.43**). These three groups contain organisms that can be found in a variety of habitats including soils, aquatic systems, and in association with eukaryotic hosts. The *Chlamydiae* and *Planctomycetes* are highly unusual among bacteria in that they lack a gene encoding the protein FtsZ, a key protein in septum formation during cell division (◀ Section 8.3). FtsZ is essential for cell division in most bacteria and hence there is great interest in understanding how *Chlamydiae* and *Planctomycetes* divide without this protein. We first consider the chlamydias, a group of small gram-negative bacteria that cause some serious human and animal diseases.

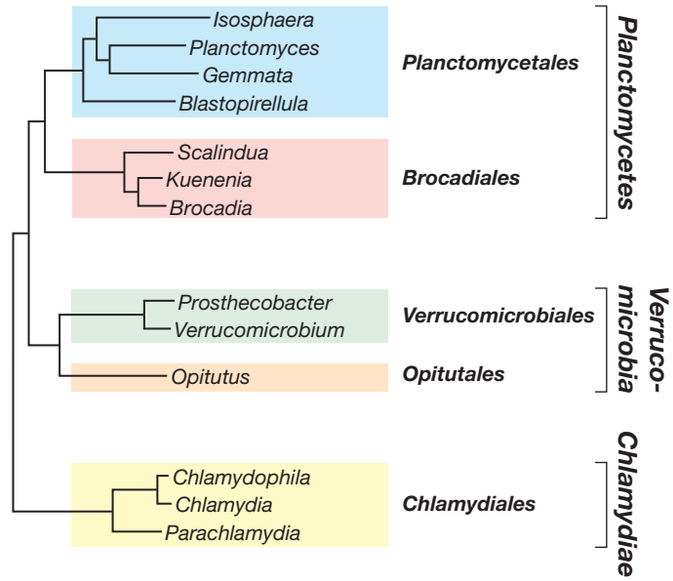


Figure 16.43 Major orders of *Chlamydiae, Planctomycetes,* and *Verrucomicrobia*. The phylogenetic tree was constructed from 16S rRNA gene sequences of representative genera of *Chlamydiae, Planctomycetes,* and *Verrucomicrobia*. Order names are shown in bold.

16.15 *Chlamydiae*

KEY GENERA: *Chlamydia, Chlamydophila, Parachlamydia*

The phylum *Chlamydiae* contains a single order, the *Chlamydiales*. The entire phylum consists of obligate intracellular parasites of eukaryotes, in particular, humans. The genomes of the chlamydias are highly reduced, being about 1 Mbp in size, and as a result they are among the most biochemically limited of all known *Bacteria*. They are unable to synthesize many of the vitamins and growth factors needed to grow and must acquire these molecules from the cytoplasm of infected host cells.

Besides their lack of the key protein FtsZ (◀ Section 8.3), many chlamydial species lack a cell wall. These organisms apparently minimize their use of peptidoglycan as a means of remaining undetected by the host immune system. Despite the complete absence of a cell wall in many species of the *Chamydiae* these organisms remain susceptible to beta-lactam antibiotics such as penicillin, a drug that prevents peptidoglycan synthesis. The answer to this conundrum is the fact that although they lack a cell wall, chlamydias still require the formation of a ringlike peptidoglycan structure to facilitate septum formation during cell division. In the absence of this structure, cell division is compromised.

Though the chlamydia that are human pathogens have been characterized in most detail, the phylum contains diverse species that interact with a wide variety of eukaryotic hosts including diverse

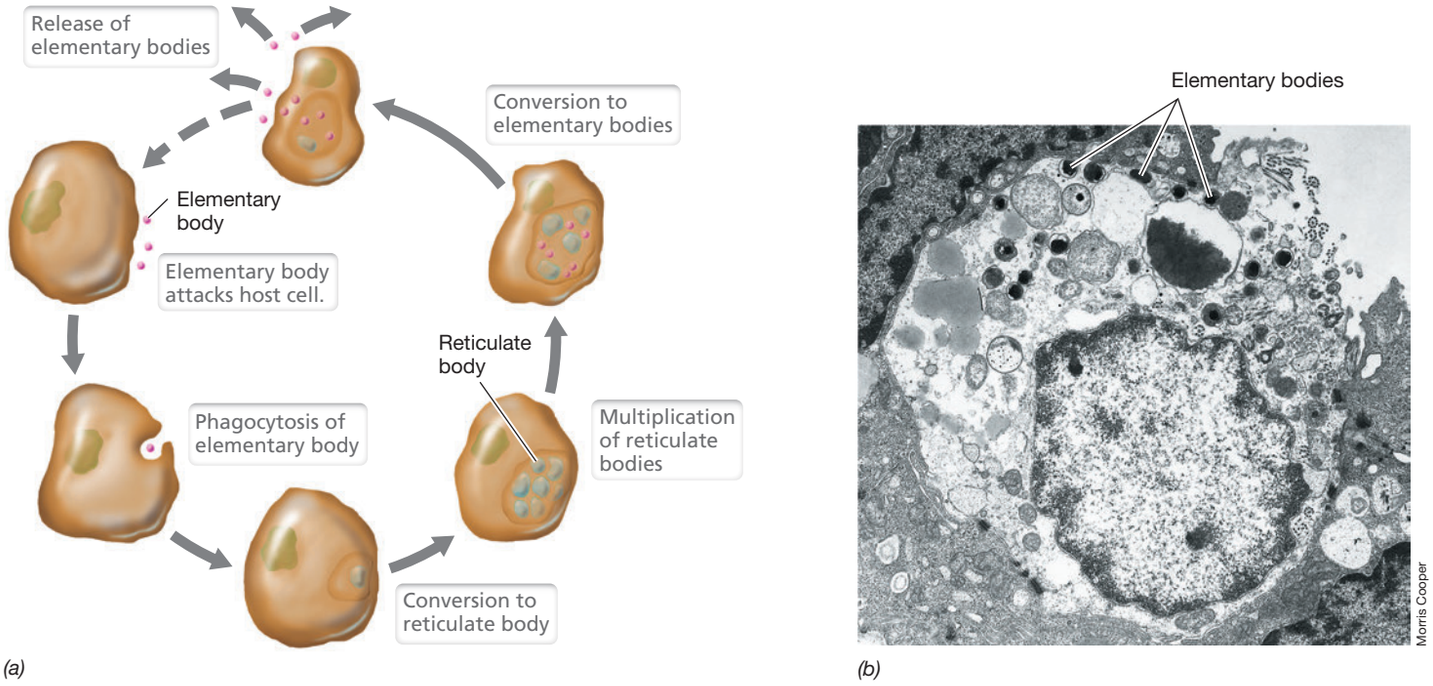


Figure 16.44 The infection cycle of a chlamydia. *(a)* Schematic diagram of the cycle: The entire cycle takes about 48 h. *(b)* Human chlamydial infection. Elementary bodies (~0.3 μm in diameter) are the infectious form and reticulate bodies (~1 μm in diameter) are the multiplying form. An infected fallopian tube cell is bursting, releasing mature elementary bodies.

animals and even many amoebae. Species are typically very small cocci, approximately 0.5 μm in diameter, and display a distinctive developmental cycle.

Life Cycle of *Chlamydiae*

Species of *Chlamydiae* demonstrate a unique chlamydial life cycle (**Figure 16.44**). *Two* types of cells are seen in the life cycle: (1) a small, dense cell, called an *elementary body*, which is relatively resistant to drying and is the means of dispersal, and (2) a larger, less dense cell, called a *reticulate body*, which divides by binary fission and is the vegetative form.

Elementary bodies are nonmultiplying cells specialized for infectious transmission. By contrast, reticulate bodies are noninfectious forms that function only to multiply inside host cells to form a large inoculum for transmission. Unlike the rickettsias, the chlamydias are not transmitted by arthropods but are primarily airborne invaders of the respiratory system—hence the significance of resistance to drying of the elementary bodies. A dividing reticulate body can be seen in **Figure 16.45**. After a number of cell divisions, these vegetative cells are converted into elementary bodies that are released when the host cell disintegrates (Figure 16.44*b*) and can then infect other nearby host cells. Generation times of 2–3 h have been measured for reticulate bodies, considerably faster than times found for the rickettsias (Section 16.1).

Notable Genera of *Chlamydiae*

Chlamydiae are particularly well adapted to invading and colonizing eukaryotic cells, and different species can infect a diverse array of eukaryotic hosts. The species *Parachlamydia acanthamoebae* infects free-living amoebae, particularly amoebae in the genus *Acanthamoeba*. *Parachlamydia* demonstrates the typical chlamydial life cycle during infection of amoebae (Figure 16.44). Most species of *Chlamydiae* can multiply or survive within free-living amoebae, and these hosts may be important for the survival and dispersal of *Chlamydiae* in nature. A diversity of 16S rRNA gene sequences from *Chlamydiae* can be detected in natural environments, suggesting that these organisms are widespread and that many of their natural hosts have yet to be identified. While free-living amoebae are the natural hosts for *P. acanthamoebae*, this species can also infect humans, although only weakly compared with *Chlamydiae* whose natural hosts are human.

Mastering Microbiology
Art Activity: Figure 16.44*a* The infection cycle of a chlamydia

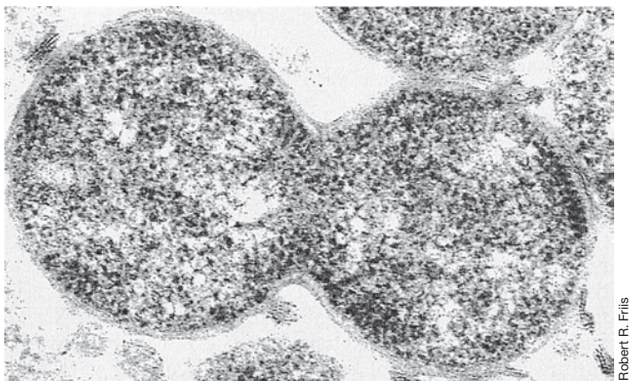


Figure 16.45 *Chlamydiae*. Thin-section electron micrograph of a dividing reticulate body of *Chlamydophila psittaci* within a mouse tissue-culture cell. A single chlamydial cell is about 1 μm in diameter.

The best-studied human pathogens are found in the genera *Chlamydia* and *Chlamydophila*. Several species are recognized within these genera: *Chlamydophila psittaci*, the causative agent of the disease psittacosis; *Chlamydia trachomatis*, the causative agent of trachoma and a variety of other human diseases; and *Chlamydophila pneumoniae*, the cause of some respiratory syndromes. Psittacosis is an epidemic disease of birds that is occasionally transmitted to humans and causes pneumonia-like symptoms. Trachoma, a debilitating disease of the eye characterized by vascularization and scarring of the cornea, is the leading cause of blindness in humans. Other strains of *C. trachomatis* infect the genitourinary tract, and chlamydial infections are currently one of the leading sexually transmitted diseases (▶ Section 31.13).

In sum, the chlamydias appear to have evolved an efficient and effective survival strategy including parasitizing the resources of the host and producing resistant cell forms specialized for transmission.

Check Your Understanding

- How are *Chlamydia* and *Mycoplasma* (Section 16.9) similar? How are they different?
- What is the difference between an elementary body and a reticulate body?

16.16 *Planctomycetes*

KEY GENERA: *Planctomyces, Blastopirellula, Gemmata, Brocadia*

The phylum *Planctomycetes* contains a diverse set of morphologically unique bacteria found primarily in two orders, *Planctomycetales* and *Brocadiales* (Figure 16.43). *Planctomycetes* are gram-negative bacteria that often have stalks or appendages, and many species form rosettes (clusters of cell typically attached at their poles). *Planctomycetes* are found widely in soils and aquatic environments and they are rarely pathogenic. Despite being observed widely in the environment, their ecology remains poorly defined. *Planctomycetes* are remarkable for having a set of unusual cellular features.

Unlike most *Bacteria*, *Planctomycetes* lack the protein FtsZ (see also *Chlamydia*, Section 16.15), and they divide by budding, much like yeast. Moreover, *Planctomycetes* were long thought to lack peptidoglycan, and transmission electron micrographs (TEM) suggested the presence of a membrane-enclosed nucleus in some species (**Figure 16.46**). This unique mix of prokaryotic and eukaryotic features has for years given the impression that *Planctomycetes* are a missing link in cellular evolution. However, the mysteries of the missing peptidoglycan and the nature of the "nucleus"—like many other microbial mysteries—were ultimately solved by advances in microbiological techniques.

Compartmentalization in *Planctomycetes*

Cryo-electron tomography (CET) is a microscopic method that generates detailed three-dimensional images with nanometer-scale resolution (◀ Section 1.10). CET scans of *Planctomycetes* revealed that what appeared to be a nuclear membrane in two-dimensional transmission electron micrographs was actually a system of deep invaginations of the cytoplasmic membrane (**Figure 16.47**). The cytoplasmic membrane of cells of *Planctopirus* is heavily invaginated, and when viewed in

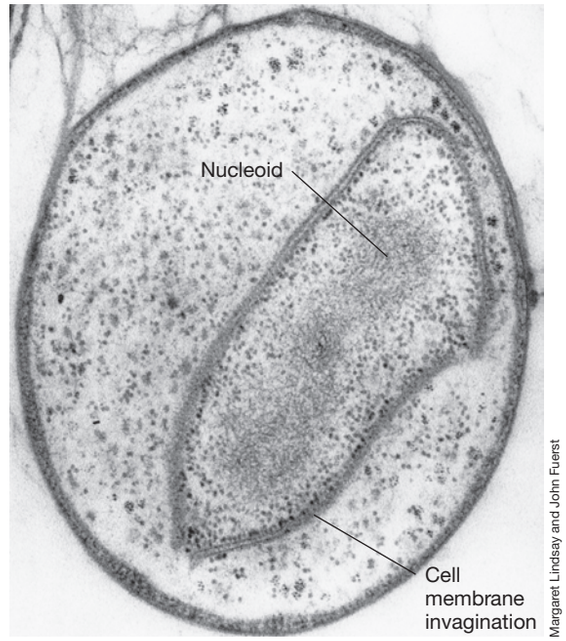


Figure 16.46 Membrane invaginations in *Gemmata*. Thin-section electron micrograph of a cell of *Gemmata obscuriglobus* showing the nucleoid surrounded by an invagination of the cell membrane (see Figure 16.47). The deep invagination of the cell membrane, when viewed in a two-dimensional section, gives the appearance of a nuclear membrane. The cell is about 1.5 μm in diameter.

Figure 16.47 Cryo-electron tomographic reconstruction of *Planctopirus*. *Planctomycetes* have a complex cell structure consisting of a series of deep and complex invaginations of the cellular membrane. Until recently, these unusual membrane structures were interpreted as evidence that *Planctomycetes* could have a membrane-bound nucleus (see Figure 16.46). The image shows the internal structure of a single cell and the outer sections of several nearby cells. The structures are color coded: outer membrane (green), a peptidoglycan-containing cell wall (orange), cytoplasmic membrane (blue), DNA-containing nucleoids (yellow), ribosomes (white) present in the cytoplasm, and crateriform structures (magenta) on the outer membrane. The presence of two nucleoids suggest the central cell is preparing to divide.

three dimensions, these invaginations can be seen to wrap around the DNA-containing nucleoid (Figure 16.47). Two-dimensional slices through this three-dimensional system of membranes gave the false appearance of a nuclear membrane (for example, see Figure 16.46). Furthermore, these images clearly reveal that *Planctomycetes* have a typical gram-negative cell envelope containing peptidoglycan.

While we now know that cells of *Planctomycetes* contain both peptidoglycan and a gram-negative cell envelope, an understanding of the remarkable membrane invaginations that appear in CET scans of cells of *Planctomycetes* remains elusive. In addition, these CET scans reveal unusual crateriform structures studding the surface of their outer membranes. It is possible that these crateriform structures mediate the transport of large molecules into the cell where they are then metabolized in the large periplasmic compartments formed by the membrane invaginations. However, the purpose of these cellular features remains unknown.

Another interesting cellular structure found in the *Planctomycetes* is present in the *Brocadiales* that carry out the anammox process (◀ Section 14.10). Anammox bacteria such as *Brocadia anammoxidans* contain a true organelle called the *anammoxosome* (◀ Figure 14.26). These bacteria catalyze the anaerobic oxidation of ammonia (NH_3) within the anammoxosome structure. The anammoxosome membrane is composed of unique lipids that form a tight seal, protecting cytoplasmic components from toxic intermediates produced during the anaerobic oxidation of ammonia (◀ Section 14.10 and Figure 14.26*c*). The evolutionary origins of this organelle and many aspects of its function remain unknown.

Planctomyces

Planctomyces is the best-characterized genus in the *Planctomycetes*. In Section 15.18 we considered the stalked proteobacterium *Caulobacter*. *Planctomyces* is also a stalked bacterium (**Figure 16.48**). However, unlike *Caulobacter*, the stalk of *Planctomyces* consists of protein and does not contain a cell wall or cytoplasm (compare Figure 16.48 with ◀ Figure 15.53). The *Planctomyces* stalk presumably functions in attachment, but it is a much narrower and finer structure than the prosthecal stalk of *Caulobacter*.

Like *Caulobacter* (◀ Figures 8.6, 8.20, and 15.54), *Planctomyces* is a budding bacterium that displays a life cycle. Its motile swarmer cells attach to a surface, grow a stalk from the attachment point, and generate a new cell from the opposite pole by budding. This daughter cell produces a flagellum, breaks away from the attached mother cell, and begins the cycle anew. Physiologically, *Planctomyces* species are facultatively aerobic chemoorganotrophs, growing either by fermentation or respiration of sugars.

Planctomyces are found primarily in soil and aquatic habitats, both freshwater and marine, and the genus *Isosphaera* is a filamentous, gliding hot spring bacterium. The isolation of *Planctomyces* and relatives, like that of *Caulobacter*, requires dilute media.

Check Your Understanding

- How does the stalk of *Planctomyces* differ from the stalk of *Caulobacter*?
- What is unusual about the bacterium *Planctopirus*?

16.17 *Verrucomicrobia*

KEY GENERA: *Verrucomicrobium, Prosthecobacter*

The phylum *Verrucomicrobia* contains at least four orders with characterized species, but most are found within the order *Verrucomicrobiales* (Figure 16.43). Species of *Verrucomicrobia* are aerobic or facultatively aerobic bacteria capable of fermenting sugars. An exception is the genus *Methylacidiphilum*, which contains aerobic methanotrophs (◀ Section 15.15). In addition, some *Verrucomicrobia* form symbiotic associations with protists. *Verrucomicrobia* are widespread in nature, inhabiting freshwater and marine environments as well as forest and agricultural soils. The *Verrucomicrobia* can have membrane-bound intracellular structures similar to those found in the *Planctomycetes*. The *Verrucomicrobia* typically form cytoplasmic appendages called *prosthecae* (◀ Section 15.18). *Verrucomicrobia* share with other prosthecate bacteria the presence of peptidoglycan in their cell walls and in this way are clearly distinct from *Planctomycetes*.

The genera *Verrucomicrobium* and *Prosthecobacter* produce two to several prosthecae per cell (**Figure 16.49**). Unlike cells of *Caulobacter* (◀ Figures 8.6, 8.20, and 15.54), which contain a single prostheca

UNIT 4

Mastering Microbiology
Art Activity: Figure 16.47 *Planctomyces maris*

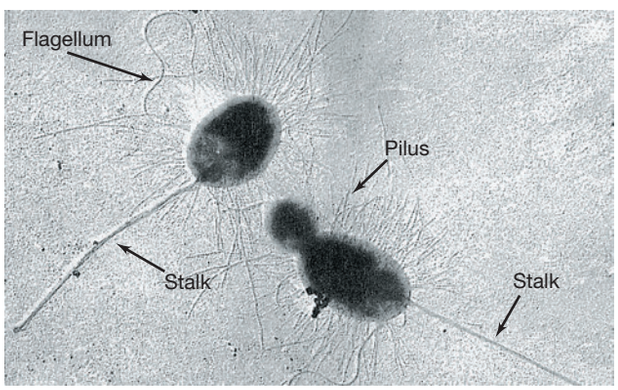


Figure 16.48 ***Planctomyces maris.*** Metal-shadowed transmission electron micrograph. A single cell is about 1–1.5 μm long. Note the fibrillar nature of the stalk. Pili are also abundant. Note also the flagella (curly appendages) on each cell and the bud that is developing from the nonstalked pole of one cell.

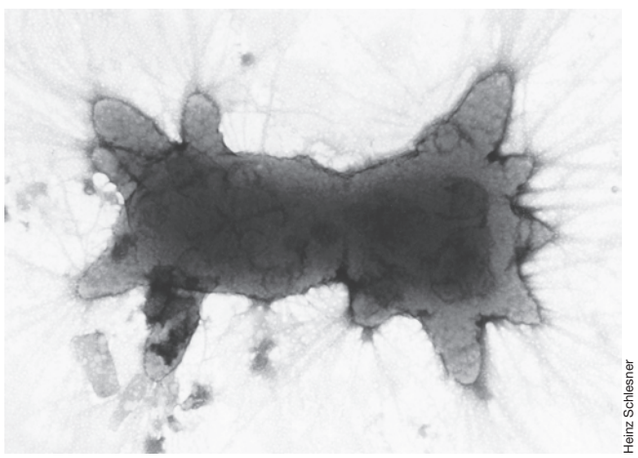


Figure 16.49 ***Verrucomicrobium spinosum.*** Negatively stained transmission electron micrograph. Note the wartlike prosthecae. A cell is about 1 μm in diameter.

and produce flagellated and nonprosthecate swarmer cells, *Verrucomicrobium* and *Prosthecobacter* divide symmetrically, and both mother and daughter cells contain prosthecae at the time of cell division. The genus name *Verrucomicrobium* derives from Greek roots meaning "warty," which is an appropriate description of cells of *Verrucomicrobium spinosum* with their multiple projecting prosthecae (Figure 16.49).

Species of the genus *Prosthecobacter* contain two genes that show significant homology to the genes that encode tubulin in eukaryotic cells. Tubulin is the key protein that makes up the cytoskeleton of eukaryotic cells (◀ Section 2.15). Although the important cell-division protein FtsZ (◀ Section 8.3) is also a tubulin homolog, the *Prosthecobacter* proteins are structurally more similar to eukaryotic tubulin than is FtsZ. The role of the tubulin proteins in *Prosthecobacter* is unknown since a eukaryotic-like cytoskeleton has not been observed in these organisms.

Check Your Understanding

- Describe two ways that *Verrucomicrobia* differ from *Planctomycetes*.

V • Hyperthermophilic *Bacteria*

***Aquificales* are common in geothermally heated aquatic systems. Hyperthermophilic phyla branch near the root of the bacterial tree, supporting the idea that Earth's first bacterial cells were thermophilic.**

Three phyla of hyperthermophilic bacteria cluster deep in the phylogenetic tree of *Bacteria*, near the root (Figure 16.1). Each group consists of one or two major genera, and a key physiological feature of most species is *hyperthermophily*—optimal growth at temperatures above 80°C (◀ Section 4.13). We begin with *Thermotoga* and *Thermodesulfobacterium*, each representative of its own lineage.

16.18 *Thermotogae* and *Thermodesulfobacteria*

KEY GENERA: *Thermotoga, Thermodesulfobacterium*

The phylum *Thermotogae* includes about 12 genera containing cultivated organisms. These species are all chemoorganotrophic anaerobes and many are thermophiles and hyperthermophiles, though some mesophiles are known. Species of the representative genus *Thermotoga* are rod-shaped hyperthermophiles that form a sheath-like envelope (called a *toga*; thus the genus name) (**Figure 16.50*a***), stain gram-negatively, and are nonsporulating. *Thermotoga* species are fermentative anaerobes, catabolizing sugars or starch and producing lactate, acetate, CO_2, and H_2 as fermentation products. The organisms can also grow by anaerobic respiration using H_2 as an electron donor and ferric iron as an electron acceptor. Species of *Thermotoga* have been isolated from terrestrial hot springs as well as marine hydrothermal vents.

Despite being bacterial, the genome of *Thermotoga* contains many genes that show strong homology to genes from hyperthermophilic *Archaea*. In fact, over 20% of the genes of *Thermotoga* probably originated from *Archaea* by horizontal gene transfers (◀ Section 13.9 and Chapter 9). Although a few archaeal-like genes have been identified in the genomes of other *Bacteria* and vice versa, only in *Thermotoga* has such large-scale horizontal transfer of genes between domains been detected thus far.

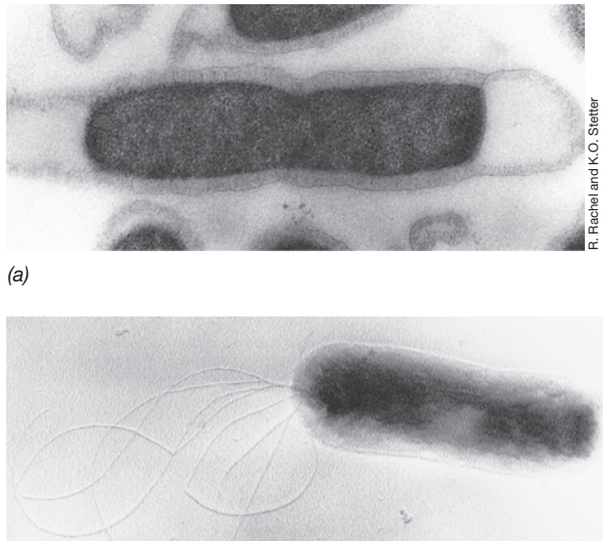


Figure 16.50 Hyperthermophilic *Bacteria*. Electron micrographs of two hyperthermophiles: *(a) Thermotoga maritima*—temperature optimum, 80°C. Note the outer covering, the toga. *(b) Aquifex pyrophilus*—temperature optimum, 85°C. Cells of *Thermotoga* measure 0.6 × 3.5 μm; cells of *Aquifex* measure 0.5 × 2.5 μm.

Thermodesulfobacterium (**Figure 16.51**) is a thermophilic sulfate-reducing bacterium, positioned on the phylogenetic tree in a separate phylum after *Thermotoga* and *Aquifex* (Figure 16.1*a*). *Thermodesulfobacterium* is a strict anaerobe that uses compounds such as lactate, pyruvate, and ethanol (but not acetate) as electron donors, as do sulfate-reducing bacteria such as *Desulfovibrio* (◀ Section 15.11), reducing SO_4^{2-} to H_2S.

An unusual biochemical feature of *Thermodesulfobacterium* is the production of *ether-linked lipids*. Recall that such lipids are a hallmark of the *Archaea* and that a polyisoprenoid C_{20} hydrocarbon (phytanyl) replaces fatty acids as the side chains in archaeal lipids (◀ Section 2.1). However, the ether-linked lipids in *Thermodesulfobacterium* are unusual because the glycerol side chains are not

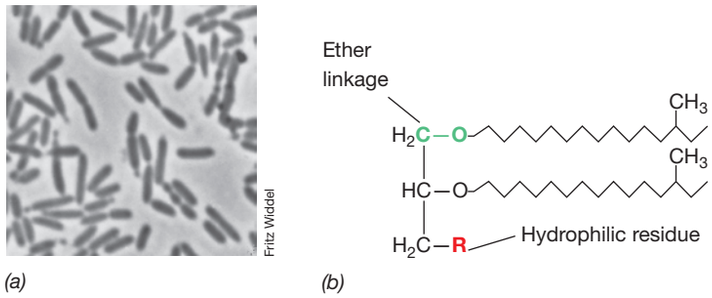


Figure 16.51 *Thermodesulfobacterium*. *(a)* Phase-contrast micrograph of cells of *Thermodesulfobacterium thermophilum*. *(b)* Structure of one of the lipids of *Thermodesulfobacterium mobile*. Note that although the two hydrophobic side chains are ether-linked, they are not phytanyl units, as in *Archaea*. The designation "R" is for a hydrophilic residue, such as a phosphate group.

phytanyl groups, as they are in *Archaea*, but instead are composed of unique C_{17} hydrocarbons along with some fatty acids (Figure 16.51*b*). Thus we see in *Thermodesulfobacterium* both a deep phylogenetic lineage (Figure 16.1) and a lipid profile that combines features of both the *Archaea* and the *Bacteria*. However, a few other *Bacteria* have also been found to contain ether-linked lipids, and thus these lipids may be more common among *Bacteria* than previously thought.

Check Your Understanding

- What is unique about the genome of *Thermotoga* and the lipids of *Thermodesulfobacterium*?

16.19 *Aquificae*

KEY GENERA: *Aquifex, Thermocrinis*

The genus *Aquifex* (Figure 16.50*b*) is an obligately chemolithotrophic and autotrophic hyperthermophile and is the most thermophilic of all known *Bacteria*. Various *Aquifex* species utilize H_2, sulfur (S^0), or thiosulfate ($S_2O_3^{2-}$) as electron donors and O_2 or nitrate (NO_3^-) as electron acceptors, and grow at temperatures up to 95°C. *Aquifex* can tolerate only very low O_2 concentrations (microaerophilic), and is unable to oxidize any tested organic compound. *Hydrogenobacter*, a relative of *Aquifex*, shows most of the same properties as *Aquifex*, but is an obligate aerobe.

Aquifex and Autotrophy

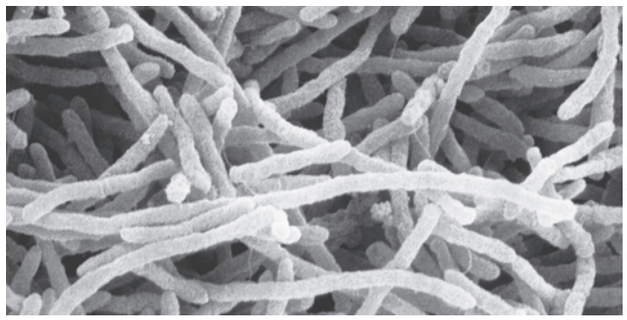
Autotrophy in *Aquifex* occurs by way of the reverse citric acid cycle, a series of reactions previously detected only in green sulfur bacteria (◀ Sections 14.2 and 15.6) within the domain *Bacteria*. The genome sequence of *Aquifex aeolicus* reveals that an entirely chemolithotrophic and autotrophic lifestyle is encoded by a genome of only 1.55 Mbp (one-third the size of the *Escherichia coli* genome). The discovery that so many hyperthermophilic species of *Archaea* and *Bacteria*, like *Aquifex*, are H_2 chemolithotrophs, coupled with the finding that they branch as very early lineages on their respective phylogenetic trees (Figure 16.1*a*), suggests that H_2 was a key electron donor for energy metabolism in primitive organisms that appeared on early Earth and also suggests that the earliest cells may have been thermophiles (◀ Section 13.1 and ▶ Section 17.14).

Thermocrinis

Thermocrinis (**Figure 16.52**) is a relative of *Aquifex* and *Hydrogenobacter*. This bacterium grows optimally at 80°C as a chemolithotroph oxidizing H_2, $S_2O_3^{2-}$, or S^0 as electron donors, with O_2 as electron acceptor. *Thermocrinis ruber*, the only known species, grows in the outflow of certain hot springs in Yellowstone National Park (Figure 16.52*a*) where it forms pink "streamers" consisting of a filamentous form of the cells attached to siliceous sinter (Figure 16.52*b*). In static culture, cells of *T. ruber* grow as individual rod-shaped cells (Figure 16.52*c*). However, when cultured in a flowing system in which growth medium is trickled over a solid glass surface to which cells can attach, *Thermocrinis* assumes the streamer morphology it forms in its constantly flowing habitat in nature.

(a)

(b)



(c)

Figure 16.52 ***Thermocrinis.*** *(a)* Octopus Spring, Yellowstone National Park (USA). The source water of this alkaline and siliceous hot spring is 92°C. *(b)* Cells of *Thermocrinis ruber* growing as filamentous streamers (arrow) attached to siliceous sinter in the outflow (85°C) of Octopus Spring (▶ Figure 17.19*b*). *(c)* Scanning electron micrograph of rod-shaped cells of *T. ruber* grown on a silicon-coated cover glass. A single cell of *T. ruber* is about 0.4 μm in diameter and from 1 to 3 μm long.

T. ruber is of historical significance in microbiology because it was one of the organisms discovered in the 1960s by Thomas Brock, a pioneer in the field of thermal microbiology and senior author of the first seven editions of this text. The discovery by Brock that the pink streamers (Figure 16.52*b*) contained protein and nucleic acids clearly indicated that they were living organisms and not just mineral debris. Moreover, the presence of streamers in 80–90°C hot spring outflow waters but not those of lower temperatures supported Brock's hypothesis that these organisms actually *required* heat for growth and were therefore likely to be present in even boiling or superheated waters. Both of these conclusions were subsequently supported by

the discovery by Brock, Karl Stetter of Germany, and many other microbiologists of dozens of genera of hyperthermophilic *Bacteria* and *Archaea* inhabiting hot springs, hydrothermal vents, and other thermal environments.More coverage of hyperthermophiles can be found in Section 4.13 and Chapter 17.

Check Your Understanding

- Of what evolutionary significance is the fact that organisms in the *Aquifex* lineage are both hyperthermophilic and H_2 chemolithotrophs?

VI • Other *Bacteria*

***Bacteria* such as *Acidobacteria* and *Fusobacteria* are difficult to cultivate but are abundant and important components of various natural habitats and the human microbiome, respectively. *Deinococcus* and *Thermus* can survive extremes of radiation and temperature, respectively.**

Thus far in this chapter we have focused on phyla that have many described species (Figure 16.1). Beyond these mainstream bacterial phyla are many others that have but one or at most a handful of characterized species (Figure 16.1*b*). In addition, many more phyla are known only from community sampling of 16S rRNA genes from nature (▶ Sections 19.6 and 19.8). We cannot cover them all. So in this final part of the chapter we consider two more phyla that have been well studied and then summarize some other phyla that are emerging into the mainstream of microbial diversity.

16.20 *Deinococcus–Thermus*

KEY GENERA: *Deinococcus, Thermus*

The *deinococci* group contains only a few characterized genera in two orders, the *Deinococcales* and the *Thermales*. Members of this phylum are typically aerobic chemoorganotrophs that metabolize sugars, amino and organic acids, or various complex mixtures. Though deinococci stain gram-positively, they have a gram-negative cell wall structure (**Figure 16.53**) made up of several layers, including an outer membrane, which is characteristic of gram-negative bacteria (◀ Section 2.3). However, unlike the outer membrane of bacteria such as *Escherichia coli*, the outer membrane of deinococci lack lipid A. Deinococci also contain an unusual form of peptidoglycan in which ornithine replaces diaminopimelic acid in the *N*-acetylmuramic acid cross-links (◀ Section 2.3).

Species of *Thermales* are typically thermophiles or hyperthermophiles and the type genus is *Thermus*. *Thermus aquaticus*, discovered in a Yellowstone National Park hot spring in the mid-1960s by Thomas Brock (Section 16.19), has been a model organism for studying life at high temperatures. *T. aquaticus* has subsequently been isolated from many thermal systems including private and commercial hot water heaters and is the source of *Taq* DNA polymerase. Because it is so heat-stable, *Taq* polymerase allowed the polymerase chain reaction (PCR) technique for amplifying DNA to be fully automated (◀ Section 12.1), an advance that revolutionized all of biology.

Radiation Resistance of *Deinococcus radiodurans*

Species of *Deinococcales* have the unusual property of being extremely radiation resistant, and *Deinococcus radiodurans* is the best-studied species in this regard. Most deinococci are red or pink due to carotenoids, and many are highly resistant to both radiation and desiccation. Resistance to ultraviolet (UV) radiation can be used to advantage in isolating deinococci. These remarkable organisms can be selectively isolated from soil, ground meat, dust, and filtered air following exposure of the sample to intense UV (or even gamma) radiation and plating on a rich medium containing tryptone and yeast extract. For example, *D. radiodurans* cells survive exposure to 15,000 grays (Gy) of ionizing radiation (1 Gy = 100 rad). This is

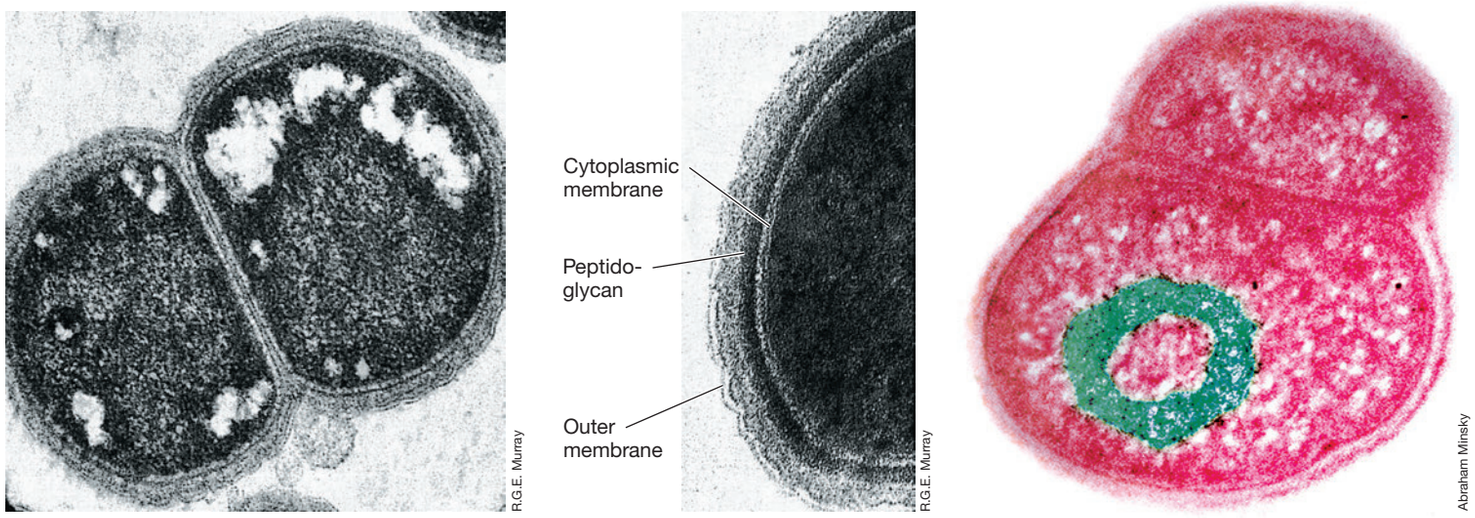


Figure 16.53 The radiation-resistant coccus *Deinococcus radiodurans*. An individual cell is about 2.5 μm in diameter. *(a)* Transmission electron micrograph of *D. radiodurans*. Note the outer membrane layer. *(b)* High-magnification micrograph of wall layer. *(c)* Transmission electron micrograph of cells of *D. radiodurans* colored to show the toroidal morphology of the nucleoid (green).

sufficient to shatter the organism's chromosome into hundreds of fragments (by contrast, some endospores are killed by lower levels, and a human can be killed by exposure to less than 10 Gy) (◀ Section 4.18).

In addition to impressive radiation resistance, *D. radiodurans* is resistant to the effects of many mutagenic agents. The only chemical mutagens that seem to work on *D. radiodurans* are agents such as nitrosoguanidine, which induces deletions in DNA. Deletions are apparently not repaired as efficiently as point mutations in this organism, and mutants of *D. radiodurans* can be isolated in this way.

DNA Repair in *Deinococcus radiodurans*

Studies of *D. radiodurans* have shown that it is highly efficient in repairing damaged DNA. Several different DNA repair enzymes exist in *D. radiodurans*. In addition to the DNA repair enzyme RecA (◀ Sections 9.4 and 9.5), several RecA-independent DNA systems exist in *D. radiodurans* that can repair breaks in single- or double-stranded DNA, and excise and repair misincorporated bases. In fact, repair processes are so effective that the chromosome can even be reassembled from a fragmented state.

It is also thought that the unique arrangement of DNA in *D. radiodurans* cells plays a role in radiation resistance. Cells of *D. radiodurans* always exist as pairs or tetrads (Figure 16.53*a*). Instead of scattering DNA within the cell as in a typical nucleoid, DNA in *D. radiodurans* is ordered into a toroidal (coiled, or stack of rings) structure (Figure 16.53*c*). Repair is then facilitated by the fusion of nucleoids from adjacent compartments, because their toroidal structure provides a platform for homologous recombination. From this extensive recombination, a single repaired chromosome emerges, and the cell containing this chromosome can then grow and divide.

Check Your Understanding

- Describe a commercial application of *Thermus aquaticus*.
- Describe an unusual biological feature of *Deinococcus radiodurans*.

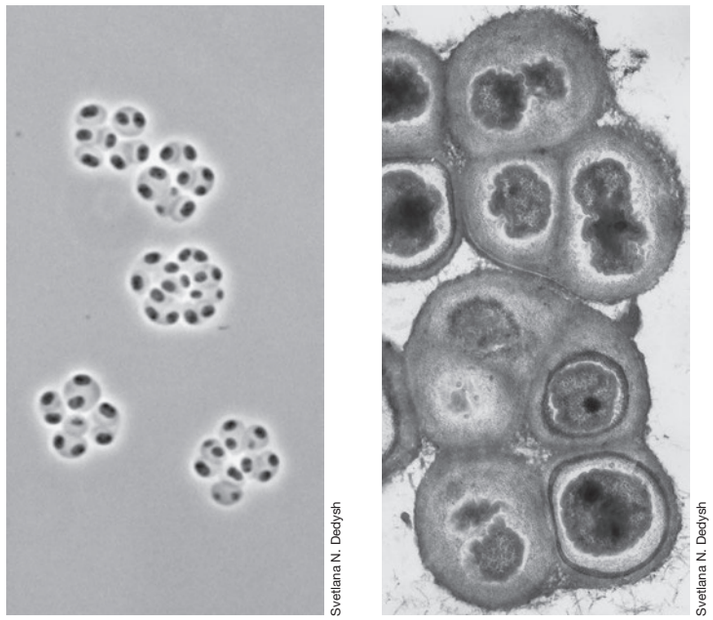
16.21 *Acidobacteria* and *Nitrospirae*

Surveys of environmental DNA (▶ Sections 19.6 and 19.8) reveal *Acidobacteria* and *Nitrospirae* to be abundant and widespread in the environment (Figure 16.1*b*). Despite their abundance, strains of these phyla have proven difficult to cultivate and much of our knowledge about the *Acidobacteria* and *Nitrospirae* comes from metagenomic surveys that detect their genes in natural samples (◀ Section 10.7). We begin with the *Acidobacteria*, which are notable for their diversity and abundance in soils (▶ Section 20.7 and Figure 20.14).

Acidobacteria

KEY GENUS: *Acidobacterium*

Acidobacteria are widespread in the environment as revealed by analyses of 16S rRNA genes (Figure 16.1*b*). *Acidobacteria* are common in soils, and especially in acid soils (pH < 6.0), where they often comprise a majority of soil microbial communities. *Acidobacteria* also inhabit freshwater, hot spring microbial mats, wastewater treatment reactors, and sewage sludge. Their abundance, widespread distribution, and phylogenetic diversity suggest they play important ecological roles, especially in soil. Unfortunately, while *Acidobacteria* are widespread in the environment, few have been cultivated (Figure 16.1*b*) and only a handful of genera have been described.



(a) (b)

Figure 16.54 Cells of *Acidisarcina polymorpha*. This species of *Acidobacteria* was isolated from acidic Russian soils and degrades both cellulose and chitin, polymers that are common in soil. Cells typically occur in cuboid packets containing to 6–8 cells encapsulated in a shared polysaccharide (see *Sarcina*, Figure 16.21). Both phase-contrast *(a)* and TEM *(b)* images are shown. Cells have an irregular shape and are 1.4–4.4 μm long.

Most described species of *Acidobacteria* are gram-negative, acid-tolerant, aerobic chemoorganotrophs. For example, *Acidobacterium capsulatum* is an acidophile isolated from acid mine drainage. An obligately aerobic encapsulated bacterium, it grows using various sugars and organic acids. *Acidisarcina polymorpha*, which grows in cuboidal packets of 6–8 cells (**Figure 16.54**), was isolated from an acidic boreal forest soil and is also acid-tolerant. This bacterium is aerobic and able to degrade polymers such as cellulose and chitin, which are common in soils. *Pyrinomonas methylaliphatogenes* is a thermophile isolated from thermally heated soil. This species is also an acidophilic aerobic chemoorganotroph. *P. methylaliphatogenes* is notable, however, because it can generate energy, but not grow, using atmospheric H_2, typically present in oxic environments in only trace amounts. It is unclear whether this ability is widespread among *Acidobacteria*, but if so, the ability to survive prolonged starvation might explain why *Acidobacteria* are so common in soils.

Acidophilic, aerobic chemoorganotrophs are common among cultivated strains of *Acidobacteria*, but this phylum contains tremendous phylogenetic diversity spanning at least 26 major subgroups. Not all of these subgroups appear to contain acidophilic organisms and not all are aerobic chemoorganotrophs. For example, *Geothrix fermentans* is a strict anaerobe capable of dissimilative iron reduction (◀ Section 15.13) and can also ferment citrate to acetate plus

succinate. *Holophaga foetida* is a strictly anaerobic acetogen (◀ Section 14.14) that grows by degrading methylated aromatic compounds to acetate. Finally, at least one genus, *Chloracidobacterium*, is phototrophic (◀ Section 15.8).

Nitrospirae

KEY GENUS: *Nitrospira*

The phylum *Nitrospirae* is named for the genus *Nitrospira*, a chemolithotroph that oxidizes nitrite to nitrate and grows autotrophically, as do species of the proteobacterium *Nitrobacter* (◀ Section 15.10). *Nitrospira* inhabits many of the same environments as *Nitrobacter*. However, environmental surveys have shown that *Nitrospira* is much more abundant than *Nitrobacter* in nature, and thus most of the nitrite oxidized in nitrogen-rich environments such as wastewater treatment plants and ammonia-rich soils is probably due to *Nitrospira*. Some species of *Nitrospira* found widely in soils have also been observed to contain the complete pathway for nitrification, oxidizing ammonia all the way to nitrate (◀ Sections 14.9, 15.10, and ▶ Section 21.3). Other key *Nitrospirae* include *Leptospirillum*, an aerobic, acidophilic, iron-oxidizing chemolithotroph (◀ Section 15.14) common in acid mine drainage associated with the mining of coal and iron (▶ Section 22.1).

Check Your Understanding

- In what kind of habitat would you expect to find *Acidobacteria* in high abundance? Make a hypothesis as to why *Acidobacteria* are favored in such habitats.
- What different reactions can *Nitrospira* species perform during the process of nitrification?

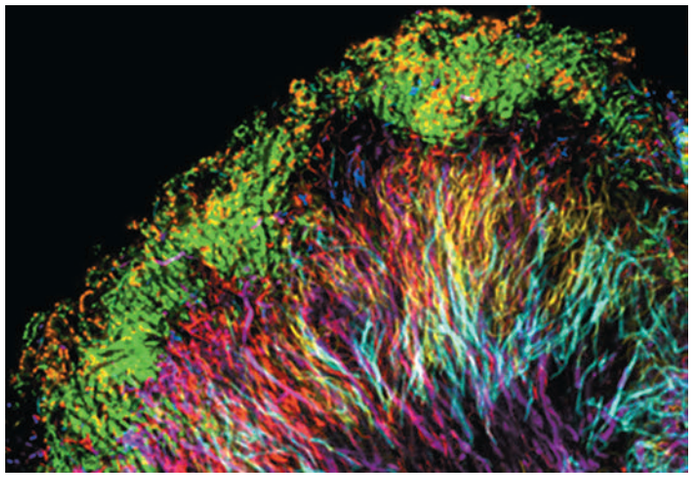
16.22 Other Notable Phyla of *Bacteria*

The basic properties of several other phyla of *Bacteria* are discussed briefly below. Although most of these phyla have few cultured representatives (Figure 16.1*b*), many may well be of considerable importance. If so, future research on their culture and ecological activities will supply the necessary proof. Until then, we paint a picture of these phyla with a broad brush to summarize their major characteristics in a general way.

Fusobacteria

Fusobacteria are gram-negative nonsporulating rods found in sediments and also the gastrointestinal systems and oral cavities of animals. *Fusobacteria* are obligate anaerobes that ferment carbohydrates, peptides, and amino acids. Species of the genus *Fusobacterium* are a common component of the human microbiome (Chapter 24). *Fusobacteria* have adhesions, molecules that allow them to form strong attachments to other cells, both bacterial and animal. These adhesions allow *Fusobacteria* to play an important structural role in the formation of biofilms called dental plaque in the oral cavity (**Figure 16.55**). *Fusobacteria* are difficult to cultivate and so their role in the human microbiome was not fully appreciated until the development of DNA-based techniques for studying microbial communities (▶ Section 19.8).

While *Fusobacteria* are common in the microbiome of healthy individuals (Chapter 24), they are also implicated in several human diseases and syndromes. In particular, *Fusobacteria* increase in abundance in individuals suffering from a range of oral diseases. Inflammation of gum tissue and tears in blood vessels allow *Fusobacteria* and other oral bacteria access to the bloodstream. Such bacteria can be found in arterial plaques and may even play a role in heart disease. In addition, *Fusobacteria* in the bloodstream have also been shown to cause adverse pregnancy outcomes. Finally, *Fusobacteria* in the gut have been shown to attach, colonize, and enhance the growth of colorectal cancer cells. In mice with such tumors, treatment with the antibiotic metronidazole both reduced the abundance of *Fusobacteria* in tumors and reduced tumor growth. It would seem that we still have a lot to learn about the *Fusobacteria*.



Jessica Mark-Welch and Gary Borisy

Figure 16.55 Cells of *Fusobacterium* in human dental plaque. The photo is a light micrograph showing a section of human dental plaque stained by fluorescence in situ hybridization (FISH, ▶ Section 19.5). While streptococci (Section 16.6), stained green here, are common in plaque and are the primary cause of dental caries (cavities, ▶ Section 24.3), plaque is a complex biofilm composed of many different species (each in different colors, see Figures 24.10 and 25.8 and the Chapter 19 MicrobiologyNow on page 648). *Fusobacterium*, stained yellow here, are filamentous bacteria with adhesins that allow them to attach to other cells. This trait allows them to colonize cellular surfaces and play a role in structurally supporting these complex biofilms. *Fusobacteria* grow within the biofilm along with other anaerobic filamentous organisms providing an anchor for lactic acid bacteria that grow closer to the biofilm surface.

Fibrobacteres and *Synergistetes*

Fibrobacteres and *Synergistetes* contain relatively few characterized species (Figure 16.1*b*), but those that have been cultured employ fermentative metabolisms similar to *Fusobacteria*. Species in these groups are often associated with the gastrointestinal tracts of animals.

While 16S ribosomal RNA genes from *Fibrobacteres* can be recovered from a wide range of habitats, the only characterized species have come from either the rumen or gastrointestinal tracts of animals. The genus *Fibrobacter* contains gram-negative fermentative strict anaerobes. However, unlike most *Fusobacteria* and *Synergistetes*, species of *Fibrobacter* are unable to ferment proteins or amino acids and specialize instead in the fermentation of carbohydrates, including cellulose. In the rumen, cellulose is the major source of energy, and in such environments it supports not only cellulolytic bacteria such as *Fibrobacter* but many noncellulolytic anaerobes that use glucose released during cellulose degradation.

Synergistetes are gram-negative nonsporulating rods found in association with animals and in anoxic environments in terrestrial and marine systems. Described species are typically obligate anaerobes that degrade proteins and are capable of fermenting amino acids. In animals they are most often found in the gastrointestinal tract; for example, *Synergistes jonesii* inhabits the rumen (▶ Section 23.15). In humans, species of *Synergistetes* have been associated with certain soft tissue wounds and abscesses, dental plaque, and periodontal conditions.

Deferribacteres and *Chrysiogenetes*

The phyla *Deferribacteres* and *Chrysiogenetes* (Figure 16.1) contain anaerobic chemoorganotrophs that display considerable metabolic diversity with respect to the electron acceptors used in anaerobic respirations (Chapter 14 and ◀ Figure 14.1). Most, though not all, species are able to grow through anaerobic respiration of nitrate to nitrite or ammonium. The *Deferribacteres* group is named for the genus *Deferribacter*, a thermophilic dissimilative ferric iron-reducer (◀ Sections 14.13 and 15.13) that can also reduce nitrate and metal oxides. *Geovibrio* is a related genus that can also grow using elemental sulfur as an electron acceptor (◀ Section 15.11). The bacterium *Chrysiogenes arsenatis* and its relatives are notable for the ability to couple the oxidation of acetate and a few other organic compounds to the reduction of arsenate as a terminal electron acceptor, reducing it to arsenite. In addition to arsenate, many species of *Chrysiogenetes* can reduce selenate, nitrite, nitrate, thiosulfate, and elemental sulfur in anaerobic respirations (◀ Section 14.13).

Check Your Understanding

- How do *Nitrospira* (Section 16.21) and *Deferribacter* differ in terms of lifestyle and metabolism?
- What metabolic characteristics are shared by most *Fusobacteria*, *Fibrobacteres*, and *Synergistetes*, and what disease in humans has been correlated with the presence of *Synergistetes* and *Fusobacteria*?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • *Proteobacteria*

16.1 The *Alphaproteobacteria* are the second largest class of *Proteobacteria* and metabolically diverse. Key genera are *Rhizobium*, *Rickettsia*, *Rhodobacter*, and *Caulobacter*.

Q Which genera of *Alphaproteobacteria* are known to form nitrogen-fixing nodules in plants?

16.2 The *Betaproteobacteria* are the third largest class of *Proteobacteria* and metabolically diverse. Key genera are *Burkholderia*, *Rhodocyclus*, *Neisseria*, and *Nitrosomonas*.

Q Which order of *Betaproteobacteria* does *Nitrosospira* belong to?

16.3 The *Gammaproteobacteria* are the largest and most diverse class of *Proteobacteria* and contain many human pathogens. The *Enterobacteriales*, or enteric bacteria, are the most heavily studied of all bacteria. Key genera are *Escherichia* and *Salmonella*.

Q What is the catalase test? What catalase reaction would you expect from an obligate aerobe? What reaction would you expect from an obligate anaerobe?

16.4 The *Pseudomonadales* and *Vibrionales* are among the most common *Gammaproteobacteria*. Key genera are *Pseudomonas* and *Vibrio*.

Q Why is *P. aeruginosa* resistant to many widely used antibiotics?

16.5 The *Deltaproteobacteria* and *Epsilonproteobacteria* are smaller and less metabolically diverse classes of *Proteobacteria*. Key genera of *Deltaproteobacteria* are *Myxococcus*, *Desulfovibrio*, and *Geobacter*. Key genera of *Epsilonproteobacteria* are *Campylobacter* and *Helicobacter*.

Q Which environments do *Epsilonproteobacteria* typically inhabit? What role do they play in these environments?

II • *Firmicutes*, *Tenericutes*, and *Actinobacteria*

16.6 Lactic acid bacteria such as *Lactobacillus* and *Streptococcus* produce lactate as the primary end product of fermentation, and they have many roles in food production and preservation. The *Firmicutes* are one of the two main phyla of gram-positive bacteria.

Q How are lactic acid bacteria different from other anaerobes, and why are they usually restricted to environments which contain sugar?

16.7 Many genera of *Firmicutes* in the orders *Bacillales* and *Clostridiales*, including *Staphylococcus*, *Listeria*, and *Sarcina*, are unable to form endospores.

Q What characteristics of *Listeria* make it a frequent cause of foodborne illness?

16.8 Production of endospores is a hallmark of the key genera *Bacillus* and *Clostridium* and is only found in the phylum *Firmicutes*.

Q What is a good strategy for isolating spore-forming bacteria from an environmental sample?

16.9 The phylum *Tenericutes* contains the mycoplasmas, organisms that lack cell walls and have very small genomes. Many species are pathogenic for humans, other animals, and plants. The key genus is *Mycoplasma*.

Q Provide an explanation for why the absence of a cell wall might be favorable to *Mycoplasma*.

16.10 *Actinobacteria* are the second major phylum of gram-positive bacteria. *Corynebacterium* and *Arthrobacter* are common gram-positive soil bacteria. *Propionibacterium* ferments lactate to propionate and is the key agent responsible for the unique flavor and texture of Swiss cheese.

Q In what sort of environment would you expect to find large numbers of *Actinobacteria*?

16.11 Species of *Actinobacteria* in the genus *Mycobacterium* are mainly harmless soil saprophytes, but *Mycobacterium tuberculosis* causes the disease tuberculosis.

Q How does the cell wall of *Mycobacterium* influence its reaction to the Gram stain and the acid-fast stain?

16.12 The streptomycetes are a large group of filamentous, gram-positive bacteria that form spores at the end of aerial filaments and are found in the phylum *Actinobacteria*. Many clinically useful antibiotics such as tetracycline and neomycin have come from *Streptomyces* species.

Q How are the spores of streptomycetes different from endospores?

III • *Bacteroidetes*

16.13 The phylum *Bacteroidetes* includes gram-negative rods that do not form spores, many of which have gliding motility. Most species in the order *Bacteroidales* are obligate anaerobes that ferment carbohydrates in anoxic environments. The genus *Bacteroides* contains species that are common in the gastrointestinal tract of animals.

Q What species of *Bacteroidetes* is most abundant in the human gastrointestinal tract, and what role does this organism play in the human gut?

16.14 The *Cytophagales* and *Flavobacteriales* are orders in the *Bacteroidetes* that include aerobic bacteria able to degrade complex polysaccharides such as cellulose. These bacteria are important in organic matter decomposition.

Q What is an exoenzyme and why are these types of organisms important in the degradation of cellulose?

IV • *Chlamydiae*, *Planctomycetes*, and *Verrucomicrobia*

16.15 The phylum *Chlamydiae* includes small obligate intracellular parasites that lack FtsZ and are adept at invading eukaryotic cells. Many species cause various diseases in humans and other animals.

Q Describe the infection cycle of *Chlamydia*.

16.16 The *Planctomycetes* are a group of stalked, budding bacteria that lack FtsZ and have extensive invaginations of the cytoplasmic membrane.

Q What is an anammoxosome and where would you expect to find one?

16.17 Species of *Verrucomicrobia* are distinguished by the multiple prosthecae on their cells and their unique phylogeny.

Q Verruca is a word of Latin origin that means "wart." How do you think the *Verrucomicrobia* got their name?

V • Hyperthermophilic *Bacteria*

16.18 *Thermotogae* and *Thermodesulfobacteria* form two deeply branching phyla within the *Bacteria*. These hyperthermophilic bacteria have proven that extensive horizontal gene transfer has occurred from *Archaea* to *Bacteria* (*Thermotoga*) and that ether-linked lipids are not limited to the *Archaea* (*Thermodesulfobacterium*).

Q How are ether-linked lipids in *Thermodesulfobacterium* unusual?

16.19 The *Aquificae* phylum contains a group of hyperthermophilic, H_2-oxidizing bacteria that form the earliest branch on the tree of the domain *Bacteria*.

Q In what environment might you observe *Thermocrinis ruber*, and what role did this organism play in the discovery of hyperthermophiles?

VI • Other *Bacteria*

16.20 *Deinococcus* and *Thermus* are the major genera in a distinct phylum of *Bacteria*. *Thermus* is the source of the key enzyme in automated PCR, whereas *Deinococcus* is the most radiation-resistant bacterium known, exceeding even many endospores in this regard.

Q What are some of the remarkable properties that allow *Deinococcus* to survive exposure to massive doses of radiation?

16.21 *Acidobacteria* are widespread in many environments, especially acidic soils. While aerobic, acidophilic, chemoorganotrophs are common, the phylum is metabolically and phylogenetically diverse. The genus *Nitrospira* includes ammonia and nitrite-oxidizing bacteria.

Q List four ways in which different species of *Acidobacteria* have been shown to generate energy.

16.22 Species of *Deferribacteres* and *Chrysiogenetes* specialize in various forms of anaerobic respiration. Species of *Synergistetes*, *Fusobacteria*, and *Fibrobacteres* are fermentative anaerobes that inhabit the gastrointestinal tract and other anoxic sites in animals.

Q How is it that *Fusobacteria* from the oral cavity are able to colonize arterial plaque in individuals with heart disease?

APPLICATION QUESTIONS

1. Enteric bacteria, lactic acid bacteria, and propionic acid bacteria have distinctive metabolic traits that can be used to characterize and identify these organisms. Describe the metabolic characteristics of these organisms, name a genus that belongs to each group, and indicate in what way these organisms can be differentiated.
2. Microorganisms have a variety of relationships with oxygen. Describe the terms used to characterize a cell's response to oxygen and give an example from this chapter of an organism that can be described by each of these terms.

CHAPTER GLOSSARY

Acid-fastness a property of *Mycobacterium* species in which cells stained with the dye basic fuchsin resist decolorization with acidic alcohol

Actinomycetes a term used to refer to aerobic filamentous bacteria in the phylum *Actinobacteria*

Coryneform bacteria gram-positive, aerobic, nonmotile, rod-shaped organisms with the characteristic of forming irregular-shaped, club-shaped, or V-shaped cell arrangements, typical of several genera of unicellular *Actinobacteria*

Enteric bacteria a large group of gram-negative, rod-shaped *Bacteria* characterized by a facultatively aerobic metabolism and commonly found in the intestines of animals

Heterofermentative in reference to lactic acid bacteria, capable of making a mixture of fermentation products typically including lactate, ethanol and CO_2

High G + C gram-positive bacteria a term that refers to bacteria in the *Actinobacteria*

Homofermentative in reference to lactic acid bacteria, producing only lactic acid as a fermentation product

Lactic acid bacteria fermentative bacteria that produce lactic acid, are found in the *Firmicutes*, and are important in the production and preservation of many foods

Low G + C gram-positive bacteria a term that refers to bacteria in the *Firmicutes*

Oligotrophic a term that refers to organisms that grow best under low-nutrient conditions

Propionic acid bacteria gram-positive fermentative bacteria that generate propionate as a fermentation end product and are important in the production of cheese

Proteobacteria the largest and most metabolically diverse phylum of bacteria

Pseudomonad a term used to refer to any gram-negative, polarly flagellated, aerobic rod able to use a diverse suite of carbon sources

17 Diversity of *Archaea*

I *Euryarchaeota* 594

II *Thaumarchaeota* and Cryptic Archaeal Phyla 603

III *Crenarchaeota* 608

IV Evolution and Life at High Temperature 614

MICROBIOLOGYNOW

Methanogens and Global Climate Change

A diversity of methanogens exists within the domain *Archaea*. Methanogens are among the oldest microbes on Earth and have a tremendous impact on our biosphere. Methanogens in nature promote the anaerobic decomposition of organic matter by consuming the products of fermentative and syntrophic metabolisms. Methanogens release methane as a waste product, and this methanogenesis is a natural part of Earth's carbon cycle. However, atmospheric methane has been increasing dramatically since the dawn of the industrial age. Methane, like CO_2, traps heat in the atmosphere, contributing to the greenhouse effect. However, molecule for molecule, methane traps far more heat than does CO_2. Moreover, whereas the increase in atmospheric CO_2 comes mostly from burning fossil fuels, the increase in atmospheric methane comes mostly from human activities that promote methanogenesis.

Human activities have created many new opportunities for methanogens. In particular, domesticated ruminants such as cows, sheep, and goats harbor methanogens within their rumens. In addition, during rice production, farmers flood the soil, generating artificial wetlands that release huge amounts of methane. These two agricultural activities alone account for a major fraction of global methane emissions. Remarkably, however, human activities have even created new habitats for methanogens under glaciers. The meltwater from this Greenland glacier (photo) is supersaturated with methane and contains DNA from cells of the *Methanomicrobiales*, *Methanosarcinales*, and *Methanobacteriales*, highly diverse groups of methanogens. As climate change accelerates glacier melting, the meltwater makes available ancient organic carbon buried beneath the ice. This meltwater creates large subglacial wetlands that are rapidly colonized by cold-active methanogens.

Massive amounts of organic carbon have been locked away for eons in frozen Arctic soils. As temperatures on Earth increase, this carbon thaws, creating new habitats for methanogens. Thus, accurately predicting the consequences of global climate change will require us to understand methanogen diversity and how humans affect it.

Source: Lamarche-Gagnon, G., et al. 2019. Greenland melt drives continuous export of methane from the ice-sheet bed. *Nature* 565: 73.

The domain *Archaea* is named for the Archaean eon, the period of geological history when life first spread across Earth (◀ Figure 13.1). In the Archaean, high temperatures and an atmosphere devoid of O_2 and thick in toxic gases enveloped Earth. *Archaea* were once thought to be remnants of this forgotten age since many *Archaea* live in extreme environments such as volcanic systems or salt ponds. We now know, however, that *Archaea* occupy a wide range of habitats and they perform important biogeochemical reactions in soil, the oceans, wetlands, and even in the guts of animals. While *Archaea* and *Bacteria* are both single-celled organisms with prokaryotic cell structure (Chapter 2), these domains are highly differentiated both genetically and physiologically. In many ways, *Archaea* share more features with *Eukarya* than with *Bacteria*. Indeed, it is likely that archaeal cells contributed fundamentally to the origin of the domain *Eukarya* (◀ Section 13.4 and Figure 13.10). In this chapter, we will learn about the enormous phylogenetic and physiological diversity found within the domain *Archaea*.

The *Archaea* are nearly as diverse as the *Bacteria*, but the vast majority of *Archaea* have thus far proven difficult to grow in culture. Hence, it is likely that we still have much to learn about the breadth of characteristics in organisms in this domain. To date, most well-characterized species of *Archaea* come from only two phyla: the *Euryarchaeota* and the *Crenarchaeota* (**Figure 17.1**). In addition, several species have been isolated from the phylum *Thaumarchaeota*. By contrast, the phyla *Korarchaeota* and *Nanoarchaeota* are represented only by strains grown in coculture or studied using enrichment techniques (▶ Section 19.1). The use of metagenomics (◀ Section 10.7) and single-cell genome sequencing (◀ Section 10.11) has led to an explosion in our understanding of the phylogenetic and physiological diversity of the archaeal domain. As a result, many new phyla of *Archaea* such as representatives of the DPANN, TACK, and Asgard groups (Figure 17.1) have been discovered relatively recently.

While all *Archaea* share certain features, this domain encompasses considerable physiological diversity. Common traits of all *Archaea* include ether-linked lipids, a lack of peptidoglycan in cell walls (Chapter 2), and structurally complex RNA polymerases that resemble those of *Eukarya* (◀ Section 6.6). These features were likely present in the last common ancestor of the *Archaea*. Genomic analyses suggest that the earliest *Archaea* lived at high temperature, oxidized H_2 as an energy source, and fixed CO_2 using the reductive acetyl-CoA pathway (◀ Section 14.14 and Figure 14.34). Despite their shared phylogenetic origin, contemporary species of *Archaea* are metabolically diverse. *Archaea* can be chemoorganotrophs or chemolithotrophs, they can be respiratory or fermentative, and they can be aerobic or anaerobic using a wide diversity of electron donors and acceptors (Chapter 14).

In addition, certain physiological capabilities are uniquely found within the domain *Archaea*. Methane production, for example, is a unique characteristic of *Archaea* called **methanogens** (◀ Section 14.15, Figures 14.38 and 14.39). Methanogenesis evolved very early within the archaeal domain, and all well-characterized methanogens belong to the phylum *Euryarchaeota*. Methanogenesis is a globally important process that has produced virtually all of the natural gas on Earth and has a significant effect on climate because methane is a strong "greenhouse gas" (▶ Sections 21.1, 21.2; and see page 592). *Archaea* are also well known for containing many examples of **extremophiles** (◀ Section 1.5 and Table 1.2) including **hyperthermophiles** (organisms with growth temperature optima above 80°C), as well as halophiles, acidophiles, and psychrophiles (Chapter 4).

With this brief background and the phylogeny of *Archaea* firmly in mind (Figure 17.1), we now consider the organismal diversity of this fascinating domain of life.

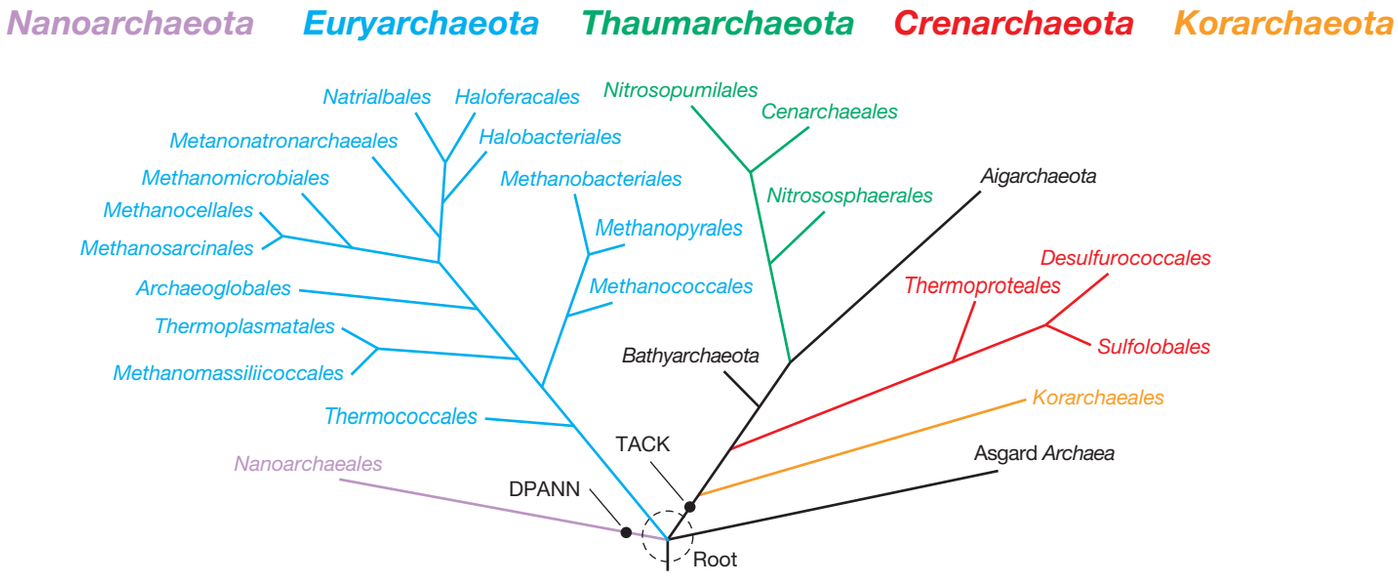


Figure 17.1 Schematic representation of the phylogeny of the major taxonomic orders within the domain *Archaea*. Five major archaeal phyla (studied in pure or highly enriched cultures) and their most representative orders are indicated in color, while other archaeal taxa are indicated in black. While archaeal phyla are nearly as numerous as *Bacteria*, far fewer *Archaea* have been cultivated. Archaeal phyla can be organized into four major branches, or superphlya, consisting of the *Euryarchaeota*, DPANN (represented here by *Nanoarchaeota*, see Section 17.6), TACK (composed of *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, *Korarchaeota*, and related lineages, see Section 17.8), and Asgard *Archaea* (see Section 17.8). The root of the archaeal tree lies between these four branches though the exact position remains uncertain.

I • *Euryarchaeota*

The phylum *Euryarchaeota* contains most cultured species of *Archaea*. The group is metabolically diverse and includes methanogens, halophiles, thermophiles, and hyperthermophiles.

Euryarchaeota comprise a large and physiologically diverse group of *Archaea*. This phylum includes methanogens as well as many genera of extremely halophilic (salt-loving) *Archaea*. As a study in physiological contrasts, these two groups are remarkable: Methanogens are the strictest of anaerobes while extreme halophiles are primarily obligate aerobes. Other groups of *Euryarchaeota* include the hyperthermophiles *Thermococcus* and *Pyrococcus*, the hyperthermophilic methanogen *Methanopyrus*, and the cell wall–less *Thermoplasma*, an organism phenotypically similar to the mycoplasmas (◀ Section 16.9). We begin our review of *Euryarchaeota* by reviewing the extremely halophilic *Archaea*.

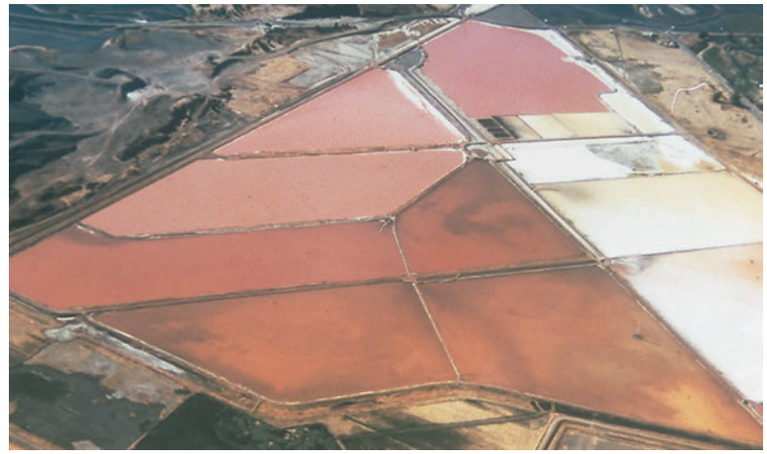
17.1 Extremely Halophilic *Archaea*

KEY GENERA: *Halobacterium, Haloferax, Natronobacterium*

Extremely halophilic *Archaea*, often given the nickname "haloarchaea," are a diverse group that inhabits environments high in salt. These include naturally salty environments, such as solar salt evaporation ponds and salt lakes, and artificial saline habitats such as the surfaces of heavily salted foods, for example, certain fish and meats. Such salty habitats are called *hypersaline* (Figure 17.2). The term **extreme halophile** is used to indicate that these organisms not only are halophilic but require a very high level of salt, in some cases at levels near saturation (◀ Section 4.15 and Figure 4.27).

An organism is considered an extreme halophile if it requires 1.5 M (about 9%) or more sodium chloride (NaCl) for growth. Most species of extreme halophiles require 2–4 M NaCl (12–23%) for

(a)



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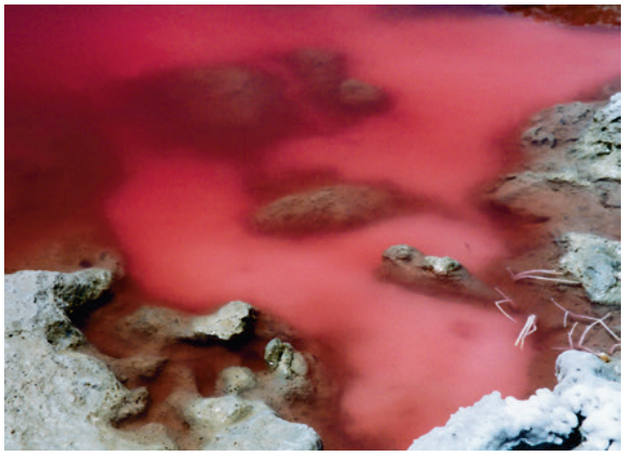

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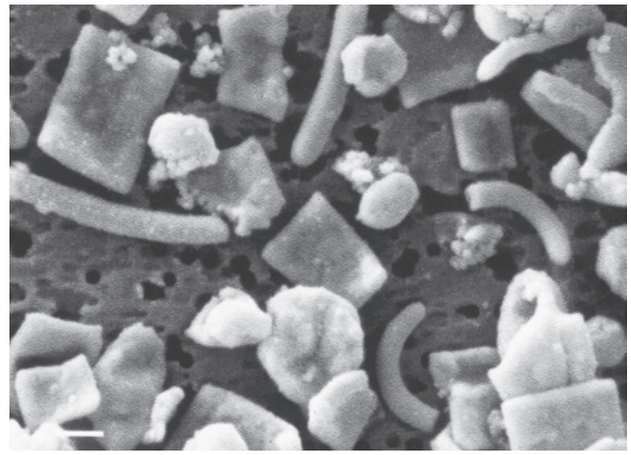


(d)

Figure 17.2 Hypersaline habitats for halophilic *Archaea*. Hypersaline habitats are home to the halophilic *Archaea*. These organisms not only tolerate salt but *require* salt, and typically in large amounts. *(a)* The north arm of Great Salt Lake, Utah, a hypersaline lake in which the ratio of ions is similar to that of seawater, but in which absolute concentrations of ions are several times that of seawater. The green color is primarily from cells of cyanobacteria and green algae. *(b)* Aerial view near San Francisco Bay, California, of a series of seawater evaporating ponds where solar salt is prepared. The red-purple color is predominantly due to bacterioruberins and bacteriorhodopsin in cells of haloarchaea. *(c)* Lake Hamara, Wadi El Natroun, Egypt. A bloom of pigmented haloalkaliphiles is growing in this pH 10 soda lake. Note the deposits of trona ($NaHCO_3 \cdot Na_2CO_3 \cdot 2\ H_2O$) around the edge of the lake. *(d)* Scanning electron micrograph of halophilic bacteria including square *Archaea* present in a saltern in Spain.

optimal growth and can grow at salinities as high as 5.5 M NaCl (32%, the limit of saturation for NaCl), although some species grow very slowly at this salinity. Some phylogenetic relatives of extremely halophilic *Archaea*, for example species of *Haloferax* and *Natronobacterium*, are able to grow at much lower salinities, such as at or near that of seawater (about 2.5% NaCl).

Hypersaline Environments: Chemistry and Productivity

Hypersaline habitats are common throughout the world, but extremely hypersaline habitats are rare. Most such environments are in hot, dry areas of the world. Salt lakes can vary considerably in ionic composition. The predominant ions in a hypersaline lake depend on the surrounding topography, geology, and general climatic conditions. Great Salt Lake in Utah (USA) (Figure 17.2*a*), for example, is essentially concentrated seawater. In this hypersaline lake the relative proportions of the various ions [e.g., sodium (Na^+), chloride (Cl^-), and sulfate (SO_4^{2-})] are those of seawater, although the overall concentration of ions is much higher. In addition, the pH of this hypersaline lake is slightly alkaline.

Soda lakes, in contrast, are highly alkaline hypersaline environments. The water chemistry of soda lakes resembles that of hypersaline lakes such as Great Salt Lake, but because high levels of carbonate minerals are also present in the surrounding strata, the pH of soda lakes is quite high. Waters of pH 10–12 are not uncommon in these environments (Figure 17.2*c*). In addition, the divalent cations Ca^{2+} and Mg^{2+} are present in only trace amounts in soda lakes because they precipitate out as carbonate salts at high pH and carbonate concentrations.

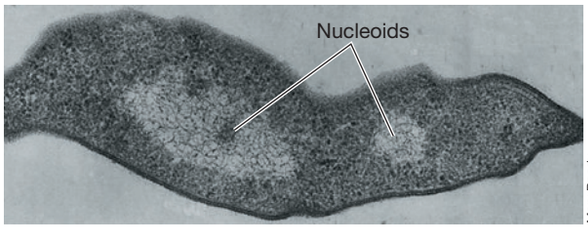
The diverse chemistries of hypersaline habitats have selected for a large diversity of halophilic microorganisms. Some organisms are unique to one environment while others are widespread. Moreover, despite their extreme conditions, salt lakes can be highly productive ecosystems (the word *productive* here means high levels of autotrophic CO_2 fixation). *Archaea* are not the only microorganisms present. The eukaryotic alga *Dunaliella* (▶ Figure 18.34*a*) is the major, if not the sole, oxygenic phototroph in most salt lakes. In highly alkaline soda lakes where *Dunaliella* is absent, anoxygenic phototrophic purple bacteria of the genera *Ectothiorhodospira* and *Halorhodospira* (◀ Section 15.4) predominate. Organic matter originating from primary production by oxygenic or anoxygenic phototrophs sets the stage for growth of haloarchaea, which are chemoorganotrophic organisms. In addition, a few extremely halophilic chemoorganotrophic *Bacteria*, such as *Halanaerobium*, *Halobacteroides*, and *Salinibacter*, thrive in such environments.

Marine salterns are also habitats for extreme halophiles. Marine salterns are enclosed basins filled with seawater left to evaporate, eventually yielding solar sea salt (Figure 17.2*b*, *d*). As salterns approach the minimum salinity limits for haloarchaea, the waters turn a reddish-purple color due to the massive growth—called a *bloom*—of cells (the red coloration apparent in Figure 17.2*b* and *c* is due to carotenoids and other pigments to be discussed later). Morphologically unusual *Archaea* are often present in salterns, including species with a square or cup-shaped morphology (Figure 17.2*d*). Extreme halophiles are also present in highly salted foods, such as certain types of sausages, marine fish, and salted pork.

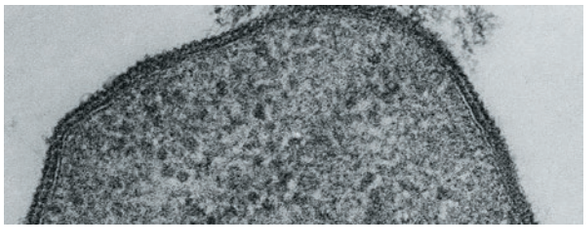
Taxonomy and Physiology of Extremely Halophilic *Archaea*

The extremely halophilic *Archaea* are found within the taxonomic orders *Halobacteriales*, *Natrialbales*, and *Haloferacales*, which share a common ancestor within the *Euryarchaeota* (Figure 17.1). These three orders constitute the haloarchaea, but they are also sometimes called "halobacteria" because the genus *Halobacterium* (**Figure 17.3**), the best-studied representative of the extreme halophiles, was discovered and characterized long before the *Archaea* were recognized as an independent domain. Many genera of *Natrialbales*, including *Natronobacterium*, *Natronomonas*, and their relatives, differ from other extreme halophiles in being extremely alkaliphilic as well as halophilic. As befits the geochemistry of their soda lake habitat (Figure 17.2*c*), these natronobacteria grow optimally at low Mg^{2+} concentrations and alkaline pH (9–11).

Haloarchaea stain gram-negatively, reproduce by binary fission, and do not form spores. Cells of the various cultured genera are rod-shaped, cocci, or cup-shaped, but even cells that form squares are known (Figure 17.2*d*). Cells of *Haloquadratum* are square and only about 0.1 μm thick. *Haloquadratum* also forms gas vesicles (◀ Section 2.7) that allow cells of this organism to float in its hypersaline habitat, probably as a means to be in contact with air since most extreme halophiles are obligate aerobes. Many other extremely halophilic *Archaea* also produce gas vesicles. A few strains of extreme halophiles are weakly motile by *archaella*, the archaeal analog of bacterial flagella, that rotate to propel the cell forward (◀ Section 2.9), but most halophiles lack archaella. The genomes of *Halobacterium* and *Halococcus* are unusual in that large plasmids (◀ Section 6.2) containing up to 30% of the total cellular DNA are present and the G + C base ratio of these plasmids (near 60% G + C) differs significantly from that of chromosomal DNA (66–68% G + C).



(a)



(b)

Figure 17.3 Electron micrographs of thin sections of the extreme halophile *Halobacterium salinarum*. A cell is about 0.8 μm in diameter. *(a)* Longitudinal section of a dividing cell showing the nucleoids. *(b)* High-magnification electron micrograph showing the glycoprotein subunit structure of the cell wall. Archaeal cell walls are discussed in Section 2.5.

Plasmids from extreme halophiles are among the largest naturally occurring plasmids known.

Most haloarchaea use amino acids or organic acids as electron donors aerobically and require a number of growth factors such as vitamins for optimal growth. A few haloarchaea oxidize carbohydrates aerobically, but this capacity is rare; sugar fermentation does not occur. Electron transport chains containing cytochromes of the *a*, *b*, and *c* types are present in *Halobacterium*, and energy is conserved via a proton motive force arising from electron transport (◀ Sections 3.8 and 3.9). Some haloarchaea can grow anaerobically, as growth by anaerobic respiration linked to the reduction of nitrate or fumarate (◀ Sections 3.10, 14.11, and 14.13) has been demonstrated in certain species.

Water Balance in Extreme Halophiles

Extremely halophilic *Archaea* require large amounts of NaCl for growth. Detailed salinity studies of *Halobacterium* have shown that the requirement for Na^+ cannot be satisfied by any other ion, even the chemically related ion potassium (K^+). However, cells of *Halobacterium* need *both* Na^+ and K^+ for growth because each plays an important role in maintaining osmotic balance.

As we learned in Section 4.15, microbes must withstand the osmotic forces they face in their habitats. To do so in a high-solute environment such as the salt-rich habitats of *Halobacterium*, organisms must either accumulate or synthesize solutes intracellularly. These solutes are called **compatible solutes** (◀ Table 4.6). These compounds counteract the tendency of the cell to become dehydrated under conditions of high osmotic strength by placing the cell in positive water balance with its surroundings. Cells of *Halobacterium*, however, do not synthesize or accumulate organic compounds but instead pump large amounts of K^+ from the environment into the cytoplasm. This ensures that the concentration of K^+ *inside* the cell is even greater than the concentration of Na^+ *outside* the cell (**Table 17.1**). This ionic condition maintains positive water balance.

The *Halobacterium* cell wall (Figure 17.3*b*) is composed of glycoprotein and is stabilized by Na^+. Sodium ions bind to the outer surface of the *Halobacterium* wall and are absolutely essential for maintaining cellular integrity. When insufficient Na^+ is present, the cell wall breaks apart and the cell lyses. This is a consequence of the exceptionally high content of the *acidic* (negatively charged) amino acids aspartate and glutamate in the glycoprotein of the *Halobacterium* cell wall. The negative charge on the carboxyl group of these amino acids is bound to Na^+; when Na^+ is diluted away, the negatively charged parts of the proteins tend to repel each other, leading to cell lysis.

Mastering Microbiology
Art Activity: Figure 17.4 Model for the mechanism of bacteriorhodopsin

TABLE 17.1 Concentration of ions in cells of *Halobacterium salinarum*[a]

Ion	Concentration in medium (M)	Concentration in cells (M)
Na^+	4.0	1.4
K^+	0.032	4.6
Mg^{2+}	0.13	0.12
Cl^-	4.0	3.6

[a]Data from Christian, J.H.B, and Waltho, J.A. 1962. *Biochim. Biophys. Acta 65*: 506.

Halophilic Cytoplasmic Components

Like cell wall proteins, cytoplasmic enzymes of *Halobacterium* are highly acidic, but it is K^+, not Na^+, that is required for activity. This makes sense because K^+ is the predominant cation in the cytoplasm of cells of *Halobacterium* (Table 17.1). Besides having a highly acidic amino acid composition, halobacterial cytoplasmic proteins typically contain lower levels of hydrophobic amino acids and lysine, a positively charged (basic) amino acid, than do proteins of nonhalophiles. This is also to be expected because in a highly ionic cytoplasm, more polar proteins would tend to remain in solution whereas less polar proteins would tend to cluster and perhaps lose activity. The ribosomes of *Halobacterium* also require high K^+ levels for stability, whereas ribosomes of nonhalophiles have no K^+ requirement.

Extremely halophilic *Archaea* are thus well adapted to life in a highly ionic environment. Cellular components exposed to the external environment require high Na^+ levels for stability, whereas cytoplasmic components require high K^+ levels. With the exception of a few extremely halophilic species of *Bacteria* that also use K^+ as a compatible solute, in no other group of prokaryotic cells do we find this unique requirement for such high amounts of specific cations.

Bacteriorhodopsin and Light-Mediated ATP Synthesis in Haloarchaea

Certain species of haloarchaea can catalyze a light-driven synthesis of ATP. This form of phototrophy is not linked to CO_2 fixation, and does not require chlorophyll pigments, and so it is not photosynthesis in the traditional sense. However, other light-sensitive pigments are present, including red and orange carotenoids—primarily C_{50} pigments called *bacterioruberins*—and inducible pigments involved in energy conservation; we discuss these pigments here.

Under conditions of low aeration, *Halobacterium salinarum* and some other haloarchaea synthesize a protein called **bacteriorhodopsin** and insert it into their cytoplasmic membranes. Bacteriorhodopsin is so named because of its structural and functional similarity to rhodopsin, the visual pigment of the eye. Conjugated to bacteriorhodopsin is a molecule of retinal, a carotenoid-like molecule that can absorb light energy and pump a proton across the cytoplasmic membrane. The retinal gives bacteriorhodopsin a purple hue. Thus cells of *Halobacterium* that are switched from growth under high-aeration conditions to O_2-limiting growth conditions (a trigger of bacteriorhodopsin synthesis) gradually change color from orange-red to purple-red (Figure 17.2) as they synthesize bacteriorhodopsin and insert it into their cytoplasmic membranes.

Bacteriorhodopsin absorbs green light around 570 nm. Following absorption, the retinal of bacteriorhodopsin, which normally exists in a *trans* configuration (Ret_T), becomes excited and converts to the *cis* (Ret_C) form (**Figure 17.4**). This transformation is coupled to the translocation of a proton across the cytoplasmic membrane. The retinal molecule then decays to the *trans* isomer along with the uptake of a proton from the cytoplasm, and this completes the cycle and resets the proton pump to repeat the process (Figure 17.4). As protons accumulate on the outer surface of the membrane, a proton motive force is generated that is coupled to ATP synthesis through the activity of a proton-translocating ATPase (Figure 17.4; ◀ Section 3.9).

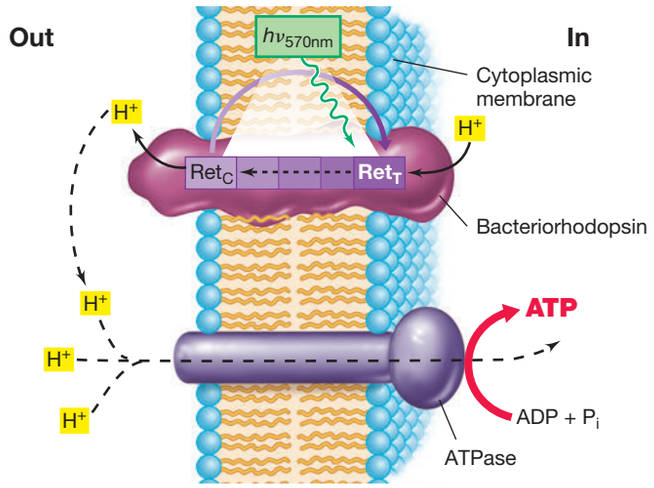


Figure 17.4 Model for the mechanism of bacteriorhodopsin. Light of 570 nm ($h\nu_{570nm}$) converts the protonated retinal of bacteriorhodopsin from the *trans* form (Ret_T) to the *cis* form (Ret_C), along with translocation of a proton to the outer surface of the cytoplasmic membrane, thus establishing a proton motive force. ATPase activity is driven by the proton motive force.

Bacteriorhodopsin-mediated ATP production in *H. salinarum* supports slow growth of this organism under anoxic conditions. The light-stimulated proton pump of *H. salinarum* also functions to pump Na^+ out of the cell by activity of a Na^+–H^+ antiport system and to drive the uptake of nutrients, including the K^+ needed for osmotic balance. Amino acid uptake by *H. salinarum* is indirectly driven by light as well, because amino acids are cotransported into the cell with Na^+ by an amino acid–Na^+ symporter (◀ Section 2.2); removal of Na^+ from the cell occurs by way of the light-driven Na^+–H^+ antiporter.

Other Rhodopsins

Besides bacteriorhodopsin, at least three other rhodopsins are present in the cytoplasmic membrane of cells of *H. salinarum*. **Halorhodopsin** is a light-driven chloride (Cl^-) pump that brings Cl^- into the cell as the anion for K^+. The retinal of halorhodopsin binds Cl^- and transports it into the cell. Two other light sensors, called *sensory rhodopsins*, are present in *H. salinarum*. These light sensors control phototaxis (movement toward light, ◀ Section 2.12) by the organism. Through the interaction of a cascade of proteins similar to those in chemotaxis (◀ Sections 2.11 and 7.6), sensory rhodopsins affect archaellar rotation, moving cells of *H. salinarum* toward light where bacteriorhodopsin can function to make ATP (Figure 17.4).

We will learn when we consider marine microbiology (▶ Sections 20.11 and 20.12) that diverse species of chemoorganotrophic *Bacteria* that inhabit the upper layers of the ocean contain bacteriorhodopsin-like proteins called *proteorhodopsins*. As far as is known, proteorhodopsin functions like bacteriorhodopsin except that different spectral forms exist, each form being tuned to the absorption of its own specific wavelength of light. Although the energy generated from proteorhodopsin alone is insufficient to sustain growth, these marine bacteria use proteorhodopsin as a supplement to the ATP they generate from respiration. Proteorhodopsin as a mechanism for energy conservation in marine bacteria makes good ecological sense because levels of dissolved organic matter in the open oceans are typically very low, and thus a strictly chemoorganotrophic lifestyle would be difficult.

Check Your Understanding

- Why do cells of *Halobacterium* require high levels of Na^+ for growth?
- What benefit does bacteriorhodopsin confer on *Halobacterium salinarum*?

17.2 Methanogenic *Archaea*

KEY GENERA: *Methanobacterium, Methanocaldococcus, Methanosarcina, Methanopyrus*

Many *Euryarchaeota* are methanogens, microorganisms that produce methane (CH_4) as an integral part of their energy metabolism (methane production is called *methanogenesis*). In Section 14.15 we considered the unique biochemistry of methanogenesis. Later, we will learn how methanogenesis is a major component of the global carbon cycle, serving as the terminal step in the biodegradation of organic matter in many anoxic habitats (▶ Sections 21.1, 21.2, and 21.9). Methanogens are important in a wide range of anoxic habitats including freshwater sediments, wetlands, rice paddies, wastewater treatment plants, geothermal systems, the subsurface of the Earth's crust, and within the guts of many animals.

Diversity and Physiology of Methanogens

Methanogens, often called by their nickname "methanoarchaea," occur in at least eight taxonomic orders, including *Methanobacteriales*, *Methanococcales*, *Methanopyrales*, *Methanomassiliicoccales*, *Methanomicrobiales*, *Methanocellales*, *Methanosarcinales*, and *Methanonatronarchaeales* (Figure 17.1). Methanogens exhibit considerable morphological and physiological diversity (**Figure 17.5** and see Table 17.2). As can be seen in Figure 17.1, methanogens are widespread within the *Euryarchaeota* and do not represent a single coherent phylogenetic group. We have already been introduced to the factors that cause inconsistency between phylogenetic diversity and functional diversity (◀ Section 15.1). In the case of methanogenesis it is most likely that the ability to reduce CO_2 to CH_4 evolved only once within the *Euryarchaeota*, and that gene loss caused lineages such as the haloarchaea and *Thermoplasmatales* to lose the capacity to produce methane. We will also see that *Archaeoglobus* (Section 17.4) still retains some of the genes encoding methanogenesis and can actually produce methane under certain growth conditions.

Methanogens have diverse physiological characteristics, but they are united by their ability to produce methane and by their intolerance to O_2. Several cofactors required by methanogenic pathways are inhibited by O_2, and hence all methanogens are obligate anaerobes. As a result, strict anoxic techniques are necessary for their cultivation in isolation. While most methanogens are mesophilic and do not live in extreme environments, a diversity of species have been described including some that grow optimally at very high (see Figure 17.7) or very low temperatures, at very high salt concentrations, or at extremes of pH.

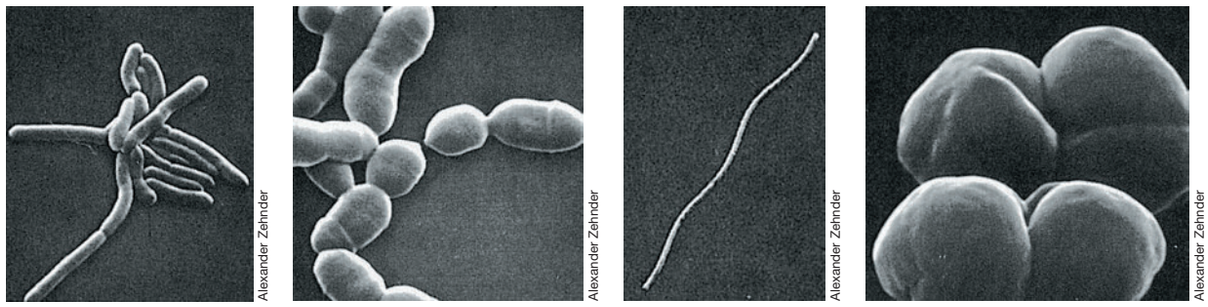


Figure 17.5 **Scanning electron micrographs of cells of diverse species of methanogenic *Archaea*.** *(a) Methanobrevibacter ruminantium.* A cell is about 0.7 μm in diameter. *(b) Methanobrevibacter arboriphilus.* A cell is about 1 μm in diameter. *(c) Methanospirillum hungatei.* A cell is about 0.4 μm in diameter. *(d) Methanosarcina barkeri.* A cell is about 1.7 μm wide.

Methanogens possess a diversity of cell envelope and cell wall configurations. *Methanobacterium* species and relatives (**Figure 17.6*a***) have cell walls composed of *pseudomurein* (◀ Section 2.3). *Methanosarcina* and relatives (Figure 17.6*b*) have cell walls composed of *methanochondroitin* (so named because of its structural resemblance to chondroitin, the connective tissue polymer of vertebrate animals). In addition, *Methanocaldococcus* (**Figure 17.7*a***) and *Methanoplanus* species have cell walls composed of protein or glycoprotein, respectively. Finally, species such as *Methanospirillum* (Figure 17.5*c*) use an S-layer (◀ Section 2.5) as their cell wall.

Methanogenic Pathways

Methanogenesis occurs through three different pathways (**Table 17.2**): CO_2 reduction (◀ Figure 14.38), methylotrophic methanogenesis (◀ Figure 14.39*a*), and acetoclastic methanogenesis (◀ Figure 14.39*b*). Each of these pathways relies on *coenzyme M*; methane is ultimately produced by reduction of methyl-CoM to methane (◀ Section 14.15). Through these pathways, methanogens convert a limited number of substrates into methane (Table 17.2). Interestingly, these substrates do not include such common compounds as glucose, organic acids (other than pyruvate), or fatty acids (other than acetate). Methanogens often form syntrophic associations with fermentative anaerobes (◀ Section 14.22, ▶ Section 21.2). In these cooperative systems, the fermentative organisms degrade a wide range of organic molecules into H_2, CO_2, and acetate, which are ultimately used as substrates for methanogenesis.

The three methanogenic pathways are distributed across different phylogenetic groups of methanogens (**Table 17.3**). Methanogenesis by CO_2 reduction occurs widely across the known diversity of methanogens, but not all methanogens can reduce CO_2. Notably, many species of *Methanosarcinales*, and all species in the *Methanomassiliicoccales*, *Methanonatronarchaeales*, and the genus *Methanosphaera* have lost the ability to produce methane by CO_2 reduction (Table 17.3). The CO_2 reduction pathway also allows some methanogens to produce methane from formate or carbon monoxide. In addition, while H_2 is the typical electron donor for methanogenesis by CO_2 reduction, this pathway also allows some methanogens to reduce CO_2 by using electrons from pyruvate or certain alcohols (Tables 17.2 and 17.3).

Acetoclastic methanogenesis is performed by few methanogens and is found exclusively within the *Methanosarcinales* (Table 17.3). Methanogenesis from acetate is a dominant source of methane production in a wide range of environments (▶ Section 21.2). In acetoclastic methanogenesis, electron flow is branched, producing both

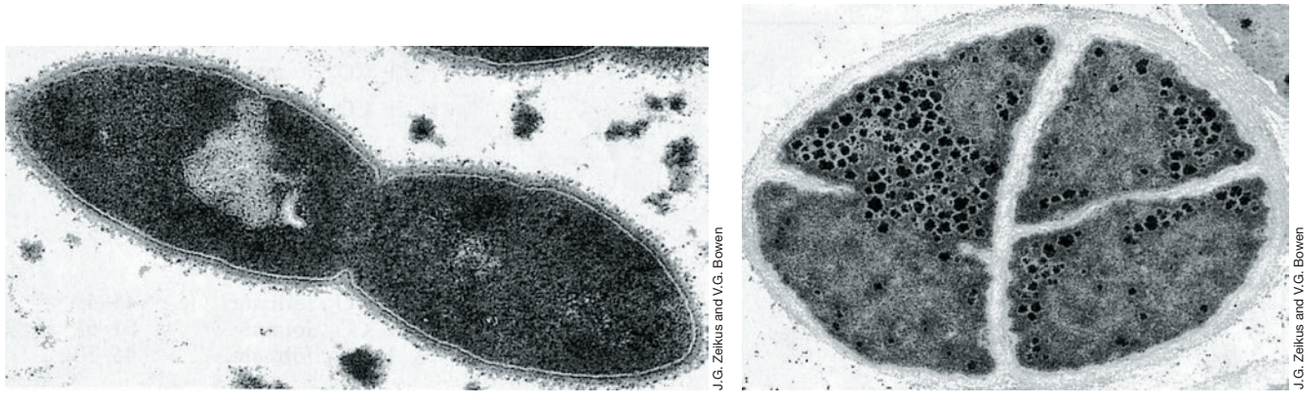


Figure 17.6 **Transmission electron micrographs of thin sections of methanogenic *Archaea*.** *(a) Methanobrevibacter ruminantium.* A cell is 0.7 μm in diameter. *(b) Methanosarcina barkeri*, showing the thick cell wall and the manner of cell segmentation and cross-wall formation. A cell is 1.7 μm in diameter.

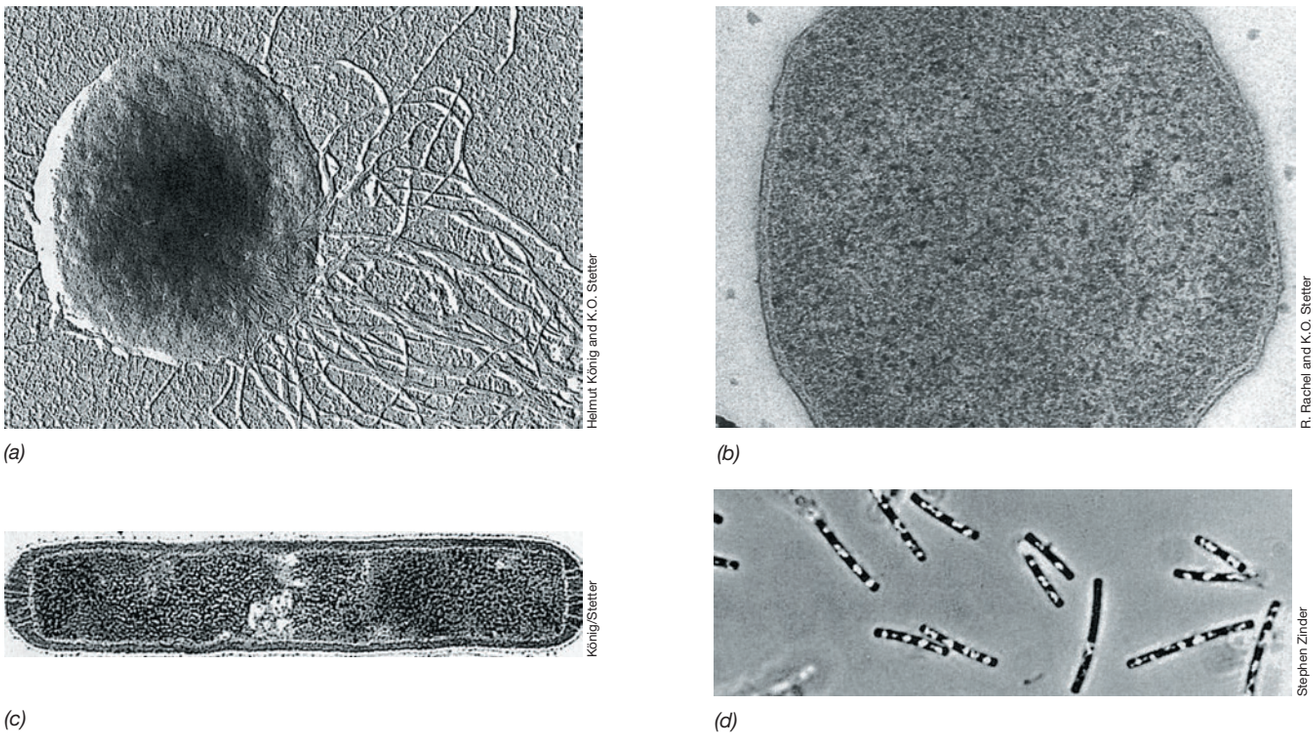


Figure 17.7 Hyperthermophilic and thermophilic methanogens. *(a) Methanocaldococcus jannaschii* (temperature optimum, 85°C), shadowed preparation electron micrograph. A cell is about 1 μm in diameter. *(b) Methanotorris igneus* (temperature optimum, 88°C), thin section. A cell is about 1 μm in diameter. *(c) Methanothermus fervidus* (temperature optimum, 88°C), thin-sectioned electron micrograph. A cell is about 0.4 μm in diameter. *(d) Methanosaeta thermophila* (temperature optimum, 60°C), phase-contrast micrograph. A cell is about 1 μm in diameter. The refractile bodies inside the cells are gas vesicles.

CO_2 and CH_4 as products. Acetoclastic methanogens perform a dismutation of acetate in which the carboxyl group is oxidized to CO_2 while the methyl group is reduced to CH_4 (Table 17.2, ◀ Figure 14.39*b*). The reductive acetyl-CoA pathway is then used to assimilate carbon into cell biomass.

Methylotrophic methanogenesis can take many forms, but all methylotrophic methanogens use one of two pathways that differ in important ways. Some methylotrophic methanogens use a branched pathway that produces both CH_4 and CO_2 as products and allows for carbon assimilation from methylated compounds. Other methylotrophic methanogens use a methyl-reducing pathway that produces only CH_4 as a product and does not allow for carbon assimilation from methylated compounds. The branched pathway is found only within the family *Methanosarcinales*. Branched pathways for methylotrophic methanogenesis are similar conceptually to the acetoclastic pathway with a major difference being that methylotrophs do not perform a dismutation, instead oxidizing one molecule to CO_2 so that they can reduce other molecules to CH_4 (Table 17.2). For example, many species of *Methanosarcinales* oxidize one molecule of CH_3OH to CO_2 to generate the six electrons needed to reduce three molecules of CH_3OH to 3 CH_4 and 3 H_2O (◀ Figure 14.39*a*). In addition, methylotrophic methanogens that use a branched pathway funnel carbon from their oxidative branch into the reductive acetyl-CoA pathway in order to assimilate carbon from methylated compounds.

The methyl-reducing pathway for methylotrophic methanogenesis, in contrast, does not result in the production of CO_2, and methanogens using the methyl-reducing pathway lack the ability to assimilate carbon from methylated compounds. Methylotrophic methanogens such as *Methanosphaera stadtmanae* are unable to oxidize methyl groups to CO_2 and therefore require an external electron donor—either H_2 or formate—to reduce the methyl group to methane (Tables 17.2 and 17.3). Hence, *M. stadtmanae* produces CH_4 but not CO_2

TABLE 17.2 The three methanogenic pathways and their substrates

I.	**CO_2 reduction pathway: electrons are typically derived from H_2**
	Carbon dioxide, CO_2
	Formate, $HCOO^-$
	Carbon monoxide, CO
II.	**Acetoclastic pathway: electrons are derived from dismutation**[a]
	Acetate, CH_3COO^-
	Pyruvate, CH_3COCOO^-
III.	**Methylotrophic pathways: electrons are derived either by oxidizing some of the methylated substrate to CO_2 (branched pathway) or by the oxidation of H_2 or formate (methyl-reducing pathway)**
	Methanol, CH_3OH
	Methylamine, $CH_3NH_3^+$
	Dimethylamine, $(CH_3)_2NH_2^+$
	Trimethylamine, $(CH_3)_3NH^+$
	Methyl mercaptan, CH_3SH
	Dimethyl sulfide, $(CH_3)_2S$

[a]In a dismutation, one part of the molecule is reduced by oxidizing another part of the same molecule.

TABLE 17.3 Characteristics of some methanogenic *Archaea*[a]

Order/Genus	Pathways[b]	Substrates for methanogenesis[c]
Methanomicrobiales	I	
Methanomicrobium	I	$H_2 + CO_2$, formate
Methanospirillum	I	$H_2 + CO_2$, formate
Methanoculleus	I	$H_2 + CO_2$, formate, alcohols + CO_2
Methanococcales	I	
Methanococcus	I	$H_2 + CO_2$, formate, pyruvate + CO_2
Methanocaldococcus	I	$H_2 + CO_2$
Methanopyrales	I	
Methanopyrus	I	$H_2 + CO_2$
Methanocellales	I	
Methanocella	I	$H_2 + CO_2$
Methanobacteriales	I, III	
Methanobacterium	I	$H_2 + CO_2$, formate
Methanobrevibacter	I	$H_2 + CO_2$, formate
Methanothermus	I	$H_2 + CO_2$
Methanothermobacter	I	$H_2 + CO_2$, formate, CO
Methanosphaera	III	H_2 + methanol
Methanosarcinales	I, II, III	
Methanosarcina	I, II, III	$H_2 + CO_2$, methanol, methylamines, acetate, CO
Methanococcoides	III	Methanol, methylamines
Methanohalophilus	III	Methanol, methylamines, methyl sulfides
Methanosaeta	II	Acetate
Methanosalsum	III	Methanol, methylamines, dimethyl sulfide
Methanimicrococcus	III	H_2 + (methanol, methylamines)
Methanomassiliicoccales	III	
Methanomassiliicoccus	III	H_2 + methanol
Methanomethylophilus	III	H_2 + methanol
Methanonatronarcheales	III	
Methanonatronarchaeum	III	H_2 + (methanol, methylamines, dimethyl sulfide)

[a]Taxonomic orders are listed in bold. An order is a taxonomic rank that consists of several families; families consist of several genera (◀Table 13.1).
[b]Methanogenic pathways I–III are summarized in Table 17.2.
[c]Methylamines can include the substrates methylamine ($CH_3NH_3^+$), dimethylamine ($(CH_3)_2NH_2^+$), and trimethylamine ($(CH_3)_3NH^+$); methyl sulfides can include dimethyl sulfide ($(CH_3)_2S$) and methyl mercaptan (CH_3SH).

from methylated substrates. In addition, although *M. stadtmanae* and metabolically similar methanogens can make methane from methylated compounds, they are unable to incorporate them as a carbon source. Instead, they typically require an organic carbon source such as acetate, though a few species can also fix CO_2. Besides *M. stadtmanae*, this pattern of methylotrophic methanogenesis is also characteristic of *Methanomicrococcus blatticola* and species in the orders *Methanomassiliicoccales* and *Methanonatronarchaeales* (Table 17.3).

Methanocaldococcus jannaschii as a Model Methanogen

The genomes of the hyperthermophilic methanogen *Methanocaldococcus jannaschii* (Figure 17.7*a*) and many other methanogens have been sequenced. The 1.66-megabase-pair (Mbp) circular genome of *M. jannaschii*, an organism that has been used as a model for the molecular study of methanogenesis and archaeal motility, contains about 1700 genes, and genes encoding enzymes of methanogenesis and several other key cell functions have been identified. Interestingly, the majority of *M. jannaschii* genes encoding functions such as central metabolic pathways and cell division are similar to those in *Bacteria*. By contrast, most of the *M. jannaschii* genes encoding core molecular processes such as transcription and translation more closely resemble those of eukaryotes. These findings reflect the various traits shared by organisms in the three cellular domains and are consistent with our understanding of how the three domains of cells evolved, as discussed in Chapter 13. However, analyses of the *M. jannaschii* genome also show that fully 40% of its genes have no counterparts in genes from either of the other domains. Some of these are genes that encode the enzymes needed for methanogenesis, of course, but many others likely encode novel cellular functions absent from cells in the other domains or may encode redundant functions carried out by classes of enzymes distinct from those found in *Bacteria* and *Eukarya*.

Methanopyrus, a Hyperthermophilic Methanogen

Methanopyrus (**Figure 17.8**), the only genus in the order *Methanopyrales*, is a rod-shaped hyperthermophilic methanogen that shares phenotypic properties with both the hyperthermophiles (see Section 17.12) and the methanogens. *Methanopyrus* was isolated from hot sediments near submarine hydrothermal vents and from the walls of "black smoker" hydrothermal vent chimneys (Section 17.11; ▶ Section 20.16 and Figure 20.46). *Methanopyrus* produces CH_4 only from $H_2 + CO_2$ and grows rapidly for an autotrophic organism

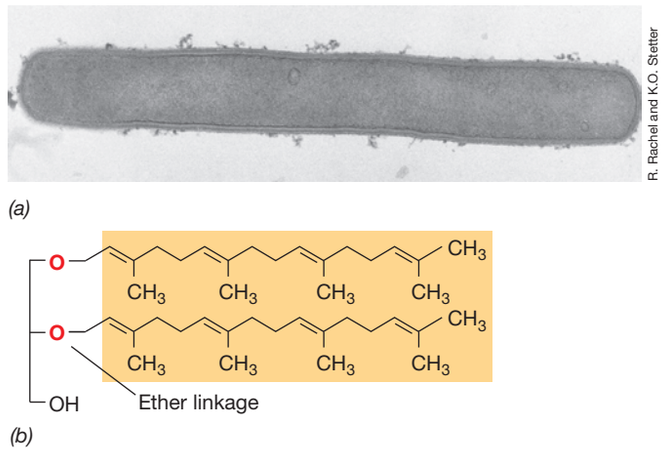


Figure 17.8 ***Methanopyrus.*** *Methanopyrus* grows optimally at 100°C and can make CH_4 only from $CO_2 + H_2$. *(a)* Electron micrograph of a cell of *Methanopyrus kandleri*, the most thermophilic of all known organisms (upper temperature limit, 122°C). This cell measures 0.5 × 8 μm. *(b)* Structure of the novel lipid of *M. kandleri*. This is the normal glycerol diether of *Archaea* (◀Figure 2.3*b*) except that the side chains are an unsaturated form of phytanyl (called geranylgeraniol) rather than phytanyl.

(generation time <1 h at 100°C). In special pressurized vessels, growth of one strain of *Methanopyrus* has been recorded at 122°C, the highest temperature yet shown to support microbial growth (Sections 17.12 and 17.13).

In addition to its remarkable tolerance to high temperature, *Methanopyrus* is also unusual because it contains membrane lipids found in no other known organism. Recall that in the lipids of *Archaea*, the glycerol side chains contain **phytanyl** rather than fatty acids bonded in ether linkage to the glycerol (◀ Section 2.1 and Figure 2.3*a*). In *Methanopyrus*, this ether-linked lipid is an *unsaturated* form of the otherwise saturated biphytanyl tetraethers found in all other hyperthermophilic *Archaea* (Figure 17.8*b*). These unusual lipids are thought to help stabilize the cytoplasmic membrane of *Methanopyrus* at its unusually high growth temperatures.

Some *Euryarchaeota* lack cell walls and we consider them next.

Check Your Understanding

- What are the three major pathways of methanogenesis and in what phylogenetic groups are they found?
- What physiological and structural features distinguish the *Methanosarcinales* from other methanogens?

17.3 *Thermoplasmatales*

KEY GENERA: *Thermoplasma, Picrophilus, Ferroplasma*

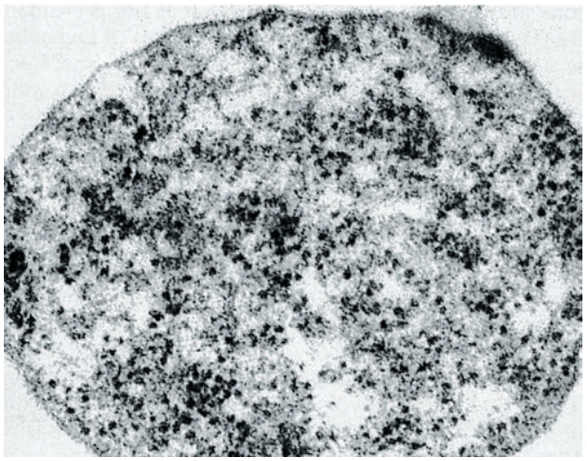
A phylogenetically distinct line of *Archaea* contains thermophilic and extremely acidophilic genera: *Thermoplasma*, *Ferroplasma*, and *Picrophilus*. These organisms are among the most acidophilic of all known microbes, with *Picrophilus* being capable of growth even below pH 0. Most are thermophilic as well. These genera also form their own taxonomic order within the *Euryarchaeota*, the *Thermoplasmatales* (Figure 17.1). We begin with a description of the mycoplasma-like organisms *Thermoplasma* and *Ferroplasma*.

Archaea Lacking Cell Walls

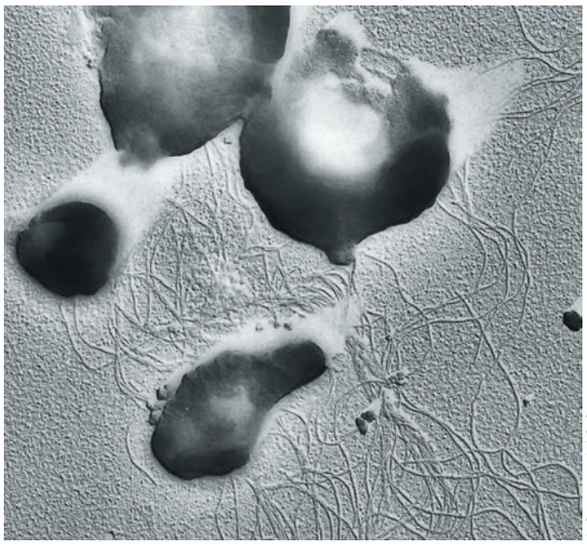
Thermoplasma and *Ferroplasma* lack cell walls, and in this respect they resemble the mycoplasmas (◀ Section 16.9). *Thermoplasma* (**Figure 17.9**) is a chemoorganotroph that grows optimally at 55°C and pH 2 in complex media. Two species of *Thermoplasma* have been described, *Thermoplasma acidophilum* and *Thermoplasma volcanium*. Species of *Thermoplasma* are facultative aerobes, growing either aerobically or anaerobically by sulfur respiration (◀ Section 14.12). Most strains of *T. acidophilum* have been obtained from self-heating coal refuse piles. Coal refuse contains coal fragments, pyrite (FeS_2), and other organic materials extracted from coal. When dumped into piles in surface mining operations, coal refuse heats as a result of microbial metabolism bringing it to combustion temperature (**Figure 17.10**). This sets the stage for growth of *Thermoplasma*, which likely metabolizes organic compounds leached from the hot coal refuse. The second species, *T. volcanium*, has been isolated in hot acidic soils throughout the world and is highly motile by multiple archaella (Figure 17.9*b*).

To survive the osmotic stresses of life without a cell wall and to withstand the dual environmental extremes of low pH and high temperature, *Thermoplasma* has evolved a unique cytoplasmic membrane structure. The membrane contains a lipopolysaccharide-like material called *lipoglycan*. This substance consists of glycolipids containing sugars such as mannose and glucose (**Figure 17.11**), and these glycolipids form a tetraether lipid monolayer membrane. The hydrophobic core of this glycolipid consists of biphytanyl (◀ Section 2.1 and Figure 2.3*b*). In *Thermoplasma* and similar organisms such as *Sulfolobus*, this basic biphytanyl structure can be modified to include one to four cyclopentane rings, with the number of rings tending to increase in proportion to the temperature of the environment. These glycolipids constitute a major fraction of the total lipids of *Thermoplasma*. The membrane also contains glycoproteins and glycophospholipids, but not sterols. These molecules render the *Thermoplasma* membrane stable to hot, acidic conditions.

Like mycoplasmas (◀ Section 16.9), *Thermoplasma* contains a relatively small genome (1.5 Mbp). In addition, *Thermoplasma* DNA is



(a)



(b)

Figure 17.9 *Thermoplasma* species. *(a) Thermoplasma acidophilum*, an acidophilic and thermophilic mycoplasma-like archaeon; electron micrograph of a thin section. The diameter of cells varies from 0.2 to 5 μm. The cell shown is about 1 μm in diameter. *(b)* Shadowed preparation of cells of *Thermoplasma volcanium* isolated from hot springs. Cells are 1–2 μm in diameter. Notice the abundant archaella (the rotating structures that confer swimming motility on *Archaea*, ◀ Section 2.9) and irregular cell morphology.

Figure 17.10 A typical self-heating coal refuse pile, habitat of *Thermoplasma*. The pile, containing coal debris, pyrite, and other microbial substrates, self-heats as a result of microbial metabolism.

complexed with a highly basic DNA-binding protein that organizes the DNA into globular particles resembling the nucleosomes of eukaryotic cells. This protein is homologous to the histone-like DNA-binding protein HU of *Bacteria*, which plays an important role in organization of the DNA in the cell. In contrast, several other *Euryarchaeota* contain basic proteins homologous to the DNA-binding histone proteins of eukaryotic cells.

Ferroplasma

Ferroplasma is a chemolithotrophic relative of *Thermoplasma*. *Ferroplasma* is a strong acidophile; however, it is not a thermophile, as it grows optimally at 35°C. *Ferroplasma* oxidizes ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) to obtain energy and uses CO_2 as its carbon source. The oxidation of Fe^{2+} results in acidification of the environment. *Ferroplasma* grows in mine tailings containing pyrite, which is its energy source. The extraordinary acidophily of *Ferroplasma* allows it to drive the pH of its habitat down to extremely acidic values. After moderate acidity is generated from Fe^{2+} oxidation by acidophilic organisms such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (▶ Sections 21.6, 22.1, and 22.2), *Ferroplasma* becomes active and subsequently generates the very low pH values typical of acid mine drainage. Acidic waters at pH 0 can be generated by the activities of *Ferroplasma*.

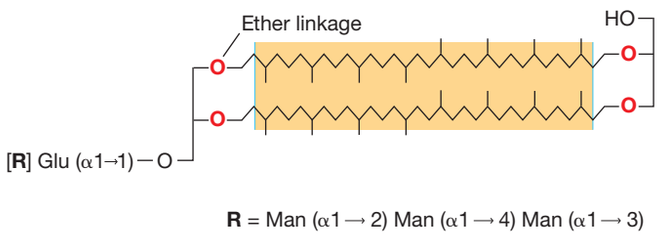


Figure 17.11 Structure of a tetraether glycolipid of *Thermoplasma acidophilum*. The dominant glycolipids of *T. acidophilum* have two polar head groups that are connected by ether linkages to a hydrophobic core. This structure causes them to form a thermostable lipid monolayer (◀ Figure 2.3*b*). One or both of the polar head groups typically contains a mono- or oligosaccharide that contains glucose (Glu), mannose (Man), and other sugars. The hydrophobic core consists of the straight-chain caldarchaeol (shown), which can be modified to include one to four cyclopentane rings (not shown).

Picrophilus

A phylogenetic relative of *Thermoplasma* and *Ferroplasma* is *Picrophilus*. Although *Thermoplasma* and *Ferroplasma* are extreme acidophiles, *Picrophilus* is even more so, growing optimally at pH 0.7 and capable of growth at pH values lower than 0. *Picrophilus* also has a cell wall (an S-layer; ◀ Section 2.5, Figures 2.14 and 2.36*a*) and a much lower DNA G + C base ratio than does *Thermoplasma* or *Ferroplasma*. Although phylogenetically related, *Thermoplasma*, *Ferroplasma*, and *Picrophilus* have quite distinct genomes. Two species of *Picrophilus* have been isolated from acidic Japanese solfataras, and like *Thermoplasma*, both grow heterotrophically on complex media.

The physiology of *Picrophilus* is of interest as a model for extreme acid tolerance. Studies of its cytoplasmic membrane point to an unusual arrangement of lipids that forms a highly acid-impermeable membrane at very low pH. By contrast, at moderate acidities such as pH 4, the membranes of cells of *Picrophilus* become leaky and disintegrate. Obviously, this organism has evolved to survive only in highly acidic habitats and shows the most extraordinary acidophily of any known microbe.

Check Your Understanding

- In what ways are *Thermoplasma* and *Picrophilus* similar? In what ways do they differ?
- How does *Thermoplasma* strengthen its cytoplasmic membrane to survive without a cell wall?

17.4 *Thermococcales* and *Archaeoglobales*

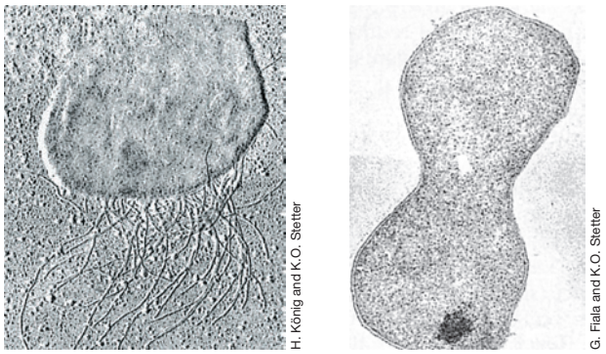
KEY GENERA: *Thermococcus, Pyrococcus, Archaeoglobus, Ferroglobus*

A few euryarchaeotes thrive in thermal environments and some are hyperthermophiles. We consider here the *Thermococcales* and *Archaeoglobales*, which are two orders of *Euryarchaeota* that contain hyperthermophilic species (Figure 17.1).

Thermococcus and *Pyrococcus*

Thermococcus and *Pyrococcus* are genera within the order *Thermococcales*. *Thermococcus* is a spherical hyperthermophilic euryarchaeote indigenous to anoxic thermal waters in various locations throughout the world. The spherical cells contain a tuft of polar archaella and are thus highly motile (**Figure 17.12*a***). *Thermococcus* is an obligately anaerobic chemoorganotroph that metabolizes proteins and other complex organic mixtures (including some sugars) with elemental sulfur (S^0) as electron acceptor at temperatures from 55 to 95°C.

Pyrococcus (Figure 17.12*b*) is morphologically similar to *Thermococcus*. *Pyrococcus* differs from *Thermococcus* primarily by its higher temperature requirements; *Pyrococcus* grows between 70 and 106°C with an optimum of 100°C. *Thermococcus* and *Pyrococcus* are also metabolically quite similar. Proteins, starch, or maltose are oxidized as electron donors, and S^0 is the terminal electron acceptor and is reduced to hydrogen sulfide (H_2S). Both *Thermococcus* and *Pyrococcus* form H_2S when S^0 is present, but form H_2 when S^0 is absent (see Table 17.4).



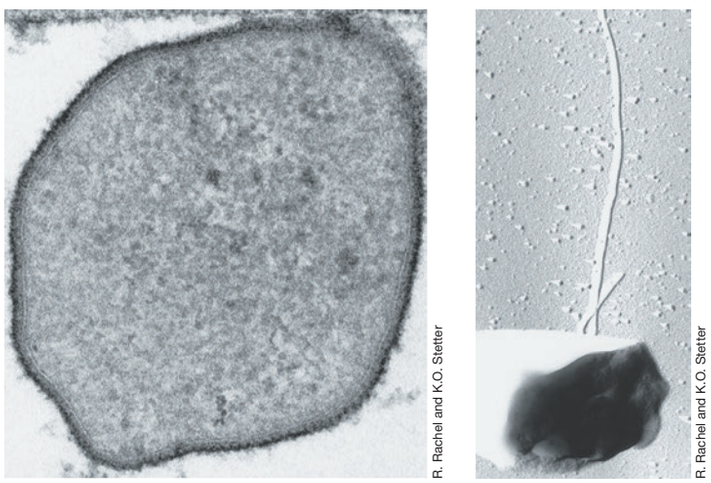
(a) (b)

Figure 17.12 Spherical hyperthermophilic *Euryarchaeota* from submarine volcanic areas. *(a) Thermococcus celer*, electron micrograph of shadowed cells (note tuft of archaella). *(b)* Dividing cell of *Pyrococcus furiosus*; electron micrograph of thin section. Cells of both organisms are about 0.8 μm in diameter.

Archaeoglobus and *Ferroglobus*

Archaeoglobus was isolated from hot marine sediments near hydrothermal vents. In its metabolism, *Archaeoglobus* couples the oxidation of H_2, lactate, pyruvate, glucose, or complex organic compounds to the reduction of SO_4^{2-} to H_2S. Cells of *Archaeoglobus* are irregular cocci (**Figure 17.13*a***) and grow optimally at 83°C.

Archaeoglobus and methanogens share some characteristics. We learned in Section 14.15 about the unique biochemistry of methanogenesis. Briefly, this process requires a series of novel coenzymes, and with rare exceptions, these coenzymes have only been found in methanogens. *Archaeoglobus*, however, also contains many of these coenzymes, and cultures of this organism can actually produce small amounts of CH_4. In addition, the genome of *Archaeoglobus*, which contains about 2400 genes, shares many genes in common with methanogens (Section 17.2). It seems likely that the ancestor of *Archaeoglobus* was a methanogen that lost many of the genes required for methanogenesis. Furthermore, genome analysis suggests that the ancestor of *Archaeoglobus* acquired genes for sulfate reduction as a result of horizontal gene transfer from sulfate-reducing bacteria within the *Deltaproteobacteria* (◀ Section 15.11).



(a) (b)

Figure 17.13 *Archaeoglobales.* *(a)* Transmission electron micrograph of the sulfate-reducing hyperthermophile *Archaeoglobus fulgidus*. The cell measures 0.7 μm in diameter. *(b)* Freeze-etched electron micrograph of *Ferroglobus placidus*, a ferrous iron–oxidizing, nitrate-reducing hyperthermophile. The cell measures about 0.8 μm in diameter.

Ferroglobus (Figure 17.13*b*) is related to *Archaeoglobus* but is not a sulfate reducer. Instead, *Ferroglobus* is an iron-oxidizing chemolithotroph, conserving energy from the oxidation of Fe^{2+} to Fe^{3+} coupled to the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) (see Table 17.4). *Ferroglobus* grows autotrophically and can also use H_2 or H_2S as electron donor in its energy metabolism. *Ferroglobus* was isolated from a shallow marine hydrothermal vent and grows optimally at 85°C.

Ferroglobus is interesting for several reasons, but especially for its ability to oxidize Fe^{2+} to Fe^{3+} under anoxic conditions. This process might help explain the origin of some Fe^{3+} found in ancient rocks dated to before the predicted appearance of cyanobacteria on Earth (◀ Section 13.2). With organisms like *Ferroglobus*, it would have been possible for Fe^{2+} oxidation to proceed without the need for O_2 as an electron acceptor. The metabolism of *Ferroglobus* thus has implications for dating the origin of cyanobacteria and the subsequent oxygenation of Earth. Certain anoxygenic phototrophic bacteria can also oxidize Fe^{2+} under anoxic conditions (◀ Section 14.8), and so several anaerobic routes to ancient Fe^{3+} are possible. This makes it difficult to estimate when cyanobacteria first appeared on Earth and to what degree nonphototrophic organisms helped trigger the Great Oxidation Event (◀ Figure 13.1).

We move on now to Part II where we survey the most recently recognized groups of *Archaea*, some of which are thermophiles but many of which inhabit common environments such as soil, lakes, and the oceans.

Check Your Understanding

- How do *Thermococcus* and *Pyrococcus* make ATP?
- Compare the energy-yielding metabolisms of *Archaeoglobus* and *Ferroglobus*.

UNIT 4

II • *Thaumarchaeota* and Cryptic Archaeal Phyla

Many *Archaea* remain uncultured and are only known from metagenomic analyses of various microbial habitats. *Thaumarchaeota*, a group of nitrifying *Archaea*, were first detected in this way, but have now been cultured and found to be the most abundant *Archaea* in nature.

Our understanding of *Archaea* has been revolutionized by the development of molecular phylogeny (◀ Sections 1.15 and 13.11) and methods for studying microorganisms without needing to cultivate them in the laboratory (▶ Sections 19.4–19.8). The discovery and characterization of *Thaumarchaeota*, *Nanoarchaeota*, and *Korarchaeota* were all enabled by the use of molecular techniques that target 16S ribosomal RNA genes. From these initial efforts, species representing each of these phyla were subsequently isolated or

at least grown in enrichment cultures. In addition, metagenomic and single-cell genome sequencing techniques (◀ Sections 10.7 and 10.11) have made it possible to obtain genome sequence data from organisms without the need to obtain cultures, and this advance has expanded greatly our knowledge of the archaeal domain. We begin our consideration of these newly discovered phyla with the *Thaumarchaeota*.

17.5 *Thaumarchaeota* and Nitrification in *Archaea*

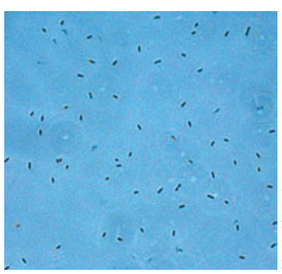
KEY GENERA: *Nitrosopumilus, Nitrososphaera*

Early surveys of 16S ribosomal RNA genes from open-ocean microbial communities resulted in the shocking conclusion that *Archaea* were abundant and widespread in the oceans. At the time, the archaeal domain was considered to contain only extremophiles and obligate anaerobes, and their presence in O_2-rich temperate and even polar oceanic environments was a major surprise. Even more remarkable, these novel *Archaea* were also discovered to be widespread in soils worldwide.

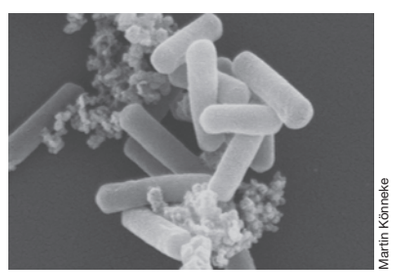
Phylogenetic analyses of 16S ribosomal RNA gene sequences initially suggested that this novel group of *Archaea* was a deeply divergent lineage of the *Crenarchaeota*, a major group of hyperthermophilic *Archaea* we will consider in Section 17.9. It was only after genome sequence analysis of the marine nitrifier *Nitrosopumilus maritimus* that it became clear that the ***Thaumarchaeota*** constitute a unique phylum of *Archaea* (Figure 17.1).

Physiological Characteristics of *Thaumarchaeota*

The physiology of *Thaumarchaeota* remained a mystery until the isolation of *Nitrosopumilus maritimus* (**Figure 17.14**). *N. maritimus* grows chemolithotrophically by oxidizing ammonia (NH_3) to nitrite (NO_2^-) aerobically, the first step in nitrification (◀ Sections 14.9, 15.10, and ▶ Section 21.3). This organism uses CO_2 as its sole carbon source, as do most nitrifying *Bacteria* (◀ Section 15.10). Prior to the discovery of *Thaumarchaeota*—for over 100 years in fact—microbiologists thought only certain species of *Bacteria* catalyzed ammonia oxidation. However, unlike ammonia-oxidizing *Bacteria* such as *Nitrosomonas*, *N. maritimus* is adapted to life under extreme nutrient limitation, as would befit an organism indigenous to open ocean waters. *N. maritimus* can grow at NH_3 concentrations that are



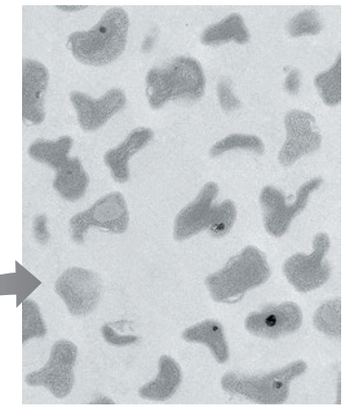
(a)



(b)

Figure 17.14 ***Nitrosopumilus maritimus*, a nitrifying species of marine *Thaumarchaeota*.** This organism can oxidize NH_3 present at the very low amounts typical of marine environments. *(a)* Phase-contrast photomicrograph. *(b)* Scanning electron micrograph. A single cell of *N. maritimus* is about 0.2 μm in diameter.

(a)



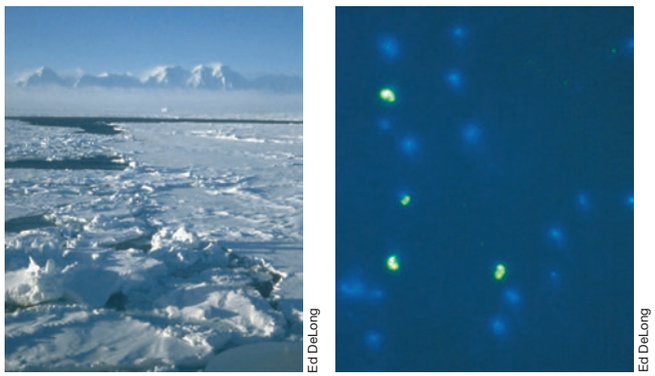
(b)

Figure 17.15 ***Nitrososphaera viennensis*, a nitrifying species of soilborne *Thaumarchaeota*.** This organism can oxidize NH_3 and urea across a wide range of concentrations. *(a)* A Viennese garden soil used to enrich *N. viennensis*. *(b)* Cells of *N. viennensis* can take on many shapes (a condition called pleomorphism), as shown in this transmission electron micrograph of sectioned cells. A single cell of *N. viennensis* is about 0.5 μm in diameter.

a hundred times lower than those required by bacterial nitrifiers, and many archaeal nitrifiers are actually inhibited at the higher NH_3 concentrations required to support growth of nitrifying species of *Bacteria*.

Several species of *Thaumarchaeota* have been isolated and characterized, revealing a number of properties common to this group. Species have been isolated from habitats including the oceans, marine sediments, estuaries, soils, and hot springs. All existing isolates are chemolithotrophic ammonia-oxidizers (◀ Section 14.9), and most species, like *N. maritimus*, are able to grow at very low concentrations of NH_3. The membranes of all *Thaumarchaeota* also contain a unique lipid called *crenarchaeol* (◀ Figure 2.3*b*, *c*), a compound limited to species of this phylum. In addition, autotrophy in *Thaumarchaeota* is supported by the 3-hydroxypropionate/4-hydroxybutyrate cycle, a finding that further distinguishes archaeal nitrifiers from nitrifying *Bacteria* that employ the Calvin cycle for CO_2 fixation (◀ Sections 3.12 and 14.2). The 3-hydroxypropionate/4-hydroxybutyrate cycle also allows for the assimilation of organic carbon, and some archaeal nitrifiers have been shown to assimilate pyruvate during mixotrophic growth. Growth temperatures of *Thaumarchaeota* vary widely, as some species thrive in polar seas while others inhabit hot spring environments up to about 75°C.

Nitrososphaera viennensis was the first species of *Thaumarchaeota* isolated from soil (**Figure 17.15**). Cells of *N. viennensis* form irregular coccoids, and the organism is both mesophilic and neutrophilic. *N. viennensis* is a mixotroph (an organism that can fix CO_2 but grows best with an organic carbon source) and it grows as an aerobic chemolithotroph using either ammonia or urea as electron donor. When growing on urea, *N. viennensis* uses the enzyme urease to produce ammonia, which is the ultimate electron donor. Like marine species of *Thaumarchaeota*, *N. viennensis* can grow at low concentrations of NH_3, but unlike archaeal marine nitrifiers, *N. viennensis* can also tolerate high levels (up to 10 mM) of ammonium at neutral pH. Hence, *N. viennensis* and related archaeal nitrifiers may be active in soils containing high levels of ammonia, and in these



(a) (b)

Figure 17.16 Cold-dwelling *Thaumarchaeota*. *(a)* Photo of the Antarctic Peninsula taken from shipboard. The frigid waters that lie under the surface ice are habitats for cold-dwelling *Thaumarchaeota*. *(b)* Fluorescence photomicrograph of seawater treated with a fluorescent FISH probe (▶ Section 19.5) specific for species of *Thaumarchaeota* (green cells). Blue cells are stained with DAPI, a fluorescent DNA stain that stains all cells.

environments, they likely compete directly with bacterial nitrifiers. *N. viennensis* was isolated from a backyard garden soil (Figure 17.15*a*) by using cultivation-independent molecular analyses to monitor its growth in different culture media and tailor enrichment media to the growth needs of the organism. This discovery reminds us that when we open our eyes to the microbial world, major discoveries are often "just underfoot."

Environmental Distribution of *Thaumarchaeota*

Thaumarchaeota are ubiquitous in soils and found throughout the marine water column from the equator to the polar seas. *Thaumarchaeota* are one of the most abundant and widespread phyla on Earth, and they are often the dominant archaeal group found in soil and marine systems. Indeed, *Thaumarchaeota* account for approximately 20% of prokaryotic cells in the oceans and 1% of all microbes in soils. With the use of fluorescent phylogenetic probes (FISH, ▶ Section 19.5), *Thaumarchaeota* have been detected in oxic marine waters worldwide, and they are highly abundant in sea ice near Antarctica (**Figure 17.16**). Marine species are planktonic (suspended freely or attached to suspended particles in the water column, Figure 17.16*b*) and present in significant numbers ($\sim 10^4$/ml) in waters that are both nutrient-poor and very cold (0–4°C in seawater and below 0°C in sea ice). Marine *Thaumarchaeota* are particularly abundant in the deep ocean, where they can also account for up to 40% of picoplankton (▶ Section 20.12). The NH_3 concentration in marine waters is often near the threshold for archaeal nitrification, suggesting that *Thaumarchaeota* may play a major role in controlling NH_3 levels in the oceans.

Thaumarchaeota are also common in soils, sometimes outnumbering nitrifying *Bacteria* by 1000-fold, and are found across a wide range of soil pH, from about 3 to 9. While widely present in soils, *Thaumarchaeota* may be particularly important in acid soils (pH <5.5), which make up more than 30% of all soils. Nitrifiers oxidize NH_3, but at low pH, NH_4^+ predominates and is thus unavailable for nitrification. While nitrification occurs in acid soils, and often at high rates, bacterial nitrifiers have not been observed to grow below pH 6.3. In contrast, the thaumarchaeotal species *Nitrosotalea devanaterra*, isolated from acidic agricultural soil, grows optimally at pH 4–5. The ability of *Thaumarchaeota* to grow at low NH_3 concentrations explains how they can be successful in acidic soils where free NH_3 is present in very low concentration.

Check Your Understanding

- How does the organism *Nitrosopumilus maritimus* conserve energy and obtain carbon?
- In what environments might you expect to find species of *Thaumarchaeota*?

17.6 *Nanoarchaeota* and the "Hospitable Fireball"

KEY GENUS: *Nanoarchaeum*

The ***Nanoarchaeota*** are represented by a single species, the highly unusual *Nanoarchaeum equitans* (**Figure 17.17**). *N. equitans* is one of the smallest cellular organisms known and has the smallest genome among species of *Archaea* (0.49 Mb). The coccoid cells of *N. equitans* are very small, about 0.4 μm in diameter, and have only about 1% of the volume of an *Escherichia coli* cell. They cannot grow in pure culture and replicate only when attached to the surface of their host organism, *Ignicoccus hospitalis* (Section 17.11), a hyperthermophilic species of *Crenarchaeota* whose name means "the hospitable fireball." *N. equitans* grows to 10 or more cells per *Ignicoccus* cell and lives an apparently parasitic lifestyle, making it the only known archaeal symbiont. Indeed, in agreement with its lifestyle, the species epithet *equitans* means "riding," as in "riding the fireball."

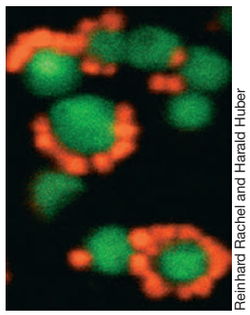
Nanoarchaeum and Its Host

N. equitans and its host *Ignicoccus* were first isolated from a submarine hydrothermal vent (▶ Section 20.16) off the coast of Iceland. However, environmental sampling of 16S ribosomal RNA genes (▶ Section 19.6) indicates that organisms phylogenetically similar to *N. equitans* exist in other submarine hydrothermal vents and in terrestrial hot springs, so *Archaea* of this kind are probably distributed worldwide in suitable hot habitats. Like its host *Ignicoccus*, *N. equitans* grows at temperatures from 70 to 98°C and optimally at 90°C.

The metabolism of *Nanoarchaeum* is not fully understood, but it likely depends on its host for many metabolic functions. *Ignicoccus* is an autotroph that uses H_2 as an electron donor and S^0 as an electron acceptor and probably supplies *N. equitans* with organic carbon. From analyses of its genome (see next subsection), *N. equitans* is incapable of metabolizing H_2 and S^0 for energy. Thus, whether it generates ATP from substances obtained from *Ignicoccus* or obtains its ATP directly from its host is unknown. The appearance of *N. equitans* cells is typical of *Archaea*, with a cell wall consisting of an S-layer (◀ Section 2.5) that overlays what appears to be a periplasmic space (Figure 17.17*b*).

The *Nanoarchaeum equitans* Genome

The sequence of the *N. equitans* genome provides insight into this organism's obligately parasitic lifestyle. Its single, circular genome is only 490,885 nucleotides long, one of the smallest cellular



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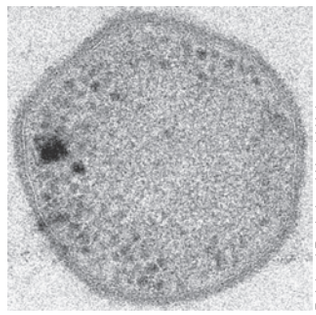


(b)

Figure 17.17 ***Nanoarchaeum equitans.*** *(a)* Fluorescence micrograph of cells of *N. equitans* (red) attached to cells of *Ignicoccus* (green). Cells were stained by FISH (▶ Section 19.5) using specific nucleic acid probes targeted to each organism. *(b)* Transmission electron micrograph of a thin section of a cell of *N. equitans*. Note the distinct cell wall. Cells of *N. equitans* are about 0.4 μm in diameter.

genomes yet sequenced (◀ Table 10.1). Genes for several important metabolic functions are missing from the *N. equitans* genome, including those for the biosynthesis of amino acids, nucleotides, coenzymes, and lipids. Also missing are genes encoding proteins for widely distributed catabolic pathways, such as glycolysis. Presumably, all of these functions are carried out for *N. equitans* by its *Ignicoccus* host, with transfer of needed substances from *Ignicoccus* to the attached *N. equitans* cells. *N. equitans* also lacks some of the genes necessary to encode ATPase, and this indicates that it may not synthesize a functional ATPase. If true, this would be the only cellular organism that lacks ATPase. If no ATPase is present and substrate-level phosphorylation does not occur (because a glycolytic pathway is lacking), then *N. equitans* would be dependent on *Ignicoccus* for energy as well as carbon.

With so many genes missing, which genes remain in the *N. equitans* genome? *N. equitans* contains genes encoding the key enzymes for DNA replication, transcription, and translation as well as genes for DNA repair enzymes. In addition to its small size, the genome of *N. equitans* is also among the most gene dense of any organism known, as over 95% of the *N. equitans* chromosome encodes proteins—a value higher than that of most all other prokaryotic cells (◀ Section 10.3).

The DPANN Superphylum

Metagenomic analyses of numerous habitats have identified diverse archaeal phyla based on their genome sequences (see Section 17.8). Many of these phyla affiliate phylogenetically with *Nanoarchaeota* in the **DPANN superphylum** (Figure 17.1). This large cluster of *Archaea* is named for the phyla **D***iapherotrites*, **P***arvarchaeota*, **A***enigmarchaeota*, **N***anohaloarchaeota*, and **N***anoarchaeota*, although recently additional phyla have been discovered to reside in this group. These *Archaea* escaped detection for so long because their 16S ribosomal RNA genes are so highly divergent from those of other *Archaea* that they were not recovered using standard metagenomic techniques for surveys of 16S rRNA genes in the environment (▶ Section 19.8 and Figure 19.21).

Members of the DPANN superphylum, like *N. equitans*, appear to be extremely small cells that have highly reduced genomes. Most have very limited metabolic capabilities lacking many core biosynthetic pathways such as those for the synthesis of certain nucleotides, amino acids, and lipids. The DPANN *Archaea* are predicted to have obligately dependent lifestyles relying on other *Archaea* or *Bacteria* to provide them with the metabolites they need to survive. Like *N. equitans*, many of the DPANN *Archaea* are likely to be symbiotic or parasitic, living on or within other cells. Sequence analyses of complete or nearly complete DPANN genomes recovered from natural samples suggest that these organisms are obligate anaerobes that rely on fermentative forms of metabolism. DPANN *Archaea* have been observed in a wide range of environments including extreme environments (geothermal, acidic, and hypersaline), temperate environments (lake and marine sediments), and the deep subsurface (▶ Section 20.8 and Figure 20.16).

Check Your Understanding

- Which aspects of the biology of *Nanoarchaeum equitans* make it especially interesting from an evolutionary point of view?
- Why can it be said that *N. equitans* is both a carbon and an energy parasite?

17.7 *Korarchaeota*, the "Secret Filament"

KEY GENUS: ***Korarchaeum***

Ribosomal RNA sequences of ***Korarchaeota*** (Figure 17.1) have been retrieved from a range of geothermal habitats, both submarine and terrestrial. However, *Korarchaeum cryptofilum*, whose name means "the secret filament of youth," is the only characterized species in the phylum *Korarchaeota*.

First observed as a 16S ribosomal RNA gene phylotype (▶ Figures 19.16 and 19.17) recovered from Obsidian Pool, a hot spring in Yellowstone National Park, USA, *K. cryptofilum* has yet to be grown in pure culture. However, its genome sequence has been determined from metagenomic analyses of a highly enriched culture. *K. cryptofilum* is an obligately anaerobic chemoorganotroph and a hyperthermophile, growing at 85°C. Cells are long, thin (<0.2-μm diameter) filaments of variable length (**Figure 17.18*a–c***), with most filaments being around 15 μm long but some reaching as much as 100 μm. Filaments of *K. cryptofilum* have a tough paracrystalline S-layer (Figure 17.18*d*), which maintains cell integrity in its extremely hot habitat.

Though *K. cryptofilum* cannot be grown in isolation, its genome sequence provides clues about its lifestyle. *K. cryptofilum* lacks the ability to perform anaerobic respiration and lives a fermentative lifestyle. Similar to other archaeal hyperthermophiles, *K. cryptofilum* grows by fermentation of peptides or amino acids (see Table 17.4). *K. cryptofilum* lacks many core genes in biosynthesis including the ability to synthesize purines, coenzyme A, and several essential cofactors. Presumably *K. cryptofilum* obtains these essential components from its environment. The inability of *K. cryptofilum* to synthesize molecules essential for its own growth may be explained by the evolution of mutual dependence in microbial communities (◀ Section 13.8 and Figure 13.18). This dependence on other members of the hot spring microbial community for key necessities may also explain why *K. cryptofilum* has not yet been obtained in pure culture.

Phylogenetic analyses indicate affinity between *Thaumarchaeota*, *Crenarchaeota*, and *Korarchaeota* (Figure 17.1). These lineages, along

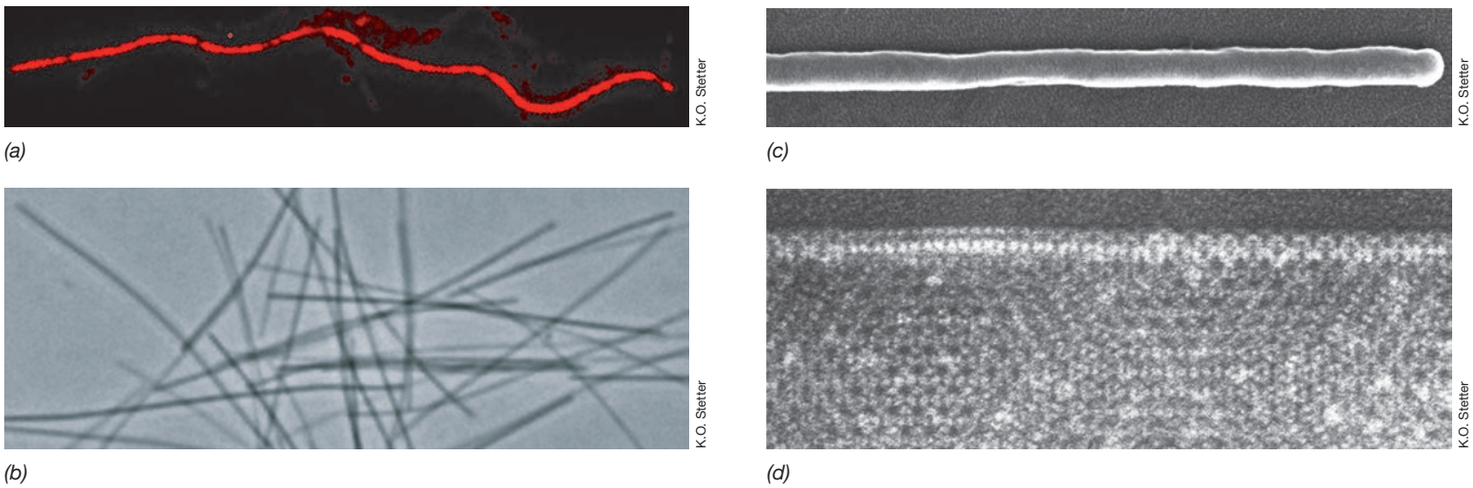


Figure 17.18 ***Korarchaeum cryptofilum.*** *(a)* Fluorescence in situ hybridization (FISH; ▶ Section 19.5) first identified the morphology of *Korarchaeota* growing in an enrichment culture at 85°C. *(b)* Phase-contrast image of filaments of *K. cryptofilum*. *(c)* Scanning electron micrograph of a *K. cryptofilum* filament. *(d)* Transmission electron micrograph of the surface of a *K. cryptofilum* filament showing the paracrystalline S-layer (◀ Section 2.5). Filaments of *K. cryptofilum* are about 0.17 μm wide and 15 μm long.

with a group called the *Aigarchaeota* (see next section), form a cluster of phyla known as the **TACK superphylum** (Figure 17.1). Like the DPANN superphylum (Section 17.6), many of the species in the TACK superphylum are only known from metagenomic analyses, but such information is often important for piecing together the nutritional secrets of uncultured microbes and obtaining their successful laboratory culture.

Check Your Understanding

- What is the most likely reason that *Korarchaeum cryptofilum* has been difficult to isolate in pure culture?
- What phyla comprise the TACK superphylum?

17.8 Other Cryptic Archaeal Phyla

Advances in DNA sequencing technologies have allowed metagenomic analyses to become almost routine. As powerful DNA sequencing approaches are applied to a wider range of habitats, more and more genomes of unusual *Archaea* are being discovered such as those just discussed in the DPANN and TACK superphyla (Sections 17.6, 17.7). These discoveries have greatly expanded our knowledge of archaeal diversity. Thus far, our only insight into these newly discovered archaeal phyla comes from the analysis of genome data assembled from metagenomes. For most of these groups, no cultures are available and there is little direct evidence pertaining to their physiology or ecology. However, metagenomic data allows us to form many hypotheses.

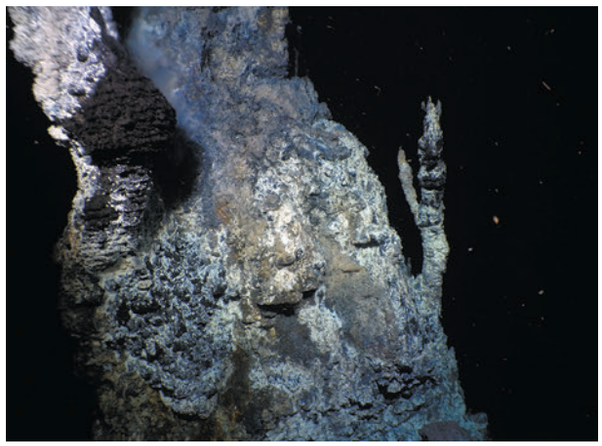
Asgard *Archaea*

The **Asgard *Archaea*** is a large group of related phyla discovered and characterized through metagenomic analyses (▶ Section 20.8 and Figure 20.16). The first of the Asgard *Archaea* to be discovered were those in the phylum *Lokiarchaeota*. *Lokiarchaeota* were discovered through metagenomic analyses of microbial communities that inhabit deep marine sediments near a highly sulfidic hydrothermal vent system known as Loki's Castle (**Figure 17.19*a***), located along the Mid-Atlantic Ridge between Greenland and Norway. Remarkably, the genomes of *Lokiarchaeota* contain a number of *eukaryotic* signature genes; in particular, genes associated with membrane remodeling and the development of a cellular cytoskeleton. These results suggest that features uniquely associated with the eukaryotic cell may also be found within the domain *Archaea*. The presence of these eukaryotic-like genes in *Lokiarchaeota* also support well-established findings that the processes of transcription and translation in *Archaea* bear greater resemblance to those in *Eukarya* than to those in *Bacteria* (◀ Section 6.6).

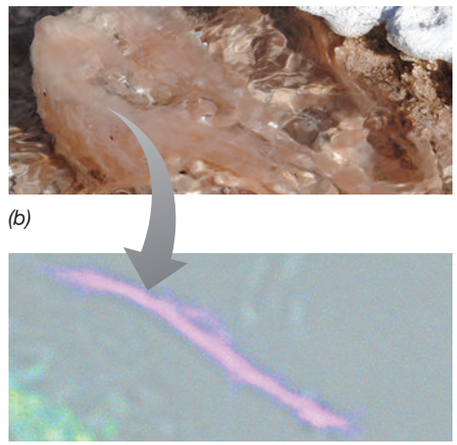
Since the discovery of the *Lokiarchaeota*, related *Archaea* have been found in many marine and freshwater sediments as well as in geothermal systems. These organisms have been given the phylum names *Thorarchaeota*, *Heimdallarchaeota*, and *Odinarchaeota* after the legendary names of Norse gods. Together, these *Archaea* comprise the Asgard *Archaea*. Like the *Lokiarchaeota*, the genomes of other Asgard *Archaea* show many features that were once thought to be uniquely eukaryal. And, since Asgard *Archaea* diverge deeply within the domain *Archaea* (Figure 17.1), it has been hypothesized that they are close relatives of *Eukarya*. Thus far we know relatively little about these mysterious organisms, although their genomes point to an anaerobic and fermentative lifestyle similar to some cultured *Archaea* and many *Archaea* known only from their genomes.

Aigarchaeota and *Bathyarchaeota*

As with the Asgard *Archaea*, all of our knowledge of *Aigarchaeota* and *Bathyarchaeota* comes from genomes assembled from metagenomic data. The *Aigarchaeota* (Figure 17.1) belong to the TACK superphylum. The first characterized genome from this group came from "*Candidatus* Caldiarchaeum subterraneum," identified in a metagenome obtained from geothermally heated water found in a Japanese gold mine deep underground. Additional genomes have been identified from other geothermal systems including hot springs. For example, in the hot water runoff channel of a Yellowstone hot spring where *Thermocrinis*, a hyperthermophilic species of *Bacteria*



(a)



(b) (c)

Figure 17.19 ***Lokiarchaeota* and *Aigarchaeota*.** *(a)* Loki's Castle, an ocean floor hydrothermal vent system at the Mid-Atlantic Ridge (depth, 2350 m). These vent chimneys are rich in iron–sulfide minerals, and when they were active, emitted water at nearly 300°C; the white areas on the surface of the mounds contain elemental sulfur and sulfur-oxidizing bacteria (see also Figure 13.11). The first genomes of *Lokiarchaeota* (Asgard *Archaea*, Figure 17.1) were assembled from metagenomic analyses of the microbial community living near this hydrothermal vent. *(b)* The "pink streamer" microbial community that develops at pH 8 and 82°C in the outflow channel of Octopus Spring, Yellowstone National Park, contains the bacterium *Thermocrinis* (◀ Section 16.19) and several *Archaea*. The mass of pink streamers is about 10 cm in diameter. *(c)* In a sample of the Octopus Spring streamers subjected to fluorescence in situ hybridization (FISH) analysis (▶ Section 19.5), a cell of *Aigarchaeota* (about 15 μm long) stains pink while cells of *Thermocrinis* stain green. Photos in *(b)* and *(c)* taken by J. Beam and W. Inskeep under research permits YELL-2012-SCI-5068 (for part *b*) and YELL-2013-SCI-5068 (for part *c*).

(◀ Section 16.19), forms pink-colored "streamers" (Figure 17.19*b*; see also Figure 16.52), a species of *Aigarchaeota* has been found that grows enmeshed in the *Thermocrinis* biomass. Although not yet cultured, this organism, given the name "*Candidatus* Calditenuis aerorheumensis" has been identified directly in natural samples using FISH probes (▶ Section 19.5). The probes revealed an organism that is a short filament (Figure 17.19*c*), and metagenomic analyses suggest that it is an aerobic chemoorganotroph capable of using a variety of organic compounds as energy sources including acetate, sugars, and fatty and amino acids and that it requires a number of vitamins and cofactors.

The *Bathyarchaeota* (Figure 17.1) are also members of the TACK superphylum. The first genomes from this phylum were discovered in metagenomes retrieved from deep-sea sediments. Additional genomes have been obtained from a range of freshwater and shallow marine sediments as well as subsurface habitats. Several genomes of *Bathyarchaeota* contain genes encoding enzymes of the reductive acetyl-CoA pathway. This pathway is used by a wide variety of microbes for CO_2 fixation, acetogenesis, and methanogenesis (◀ Section 14.14 and Figure 14.34). It has been hypothesized that some *Bathyarchaeota* are indeed methanogens, although this remains to be proven. The genomes of *Bathyarchaeota* that have been characterized suggest this phylum likely contains a diversity of metabolic types, including some species capable of degrading complex polymers as well as others that might metabolize C_1 compounds (methylotrophy, ◀ Section 14.16).

Check Your Understanding

- What unique feature of the Asgard *Archaea* could provide insight on the origin of the domain *Eukarya*?
- What technique has led to the recent discovery and characterization of many new phyla of *Archaea*?

III • *Crenarchaeota*

The phylum *Crenarchaeota* contains many cultured isolates, most of which are thermophiles or hyperthermophiles that use sulfur compounds as either electron donors or electron acceptors to thrive in their punishing environments.

Among *Archaea* in laboratory culture, the ***Crenarchaeota*** are mostly hyperthermophiles and include species growing optimally above the boiling point of water. Many hyperthermophiles are chemolithotrophic autotrophs, and because no phototrophs can survive such temperatures, these organisms are the sole primary producers in these habitats.

17.9 Habitats and Energy Metabolism of *Crenarchaeota*

Most hyperthermophilic *Archaea* have been isolated from geothermally heated soils or waters containing S^0 and H_2S, and most species metabolize sulfur in one way or another. In terrestrial environments, sulfur-rich springs, boiling mud, and soils may have temperatures up to 100°C and are mildly to extremely acidic owing to the production of sulfuric acid (H_2SO_4) from the biological oxidation of H_2S and S^0(◀ Section 14.7 and ▶ Section 21.4). Such hot, sulfur-rich environments, called **solfataras**, are found throughout the world (**Figure 17.20**), including Italy, Iceland, New Zealand, and Yellowstone National Park in Wyoming. Depending on the surrounding geology, solfataras can be mildly acidic to slightly alkaline (pH 5–8) or extremely acidic, with pH values below 1. Hyperthermophilic crenarchaeotes have been obtained from all of these environments, but most inhabit neutral or weakly acidic thermal habitats.

(a)

(b)

(c)

(d)

Figure 17.20 **Terrestrial habitats of hyperthermophilic *Archaea*: Yellowstone National Park.** *(a)* A typical solfatara; steam rich in H_2S rises to the surface. *(b)* Sulfur-rich hot spring, a habitat containing dense populations of *Sulfolobus*. The acidity in solfataras and sulfur springs comes from the oxidation of H_2S and S^0 to H_2SO_4 (sulfuric acid) by *Sulfolobus* and related sulfur-oxidizing microbes. *(c)* A typical neutral pH boiling spring, Imperial Geyser. Many different species of hyperthermophilic *Archaea* may reside in such a habitat. *(d)* An acidic iron-rich geothermal spring, another *Sulfolobus* habitat; here the oxidation of Fe^{2+} to Fe^{3+} generates acidity (▶ Section 22.2 and Figure 22.4*c*).

Hyperthermophilic *Crenarchaeota* also inhabit undersea hot springs called **hydrothermal vents**. We discuss the geology and microbiology of these fascinating microbial habitats in Section 20.16. Here we only note that submarine waters can be much hotter than surface waters because the water is under hydrostatic pressure. Indeed, all hyperthermophiles with growth temperature optima above 100°C originate from submarine sources. These sources include shallow (2–10 m depth) vents such as those off the coast of Vulcano, Italy, as well as deep (2000–4000 m depth) vents near ocean-spreading centers (see Figure 17.26). Deep hydrothermal vents are the hottest habitats so far known to yield viable life forms.

Most hyperthermophilic *Crenarchaeota* are obligate anaerobes. Their energy-yielding metabolism is either chemoorganotrophic or chemolithotrophic (or both, for example, in *Sulfolobus*) and is dependent on diverse electron donors and acceptors. Fermentation is rare and most bioenergetic strategies employ anaerobic respiration (**Table 17.4**). Energy is conserved during these respiratory processes by the same general mechanism widespread in *Bacteria*: electron transfer within the cytoplasmic membrane leading to the formation of a proton motive force from which ATP is made by way of proton-translocating ATPases (◀ Sections 3.8–3.10).

A variety of metabolisms are practiced by crenarchaeotes, and all of these were discussed in Chapter 14. For example, many hyperthermophilic crenarchaeotes grow chemolithotrophically under anoxic conditions with H_2 as the electron donor and S^0 or NO_3^- as the electron acceptor; a few can also oxidize H_2 aerobically (Table 17.4). H_2 respiration with ferric iron (Fe^{3+}) as electron acceptor also occurs in several hyperthermophiles. Other chemolithotrophic lifestyles include the oxidation of S^0 and Fe^{2+} aerobically or Fe^{2+} anaerobically with NO_3^- as the acceptor, and a variety of organic compounds are oxidized by one or another species of crenarchaeote (Table 17.4). Only one sulfate-reducing hyperthermophile is known (the euryarchaeote *Archaeoglobus*, Section 17.4). The only bioenergetic option apparently absent from hyperthermophilic crenarchaeotes is photosynthesis, a means of energy conservation that is apparently limited to temperatures below 74°C (see Figure 17.30). In the next two sections we will meet some of these fascinating microbes, microbes that have contributed significantly to our understanding of the physiochemical limits to life.

TABLE 17.4 Energy-yielding reactions of hyperthermophilic *Archaea* by nutritional class

Energy-yielding reaction	Metabolic type[a]	Example genera[b]
Chemoorganotrophic		
Organic compound + $S^0 \rightarrow H_2S + CO_2$	AnR	*Thermoproteus, Thermococcus, Desulfurococcus, Thermofilum, Pyrococcus, Pyrobaculum, Staphylothermus*
Organic compound + $SO_4^{2-} \rightarrow H_2S + CO_2$	AnR	*Archaeoglobus*
Organic compound + $O_2 \rightarrow H_2O + CO_2$	AeR	*Sulfolobus*
Organic compound $\rightarrow CO_2 + H_2$ + fatty acids	F	*Pyrodictium*
Organic compound + $Fe^{3+} \rightarrow CO_2 + Fe^{2+}$	AnR	*Pyrodictium*
Organic compound + $NO_3^- \rightarrow CO_2 + N_2$	AnR	*Pyrobaculum*
Pyruvate $\rightarrow CO_2 + H_2$ + acetate	F	*Pyrococcus, Thermococcus*
Peptides $\rightarrow CO_2$ + acetate + butanol	F	*Hyperthermus, Korarchaeum*
Chemolithotrophic		
$H_2 + S^0 \rightarrow H_2S$	AnR	*Acidianus, Pyrodictium, Thermoproteus, Stygiolobus, Ignicoccus*
$H_2 + NO_3^- \rightarrow NO_2^- + H_2O$ (NO_2^- is reduced to N_2 by some species)	AnR	*Pyrobaculum*
$4\ H_2 + NO_3^- + H^+ \rightarrow NH_4^+ + 2\ H_2O + OH^-$	AnR	*Pyrolobus*
$H_2 + 2\ Fe^{3+} \rightarrow 2\ Fe^{2+} + 2\ H^+$	AnR	*Pyrobaculum, Pyrodictium, Archaeoglobus*
$2\ H_2 + O_2 \rightarrow 2\ H_2O$	AeR	*Acidianus, Sulfolobus, Pyrobaculum*
$2\ S^0 + 3\ O_2 + 2\ H_2O \rightarrow 2\ H_2SO_4$	AeR	*Sulfolobus, Acidianus*
$2\ FeS_2 + 7\ O_2 + 2\ H_2O \rightarrow 2\ FeSO_4 + 2\ H_2SO_4$	AeR	*Sulfolobus, Acidianus, Metallosphaera*
$2\ FeCO_3 + NO_3^- + 6\ H_2O \rightarrow 2\ Fe(OH)_3 + NO_2^- + 2\ HCO_3^- + 2\ H^+ + H_2O$	AnR	*Ferroglobus*
$4\ H_2 + SO_4^{2-} + 2\ H^+ \rightarrow 4\ H_2O + H_2S$	AnR	*Archaeoglobus*
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	AnR	*Methanopyrus, Methanocaldococcus, Methanothermus*

[a]AnR, anaerobic respiration; AeR, aerobic respiration; F, fermentation.
[b]Most are *Crenarchaeota*.

Check Your Understanding

- What form of energy metabolism is widespread among hyperthermophiles?
- How might the temperature and pH tolerance of a hyperthermophile living in a solfatara differ from that of a hyperthermophile living in a hydrothermal vent?

17.10 *Crenarchaeota* from Terrestrial Volcanic Habitats

KEY GENERA: *Sulfolobus, Acidianus, Thermoproteus, Pyrobaculum*

Terrestrial volcanic habitats can have temperatures as high as 100°C and are thus suitable for hyperthermophilic *Archaea*. Two phylogenetically related organisms isolated from these environments are *Sulfolobus* and *Acidianus*. These genera form the heart of an archaeal order called the *Sulfolobales* (Table 17.5). In addition, *Sulfolobus* has been a model organism for molecular biology studies of *Archaea*.

Sulfolobales

Sulfolobus grows in sulfur-rich acidic thermal areas (Figure 17.20) at temperatures up to 90°C and at pH values of 1–5. Species of *Sulfolobus* are typically aerobic chemoorganotrophs, though some can also grow as chemolithotrophs by oxidizing H_2S or S^0 to H_2SO_4 and fixing CO_2. Cells of *Sulfolobus* are more or less spherical but contain distinct lobes (Figure 17.21*a*). Cells adhere tightly to sulfur crystals, where they can be seen with a microscope after staining with fluorescent dyes (◀ Figure 14.19*b*). Besides the aerobic respiration of sulfur or organic compounds, some *Sulfolobus* can also oxidize Fe^{2+} to Fe^{3+}, and this ability has been harnessed for the high-temperature leaching of iron and copper ores (▶ Sections 21.6 and 22.1 and 22.2).

A facultative aerobe resembling *Sulfolobus* also lives in acidic solfataric springs. This organism, *Acidianus* (Figure 17.21*b*), differs from *Sulfolobus* most clearly by its ability to grow using S^0 both anaerobically as well as aerobically. Under *aerobic* conditions the organism uses S^0 as an electron *donor*, oxidizing S^0 to H_2SO_4, with O_2 as an electron acceptor. Under *anaerobic* conditions, by contrast, *Acidianus* uses S^0 as an electron *acceptor* with H_2 as the electron donor, forming H_2S as the reduced product. Thus, the metabolic fate of S^0 in cultures of *Acidianus* depends on the presence or absence of O_2. Like *Sulfolobus*, *Acidianus* is roughly spherical in shape but is not as lobed (Figure 17.21*b*). It grows at temperatures from 65°C up to a maximum of 95°C. As a group, then, the *Sulfolobales* contain the most thermophilic of all highly acidophilic *Archaea*.

Thermoproteales

Key genera within the *Thermoproteales* are *Thermoproteus*, *Thermofilum*, and *Pyrobaculum*. The genera *Thermoproteus* and *Thermofilum* consist of rod-shaped cells that inhabit neutral or slightly acidic hot springs. Cells of *Thermoproteus* are rigid rods about 0.5 μm in

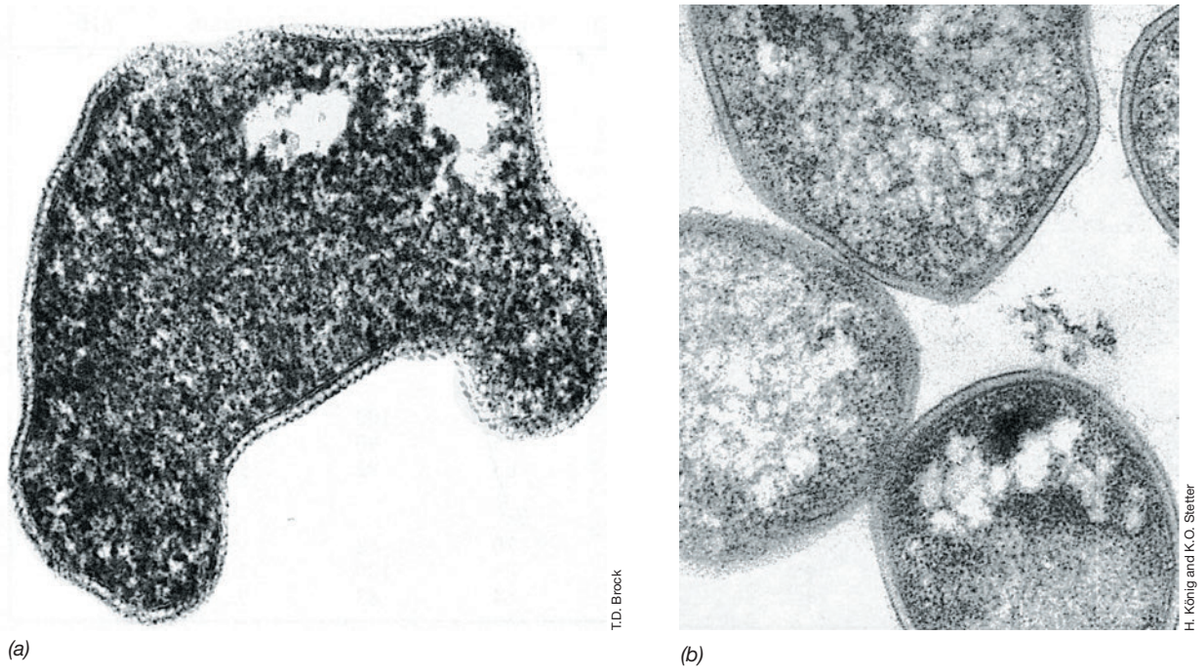
TABLE 17.5 Properties of some well-characterized hyperthermophilic *Crenarchaeota*

Genus	Morphology	Relationship to O_2[a]	Temperature		
			Minimum	Maximum	Optimum pH
Sulfolobus	Lobed coccus	Ae	55	90	2–3
Acidianus	Coccus	Fac	60	95	2
Metallosphaera	Coccus	Ae	50	80	2
Sulfurococcus	Coccus	Ae	40	85	2.5
Thermoproteus	Rod	An	60	96	6
Pyrobaculum	Rod	Fac	74	102	6
Desulfurococcus	Coccus	An	70	95	6
Staphylothermus	Cocci in clusters	An	65	98	6–7
Pyrodictium	Disk-shaped with filaments	An	82	110	6
Pyrolobus	Lobed coccus	Fac	90	113	5.5
Ignicoccus	Irregular coccus	An	65	103	5
Hyperthermus	Irregular coccus	An	75	108	7
Stetteria	Coccus	An	68	102	6
Strain 121[b]	Coccus	An	85	121	7

[a]Ae, aerobe; An, anaerobe; Fac, facultative.
[b]Also known by the unofficial taxonomic name of *"Geogemma barossii."*

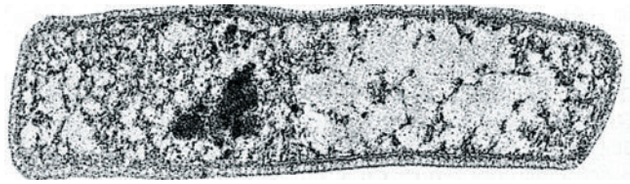
diameter and highly variable in length, ranging from short cells of 1–2 μm (**Figure 17.22*a***) up to filaments 70–80 μm long. Filaments of *Thermofilum* are thinner, some as little as 0.17–0.35 μm wide, with filament lengths ranging up to 100 μm (Figure 17.22*b*).

Both *Thermoproteus* and *Thermofilum* are strict anaerobes that carry out an S^0-based anaerobic respiration (Table 17.4). Most *Thermoproteus* isolates can grow chemolithotrophically on H_2 or chemoorganotrophically on complex carbon substrates such as yeast extract, small peptides, starch, glucose, ethanol, malate, fumarate, or formate (Table 17.4). *Pyrobaculum* (Figure 17.22*c*) is a rod-shaped hyperthermophile but is physiologically distinct from other *Thermoproteales* in that some species of *Pyrobaculum* can respire aerobically.

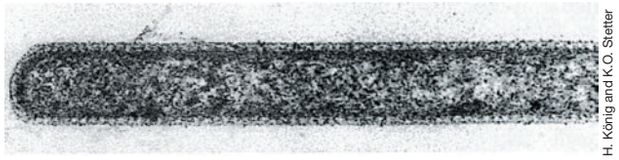


(a) (b)

Figure 17.21 Acidophilic hyperthermophilic *Archaea*, the *Sulfolobales*. *(a) Sulfolobus acidocaldarius*; electron micrograph of a thin section. *(b) Acidianus infernus*; electron micrograph of a thin section. Cells of both organisms vary from 0.8 to 2 μm in diameter. *Sulfolobales* typically show temperature optima below 90°C (Table 17.5).



(a)



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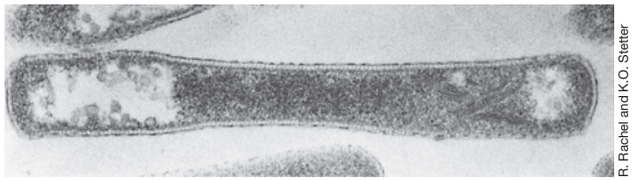


(c)

Figure 17.22 **Rod-shaped hyperthermophilic *Archaea*, the *Thermoproteales*.** *(a) Thermoproteus neutrophilus*; electron micrograph of a thin section. A cell is about 0.5 μm in diameter. *(b) Thermofilum librum*. A cell is about 0.25 μm in diameter. *(c) Pyrobaculum aerophilum*. Transmission electron micrograph of a thin section; a cell measures 0.5 × 3.5 μm. Although the temperature optimum of *P. aerophilum* is 100°C, optima for other *Thermoproteales* are all below 90°C (Table 17.5).

However, *Pyrobaculum* can also grow by anaerobic respiration with NO_3^-, Fe^{3+}, or S^0 as electron acceptors and H_2 as an electron donor (that is, they can grow chemolithotrophically and autotrophically). Other species of *Pyrobaculum* can grow anaerobically on organic electron donors, reducing S^0 to H_2S. The growth temperature optimum of *Pyrobaculum* is 100°C, and species of this organism have been isolated from both terrestrial hot springs and hydrothermal vents.

Check Your Understanding

- What are the similarities and differences between *Sulfolobus* and *Pyrobaculum*?
- Among *Thermoproteales*, what is unusual about the metabolism of *Pyrobaculum*?

17.11 *Crenarchaeota* from Submarine Volcanic Habitats

KEY GENERA: *Pyrodictium, Pyrolobus, Ignicoccus, Staphylothermus*

We now consider the microbiology of submarine volcanic habitats, homes to the most thermophilic of all known *Archaea*. These habitats include both shallow-water thermal springs and deep-sea hydrothermal vents. We discuss the geology of these fascinating microbial habitats in Section 20.16 and the interesting animal communities that develop there in Section 23.11. The organisms to be described here constitute an order of *Archaea* called the *Desulfurococcales* (Table 17.5).

Pyrodictium and *Pyrolobus*

Pyrodictium and *Pyrolobus* are examples of microorganisms whose growth temperature optimum lies above 100°C; the optimum for *Pyrodictium* is 105°C and for *Pyrolobus* is 106°C. Cells of *Pyrodictium* are irregularly disk-shaped and grow in culture in a mycelium-like layer attached to crystals of S^0. The cell mass consists of a network of fibers to which individual cells are attached (**Figure 17.23*a***). The fibers are hollow and consist of protein arranged in a fashion similar to that of bacterial flagella (◀ Section 2.9). However, the filaments do not function in motility but instead as organs of attachment. The cell walls of *Pyrodictium* are composed of glycoprotein. Physiologically, *Pyrodictium* is a strict anaerobe that grows chemolithotrophically on H_2 as an electron donor and S^0 as an electron acceptor (◀ Section 14.12) or chemoorganotrophically on complex mixtures of organic compounds (Table 17.4).

Pyrolobus fumarii (Figure 17.23*c*) is one of the most thermophilic of the hyperthermophiles. Its growth temperature maximum is 113°C (Table 17.5). *P. fumarii* lives in the walls of "black smoker" hydrothermal vent chimneys (▶ Section 20.16 and Figures 20.43, 20.44, and 20.46) where its autotrophic abilities contribute organic carbon to this otherwise inorganic environment. *P. fumarii* cells are coccoid-shaped (Figure 17.23*c*), and the cell wall is composed of protein. The organism is an obligate H_2 chemolithotroph, growing by the oxidation of H_2 coupled to the reduction of NO_3^- to ammonium (NH_4^+), thiosulfate ($S_2O_3^{2-}$) to H_2S, or very low concentrations of O_2 to H_2O. Besides its extremely thermophilic nature, *P. fumarii* can withstand temperatures substantially above its growth temperature maximum. For example, cultures of *P. fumarii* survive autoclaving (121°C, ◀ Section 4.17) for 1 h, a condition that even bacterial endospores (◀ Section 2.8) cannot withstand.

Another organism in this group shares with *Pyrolobus* a growth temperature optimum of 106°C. However, "Strain 121," as this organism has been called, actually shows weak growth at 121°C, and cells remain viable for 2 h at 130°C. Only *Methanopyrus*, a hyperthermophilic methanogen, can grow at a higher temperature (122°C, Section 17.2). Strain 121 consists of coccoid cells that are motile by rotating archaella (Figure 17.23*d*); the organism is also a strict anaerobe and grows chemolithotrophically and autotrophically with Fe^{3+} as electron acceptor and formate or H_2 as electron donors. Thus, it is clear that the *Pyrodictium*/*Pyrolobus* group collectively contains many of the most hyperthermophilic examples of all known microbes.

Desulfurococcus and *Ignicoccus*

Other notable members of the *Desulfurococcales* include *Desulfurococcus*, the genus for which the order is named (**Figure 17.24*a***), and *Ignicoccus*. *Desulfurococcus* is a strictly anaerobic S^0-reducing organism like *Pyrodictium*, but differs from this organism in its phylogeny and the fact that it is much less thermophilic, growing optimally at about 85°C.

Ignicoccus species grow optimally at about 90°C, and their energy metabolism is based on H_2 as an electron donor and S^0 as an electron acceptor, as is that of so many hyperthermophilic *Archaea* (Table 17.4). Some *Ignicoccus* species are hosts to the small parasitic archaeon *Nanoarchaeum equitans* (Section 17.6). *Ignicoccus*

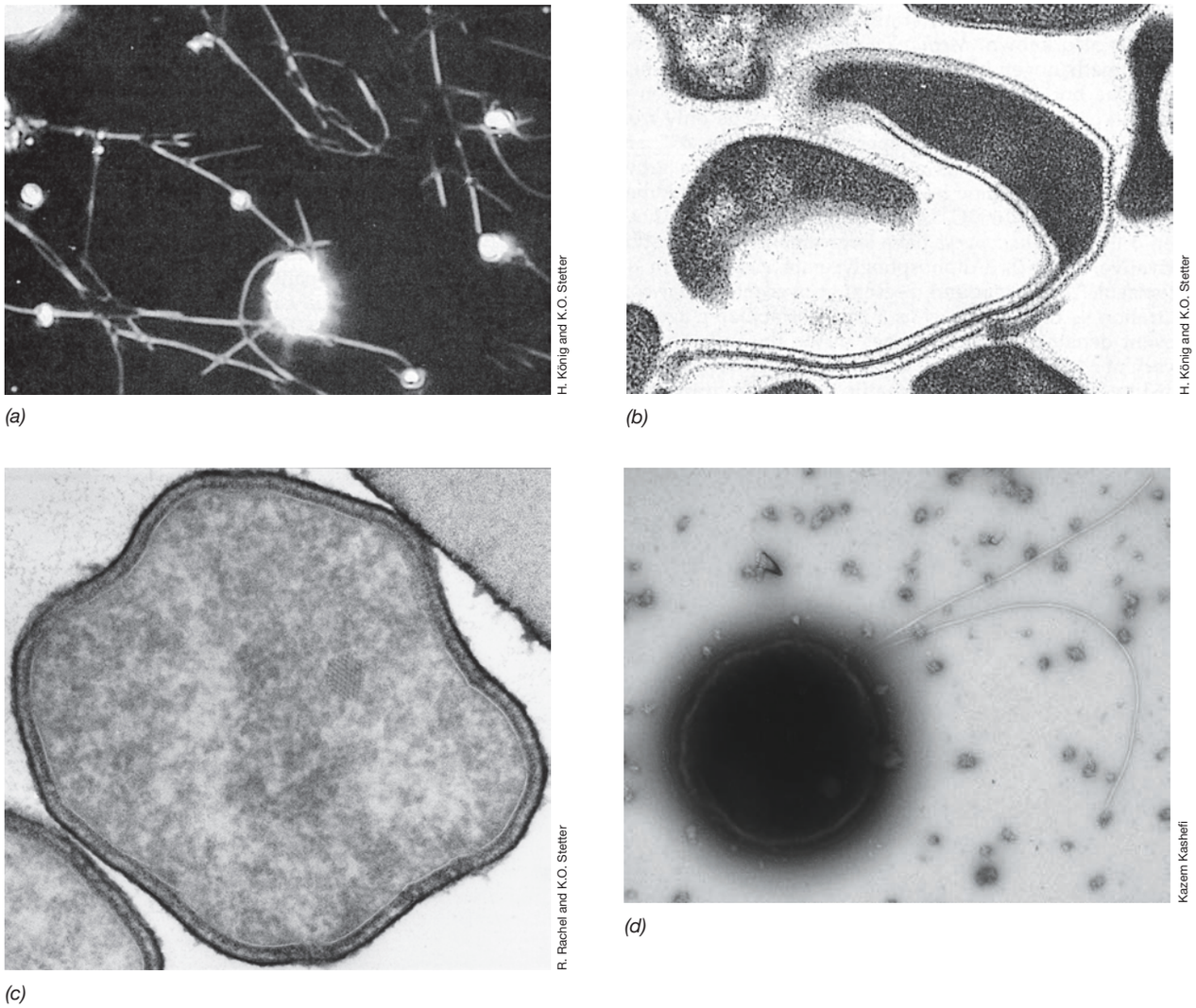


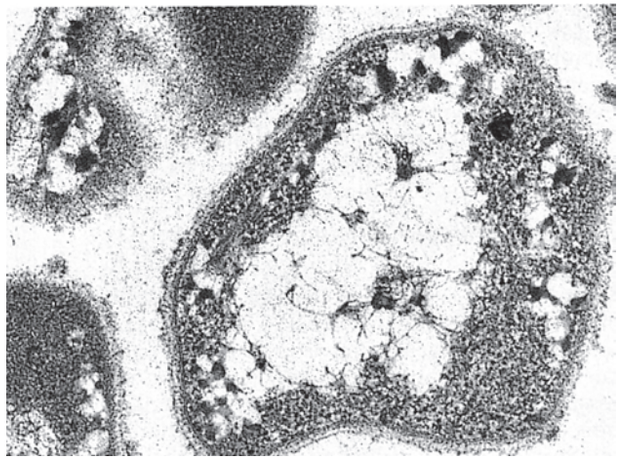
Figure 17.23 ***Desulfurococcales*** **with growth temperature optima > 100°C.** *(a) Pyrodictium occultum* (growth temperature optimum, 105°C), dark-field micrograph. *(b)* Thin-section electron micrograph of *P. occultum*. Cells are highly variable in diameter from 0.3 to 2.5 μm. *(c)* Thin section of a cell of *Pyrolobus fumarii*, one of the most thermophilic organisms ever described (growth temperature optimum, 106°C); a cell is about 1.4 μm in diameter. *(d)* Negative stain of a cell of "Strain 121," capable of growth at 121°C; a cell is about 1 μm wide. Although the *Desulfurococcales* contain the greatest number of hyperthermophiles capable of growth above 100°C, the most thermophilic of all known *Archaea* is actually a euryarchaeote, *Methanopyrus* (Section 17.2).

(Figure 17.24*b*) has a novel cell structure that lacks an S-layer and possesses a unique *outer cellular membrane*. This outer cellular membrane is distinct in several ways from the outer membrane of gram-negative *Bacteria* (◀ Section 2.4). Most notably, the outer cellular membrane of *Ignicoccus* contains ATPase and is the site of energy conservation. *Ignicoccus* also contains an inner cellular membrane that contains the cytoplasm and the enzymes responsible for biosynthesis and information processing. In this way neither the outer nor inner cellular membrane satisfies the typical definition of a cytoplasmic membrane (◀ Section 2.1).

Between the inner and outer cellular membranes of *Ignicococcus* is a large *intermediate compartment* that is analogous to the periplasm of gram-negative *Bacteria* but is much larger in volume, representing some two to three times the volume of the cytoplasm (Figure 17.24*b*). The periplasm of *Ignicoccus* also contains membrane-bound vesicles (Figure 17.24*b*) that may function in exporting substances outside the cell. In this way, the cell structure of *Ignicoccus* resembles that of *Eukarya*. Because of this, *Ignicoccus* has been proposed to be a modern descendant of the ancestral cell type that gave rise to the origin of eukaryotic cells, a topic we covered earlier (◀ Section 13.4 and Figure 13.10).

Staphylothermus

A morphologically unusual member of the order *Desulfurococcales* is the genus *Staphylothermus* (**Figure 17.25**). Cells of *Staphylothermus* are spherical, about 1 μm in diameter, and form aggregates of up to 100 cells, much like its morphological but mesophilic counterpart among the *Bacteria*, *Staphylococcus* (◀ Figure 16.20 and ▶ Figure 31.28). Unlike many hyperthermophiles, *Staphylothermus* is not a chemolithotroph



R. Rachel and K.O. Stetter

(a)

Reinhard Rachel and Karl O. Stetter

(b)

Figure 17.24 ***Desulfurococcales*** **with growth temperature optima < 100°C.** *(a)* Thin section of a cell of *Desulfurococcus saccharovorans*; a cell is 0.7 μm in diameter. *(b)* Thin section of a cell of *Ignicoccus islandicus*; the cell proper is surrounded by an extremely large periplasmic compartment. The cell itself measures about 1 μm in diameter and the cell plus periplasm measures 1.4 μm. *Ignicoccus* is a host for the tiny parasitic microbe *Nanoarchaeum* (see Figure 17.17).

but instead a chemoorganotroph, with species that grow optimally at 92°C. Organic carbon sources are used as electron donors in sulfur reduction, producing H_2S, acetate, and isovalerate as products (Table 17.4).

Isolates of *Staphylothermus* have been obtained from both shallow marine hydrothermal vents and very hot black smokers (see Figure 17.26; ▶ Section 20.16). This organism is apparently widely distributed in submarine thermal areas, where it is likely to play a significant role in consuming proteins released from dead organisms.

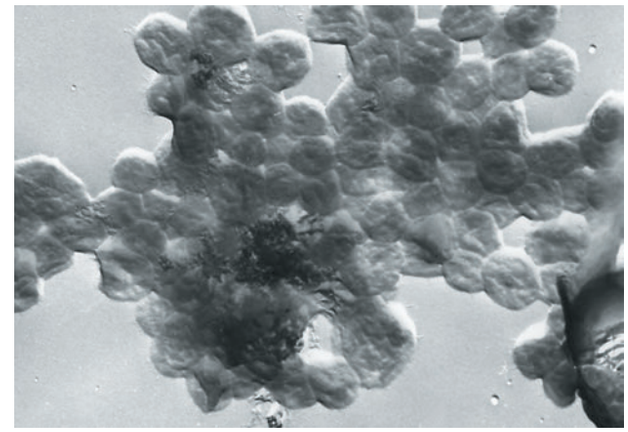


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Figure 17.25 **The hyperthermophile** ***Staphylothermus marinus.*** Electron micrograph of shadowed cells. A single cell is about 1 μm in diameter.

Check Your Understanding

- What archaeal genera contain species having growth optima at temperatures greater than 100°C?
- What unusual structural features are present in *Ignicoccus* and *Staphylothermus*?

IV • Evolution and Life at High Temperature

The ancestor of all *Archaea* was likely a hyperthermophile, and virtually all such organisms are *Archaea*. Hyperthermophilic *Archaea* have several biochemical adaptations that make life possible at high temperatures, and the widespread use of H_2 in their metabolisms may reflect early Earth conditions.

Most of the hyperthermophiles discovered so far are species of *Archaea* and some grow near to what may be the upper temperature limit for life. Here we consider the major factors that likely define the upper temperature limit for life and the biological adaptations of hyperthermophiles that permit them to exist at the exceptionally high temperatures of 100°C and higher. We end with a discussion of the importance of hydrogen (H_2) metabolism to the biology of hyperthermophiles.

17.12 An Upper Temperature Limit for Microbial Life

Habitats that contain liquid water—a prerequisite for cellular life—and that have temperatures higher than 100°C are only found where geothermally heated water flows out of vents or rifts in the ocean floor (◀ Figure 13.3 and ▶ Figures 20.43 and 20.44). The hydrostatic pressure that overlies the water keeps it from boiling, allowing it to reach temperatures of up to about 400°C in vents at several thousand meters' depth. In contrast, terrestrial hot springs can boil and therefore only attain temperatures near 100°C. It is not surprising, then,

that hydrothermal vents have been rich sources of hyperthermophilic *Archaea* with growth temperature optima above 100°C (Table 17.5).

Black smokers emit hydrothermal vent fluid at 250–350°C or higher. Metallic mounds or more upright structures called *chimneys* form from the metal sulfides that precipitate out of the hot fluid as it mixes with the surrounding, much cooler seawater (Figure 17.19*a* and **Figure 17.26**). As far as is known, the superheated vent water itself is sterile. However, hyperthermophiles thrive in mounds or smoker chimney walls where temperatures are compatible with their survival and growth (▶ Figures 20.45 and 20.46). By studying structures such as these, we can address the question, "What is the upper temperature for microbial (and presumably all forms of) life?"

What Is the Upper Temperature Limit for Life?

How high a temperature can hyperthermophiles withstand? Over the past several decades, the known upper temperature limit for life has been pushed higher and higher with the isolation and characterization of new species of thermophiles and hyperthermophiles (**Figure 17.27**). For some time the record holder was *Pyrolobus fumarii* (Figure 17.23*c*), with its upper temperature limit for growth of 113°C. The current record holder, *Methanopyrus* (Section 17.2 and Figure 17.8), however, has pushed the limit somewhat higher, with the ability to grow at 122°C and to survive substantial periods at even higher temperatures. Given the trend over the past several years (Figure 17.27), one can predict that *Archaea* even more hyperthermophilic than *Methanopyrus* may inhabit hydrothermal environments but have yet to be isolated. Indeed, many experts predict that the upper temperature limit for *Archaea* is likely to exceed 140°C, perhaps even 150°C, and that the maximum temperature allowing survival but not growth is hotter yet.

Biochemical Problems at Supercritical Temperatures

Whatever the upper temperature limit is for life, it is likely to be defined by one or more biochemical challenges that evolution has been unable to solve. There is obviously an upper limit, but we do not yet know what it is. Water samples taken directly from superheated (>250°C) hydrothermal vent discharges are devoid of measurable biochemical markers (DNA, RNA, and protein) that would signal life as we know it, while vents emitting water at temperatures below about 150°C yield evidence of macromolecules. These results are consistent with laboratory experiments on the stability of key biomolecules. For example, ATP is degraded almost instantly at 150°C. Thus, above 150°C, any life forms would have to deal with the heat lability of a molecule that is, as far as is known, a universal energy currency in all cells.

This conclusion has a caveat, however, in that it is possible that the stability of small molecules such as ATP may be significantly greater under cytoplasmic conditions of high levels of dissolved solutes than in pure solutions tested in the laboratory. Nevertheless, if life forms exist at temperatures above 150°C, they must be unique in at least one of two ways—they must either produce novel biomolecules absent from cells as we know them or deploy protective systems that maintain known cellular molecules in a sufficiently stable state to support biochemistry. Only time and more microbiological research and exploration for new organisms growing at ever higher temperatures will reveal which of these possibilities (or perhaps some of both) is correct.

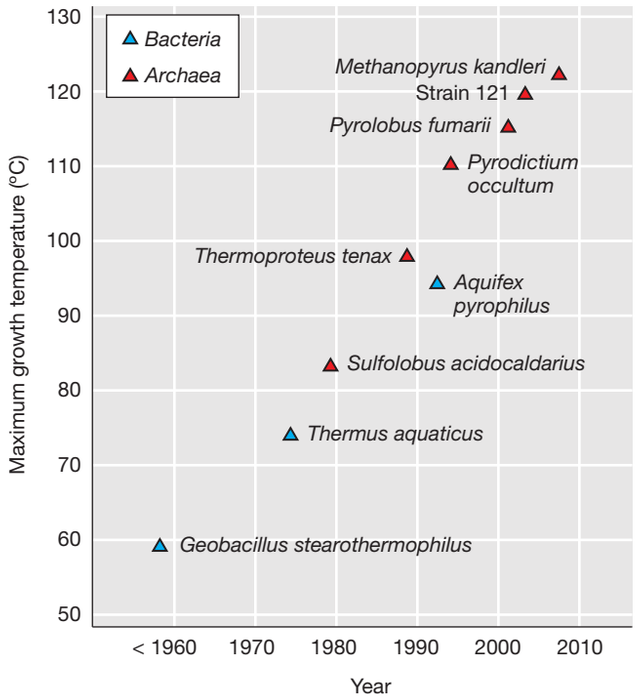
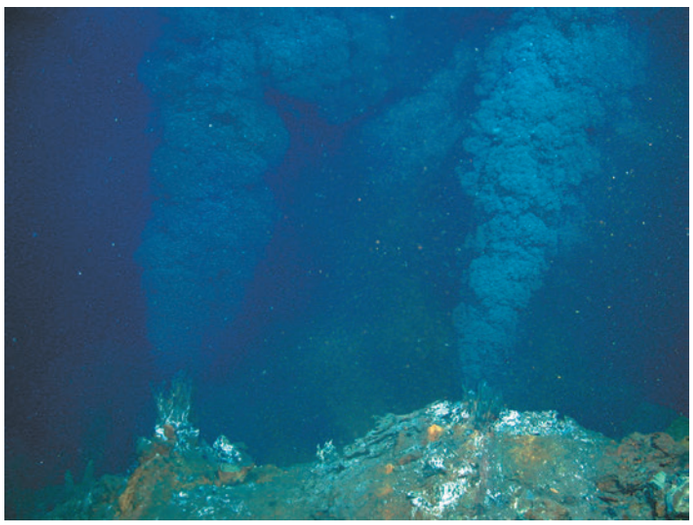


Figure 17.27 **Thermophilic and hyperthermophilic *Bacteria* and *Archaea*.** The graph gives the species that were, in turn, the record holders for growing at the highest temperature, from before 1960 to the present.

Mastering Microbiology
Art Activity: Figure 17.25 Thermophilic and hyperthermophilic *Bacteria* and *Archaea*



Anna-Louise Reysenbach and Woods Hole Oceanographic Institution

Figure 17.26 **Hydrothermal vents.** Hydrothermal mound from the Rainbow vent field, Mid-Atlantic Ridge hydrothermal system. The hydrothermal fluid emitting from the two short chimneys is > 300°C and rich in sulfidic minerals.

Check Your Understanding

- Where would you expect to find hyperthermophiles that grow at temperatures above 100°C? Why would it be impossible for organisms to grow at 200 or 300°C?

17.13 Molecular Adaptations to Life at High Temperature

Because all cellular structures and activities are affected by heat, hyperthermophiles are likely to exhibit multiple adaptations to the exceptionally high temperatures of their habitats. Here we briefly examine some adaptations employed by hyperthermophiles to protect their proteins and nucleic acids at high temperatures.

Protein Folding and Thermostability

Because most proteins denature at high temperatures, much research has been done to identify the properties of thermostable proteins. Protein thermostability derives from the folding of the molecule itself (◀ Figure 6.29), not because of the presence of any special amino acids. Perhaps surprisingly, however, the amino acid composition of thermostable proteins is not particularly unusual except perhaps in their slight bias for increased levels of amino acids that promote alpha-helical secondary structures. In fact, many enzymes from hyperthermophiles contain the same major structural features in both primary and higher-order structure (◀ Section 6.7) as their heat-labile counterparts from organisms that grow best at much lower temperatures.

Thermostable proteins typically do display some structural features that likely improve their thermostability. These include having highly hydrophobic cores, which decrease the tendency of the protein to unfold in an ionic environment, and more ionic interactions on the protein surfaces, which also help hold the protein together and work against unfolding. Ultimately, it is the *folding* of the protein that most affects its heat stability, and noncovalent ionic bonds called *salt bridges* on a protein's surface likely play a major role in maintaining the biologically active structure. But, as previously stated, many of these changes are possible with only minimal changes in primary structure (amino acid sequence), as seen when thermostable and heat-labile forms of the same protein are compared.

Chaperones: Assisting Proteins to Remain in Their Native State

Earlier we discussed a class of proteins called *chaperones* (heat shock proteins; ◀ Section 6.11) that function to refold partially denatured proteins. Hyperthermophilic *Archaea* have special classes of chaperones that function only at the highest growth temperatures. In cells of *Pyrodictium abyssi* (**Figure 17.28**), for example, a major chaperone is the protein complex called the **thermosome**. This complex keeps other proteins properly folded and functional at high temperature, helping cells survive even at temperatures above their maximal growth temperature. Cells of *P. abyssi* grown near its maximum temperature (110°C) contain high levels of the thermosome. Possibly because of this, the cells can remain viable following a heat shock, such as a 1-h treatment in an autoclave (121°C). In cells experiencing such a treatment and then returned to the optimum temperature, the thermosome, which is itself quite heat resistant, is thought to refold sufficient copies of key denatured proteins that *P. abyssi* can once again begin to grow and divide. Thus, as a result of chaperone activity, the upper temperature limit at which many hyperthermophiles can *survive* is higher than the upper temperature at which they can *grow*. The "safety net" of chaperone activity probably ensures that cells in nature that briefly experience temperatures above their growth temperature maximum are not killed by the exposure.

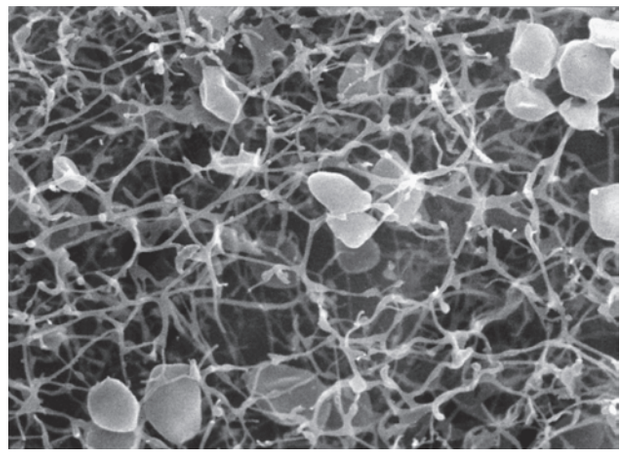


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Figure 17.28 ***Pyrodictium abyssi*, scanning electron micrograph.** *Pyrodictium* has been studied as a model of macromolecular stability at high temperatures. Cells are enmeshed in a sticky glycoprotein matrix that binds them together.

DNA Stability: Solutes, Reverse Gyrase, and DNA-Binding Proteins

What keeps DNA from melting at high temperatures? Various mechanisms are known to contribute. One such mechanism increases cellular solute levels, in particular potassium (K^+) or compatible organic compounds. For example, the cytoplasm of the hyperthermophilic methanogen *Methanopyrus* (Section 17.2) contains greater than 1 M potassium cyclic 2,3-diphosphoglycerate. This solute prevents chemical damage to DNA, such as depurination or depyrimidization (loss of a nucleotide base through hydrolysis of the glycosidic bond) from high temperatures, events that can lead to mutation (◀ Section 9.2). This compound and other compatible solutes such as potassium di-*myo*-inositol phosphate, which protects against osmotic stress, and the polyamines putrescine and spermidine, which stabilize both ribosomes and nucleic acids at high temperature, help maintain key cellular macromolecules in hyperthermophiles in their active forms.

A unique protein found *only* in hyperthermophiles is responsible for DNA stability in these organisms. All hyperthermophiles produce a special DNA topoisomerase called **reverse DNA gyrase**. This enzyme introduces positive supercoils into the DNA of hyperthermophiles (in contrast to the negative supercoils introduced by DNA gyrase present in *Bacteria* and most *Archaea*; ◀ Section 6.1). Positive supercoiling stabilizes DNA to heat and thereby prevents the DNA helix from spontaneously unwinding. The noticeable absence of reverse DNA gyrase in organisms whose growth temperature optima lie below 80°C strongly suggests a specific role for this enzyme in DNA stability at high temperatures.

Most *Archaea* also contain highly basic (positively charged) DNA-binding proteins that are remarkably similar in amino acid sequence and folding properties to the core histones of the *Eukarya*

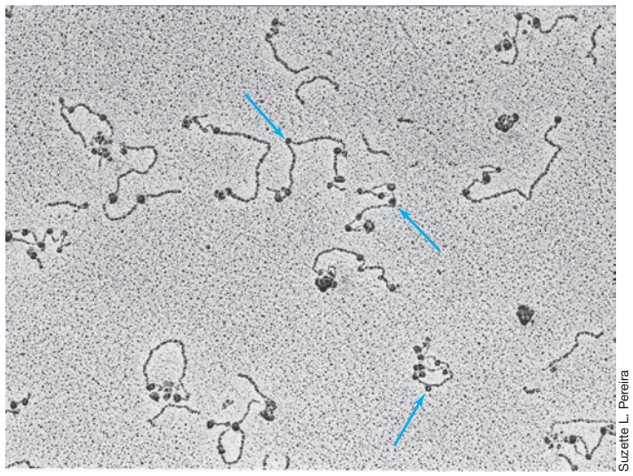

Figure 17.29 Archaeal histones. Electron micrograph of linearized plasmid DNA wrapped around copies of archaeal histone Hmf (from the hyperthermophilic methanogen *Methanothermus fervidus*) to form the roughly spherical, darkly stained nucleosome structures (arrows).Compare this micrograph with an artist's depiction of the histones and nucleosomes of *Eukarya* shown in Figure 2.43*b*.

(◀ Figure 2.43*b*). Archaeal histones from the hyperthermophilic methanogen *Methanothermus fervidus* (Figure 17.7*c*) have been particularly well studied. These proteins wind and compact DNA into nucleosome-like structures (**Figure 17.29**) and maintain the DNA in a double-stranded form at very high temperatures. Archaeal histones are found in most *Euryarchaeota*, including extremely halophilic *Archaea*, such as *Halobacterium*. However, because the extreme halophiles are not thermophiles, archaeal histones may have other functions besides DNA stability, in particular in assisting in gene expression by opening the helix to allow transcriptional proteins to bind.

Lipid and Ribosomal RNA Stability

How have the lipids and the protein-synthesizing machinery of hyperthermophiles adjusted to high temperatures? Virtually all hyperthermophilic *Archaea* synthesize lipids of the biphytanyl tetraether type (◀ Section 2.1). These lipids are naturally heat resistant because the phytanyl units forming each half of the membrane structure are covalently bonded to one another; this yields a *lipid monolayer* membrane instead of the normal lipid bilayer (◀ Figure 2.3). This structure resists the tendency of heat to pull apart a lipid bilayer constructed of fatty acid or phytanyl side chains that are not covalently bonded.

A final point on molecular adaptations to life at high temperatures is the base composition of ribosomal RNAs. Ribosomal RNAs are key structural and functional components of the ribosome, the cell's protein-synthesizing apparatus (◀ Section 6.10). Hyperthermophilic species of both *Bacteria* and *Archaea* show as much as a 15% greater proportion of G + C base pairs in their small ribosomal subunit RNAs compared with organisms that grow at lower temperatures. G + C base pairs form three hydrogen bonds compared to the two of AU base pairs (◀ Figure 6.1*c*), and thus the higher G + C content of the ribosomal RNAs should confer greater thermal stability on the ribosomes of these organisms and this should assist protein synthesis at high temperatures. By contrast to ribosomal RNAs, the G + C content of genomic DNA of hyperthermophiles is often rather low, which suggests that the thermal stability of ribosomal RNA might be an especially significant factor for life under hyperthermophilic conditions.

Check Your Understanding

- How do hyperthermophiles keep proteins and DNA from being destroyed by high heat?
- How are the lipids and ribosomes of hyperthermophiles protected from heat denaturation?

17.14 Hyperthermophilic *Archaea*, H_2, and Microbial Evolution

When cellular life first arose on Earth nearly 4 billion years ago (◀ Figures 1.10*a* and 13.1), temperatures almost certainly were far hotter than they are today. Thus, for hundreds of millions of years, Earth may have been suitable only for hyperthermophiles. Given the discussion above on the temperature limits to life, it has been hypothesized that biological molecules, biochemical processes, and the first cells arose on Earth around hydrothermal springs and vents on the seafloor as they cooled to temperatures compatible with biological molecules (◀ Section 13.1 and Figures 13.3 and 13.4). The phylogeny of modern hyperthermophiles (Figure 17.1), as well as the similarities in their habitats and metabolism to those of early cells on Earth, suggests that hyperthermophiles may be the closest remaining descendants of ancient cells and are a living window into the biology of ancient microbial life.

UNIT 4

Hyperthermophilic Habitats and H_2 as an Energy Source

The oxidation of H_2 linked to the reduction of Fe^{3+}, S^0, NO_3^-, or, rarely, O_2 is a widespread mechanism of energy conservation in hyperthermophiles (Table 17.4 and **Figure 17.30**). This, coupled with the likelihood that these hyperthermophiles best characterize early Earth phenotypes, points to the important role H_2 has played in the evolution of microbial life. Hydrogen metabolism may have evolved in primitive organisms because of the ready availability of H_2 and suitable inorganic electron acceptors in their primordial environments, but also because known examples of H_2-based energy conservation require relatively few proteins (◀ Figure 13.5). As chemolithotrophs, these organisms may have obtained all of their carbon from CO_2 or might have assimilated available organic compounds directly for biosynthetic needs. Either way, it is likely that the oxidation of H_2 was the energetic driving force for maintaining life processes.

Mastering Microbiology
Art Activity: Figure 17.28 Upper temperature limits for energy metabolism

If one compares microbial energy conservation mechanisms as a function of temperature from data of cultured *Bacteria* and *Archaea*, only chemolithotrophic organisms are known at the hottest temperatures (Figure 17.30). Chemoorganotrophy occurs up to at least 110°C, as this is the upper temperature limit for growth of *Pyrodictium occultum*, an organism that can conserve energy and grow by fermentation and by chemolithotrophic growth on H_2 with S^0 as electron acceptor (Table 17.4). Photosynthesis is apparently the least heat-tolerant of all bioenergetic processes, with no hyperthermophilic representatives known and an upper temperature limit of 73°C. This is consistent with the conclusion that

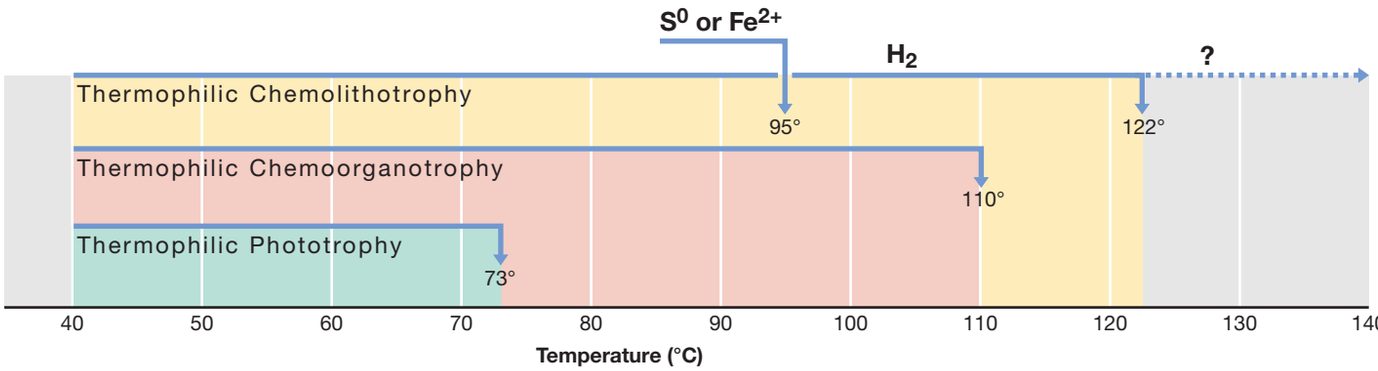

Figure 17.30 Upper temperature limits for energy metabolism. The record holder for phototrophy is *Synechococcus lividus* (*Bacteria*, cyanobacteria); for chemoorganotrophy, *Pyrodictium occultum* (*Archaea*); for chemolithotrophy with S^0 as electron donor, *Acidianus infernus* (*Archaea*); for chemolithotrophy with Fe^{2+} as electron donor, *Ferroglobus placidus* (*Archaea*); and for chemolithotrophy with H_2 as electron donor, *Methanopyrus kandleri* (*Archaea*, 122°C).

anoxygenic photosynthesis first appeared on Earth some hundreds of millions of years after the first life forms are thought to have appeared (◀ Figure 13.1).

Comparisons of bioenergetic options as a function of temperature (Figure 17.30) point to the H_2-oxidizing hyperthermophilic *Archaea* and *Bacteria* as the most likely extant examples of Earth's earliest cellular life forms. More so than any other microbes, these organisms retain the metabolic and physiological traits one would predict to be necessary for existence on a hot early Earth.

Check Your Understanding

- What phylogenetic and physiological evidence suggests that today's hyperthermophiles are the closest living links to Earth's earliest cells?
- Which mechanism of energy conservation is least heat tolerant?
- Which chemolithotrophic lifestyle seems best suited to life at the highest temperatures?

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CHAPTER REVIEW

I • *Euryarchaeota*

17.1 Extremely halophilic *Archaea* require large amounts of NaCl for growth and accumulate large levels of K^+ in their cytoplasm as a compatible solute. These salts affect cell wall stability and enzyme activity. The light-mediated proton pump bacteriorhodopsin helps extreme halophiles make ATP.

Q Describe how the light-mediated proton motive force is established in *Halobacterium salinarum*.

17.2 Methanogenic *Archaea* are strict anaerobes whose metabolism is linked to the production of CH_4. Methane can be produced by CO_2 reduction by H_2, from methyl substrates such as CH_3OH, or from acetate.

Q What are two unique features of the methanogen *Methanopyrus*?

17.3 *Thermoplasma*, *Ferroplasma*, and *Picrophilus* are extremely acidophilic thermophiles that form their own phylogenetic family of *Archaea*. Cells of *Thermoplasma* and *Ferroplasma* lack cell walls, resembling the mycoplasmas in this regard.

Q What two major physiological features unify species of *Thermoplasmatales*? Why does this allow some of them to successfully colonize coal refuse piles?

17.4 *Archaeoglobus* and *Ferroglobus* are related anaerobic *Archaea* that carry out different anaerobic respirations. *Archaeoglobus* is a sulfate reducer and *Ferroglobus* is a nitrate reducer that oxidizes ferrous iron.

Q What metabolic property is shared by the archaean *Archaeoglobus* and the bacterium *Desulfovibrio*?

II • *Thaumarchaeota* and Cryptic Archaeal Phyla

17.5 *Thaumarchaeota* are widespread and abundant in soils and marine environments. All cultivated species of thaumarchaea are autotrophic ammonia-oxidizers and these organisms are important in the global nitrogen cycle.

Q What is physiologically unusual about the thaumarchaeotal species *Nitrosopumilus maritimus*?

17.6 *Nanoarchaeum equitans* is a hyperthermophile that forms its own phylum, the *Nanoarchaeota*, and is a parasite of the crenarchaeote *Ignicoccus*. *N. equitans* has a tiny, highly compact genome and depends on *Ignicoccus* for most of its cellular needs, including both carbon and energy.

Q What features of *Nanoarchaeota* and other members of the DPANN superphylum distinguish them from other *Archaea*?

17.7 *Korarchaeum cryptofilum* forms its own phylum, the *Korarchaeota*, and is a hyperthermophile that lacks important biosynthetic pathways, obtaining key building blocks from its environment.

Q What is a likely explanation for the fact that organisms like *Korarchaeum cryptofilum* have proven so difficult to cultivate in pure culture?

17.8 *Lokiarchaeota* and other Asgard *Archaea* have eukaryotic signature genes that were once thought to be unique to the domain *Eukarya*. *Bathyarchaeota* and *Aigarchaeota* are members of the TACK superphylum known only from their genome sequences obtained from metagenomic studies.

Q What pathway, observed in the genomes of certain *Bathyarchaeota*, has led to the hypothesis that these organisms are capable of acetogenesis or possibly methanogenesis?

III • *Crenarchaeota*

17.9 A wide variety of chemoorganotrophic and chemolithotrophic energy metabolisms have been found in hyperthermophilic *Crenarchaeota*, including fermentation and anaerobic respirations. Strictly autotrophic lifestyles are common, but photosynthesis is absent.

Q What forms of energy metabolism are present in *Crenarchaeota*? What form is not present?

17.10 Hyperthermophilic *Crenarchaeota* thrive in terrestrial hot springs of various chemistries. These include in particular organisms such as *Sulfolobus*, *Acidianus*, *Thermoproteus*, and *Pyrobaculum*.

Q What is unusual about the metabolism of S^0 by *Acidianus*?

17.11 In deep-sea hydrothermal systems, *Crenarchaeota* such as *Pyrolobus*, *Pyrodictium*, *Ignicoccus*, and *Staphylothermus* thrive. With the exception of the methanogen *Methanopyrus* (*Euryarchaeota*), species of these genera grow at the highest temperatures of all *Archaea*, in many cases well above the boiling point of water.

Q Why is it likely that organisms such as *Pyrolobus fumarii* are limited to hydrothermal habitats rather than terrestrial hot springs?

IV • Evolution and Life at High Temperature

17.12 Life as we know it is probably limited to temperatures below 150°C. Key small molecules, such as ATP, are quickly destroyed above this temperature.

Q What organism is the current record holder for the upper temperature limit for growth? What is predicted to be the absolute upper temperature limit for growth, and why?

17.13 Macromolecules in hyperthermophiles are protected from heat denaturation by their heat-stable folding patterns (proteins), solutes and binding proteins (DNA), unique monolayer membrane architecture (lipids), and the high GC content of their ribosomal RNAs.

Q What is reverse DNA gyrase and why is it important to hyperthermophiles?

17.14 Hydrogen metabolism is likely to have been the driving force behind the energetics of the earliest cells on Earth. Chemolithotrophic metabolisms based on H_2 as an electron donor are found in the most heat-tolerant of all known *Bacteria* and *Archaea*.

Q Why might H_2 metabolism have evolved as a mechanism for energy conservation in the earliest organisms on Earth?

APPLICATION QUESTIONS

1. Using the phylogenetic tree in Figure 17.1 as a guide, discuss what indicates that bacteriorhodopsin may have been a late evolutionary invention and that anaerobic respiration with S^0 as electron acceptor might have been an early evolutionary invention.
2. Use Figure 17.1 to make a hypothesis about the distribution of methanogenesis in the domain *Archaea*. When do you think this trait evolved? What evolutionary processes have resulted in the distribution of this trait in the archaeal family tree?

CHAPTER GLOSSARY

Asgard *Archaea* a large collection of related archaeal phyla including the *Lokiarchaeota*, members of which are notable for containing many eukaryotic signature genes

Bacteriorhodopsin a protein containing retinal that is found in the membranes of certain extremely halophilic *Archaea* and that is involved in light-mediated ATP synthesis

Compatible solute a molecule that is accumulated in the cytoplasm of a cell for adjustment of water activity but that does not inhibit biochemical processes

Crenarchaeota a large phylum of *Archaea* that contains hyperthermophilic organisms

DPANN superphylum a large collection of related archaeal phyla characterized by extremely small cells and reduced genomes, *Nanoarchaeum equitans* is the best-characterized species

Euryarchaeota a large phylum of *Archaea* that contains primarily methanogens, extreme halophiles, *Thermoplasma*, and some marine hyperthermophiles

Extreme halophile a microorganism that requires very large amounts of NaCl for growth, usually greater than 10% and in some cases near saturation, for growth

Extremophiles microorganisms that inhabit environments characterized by extremes of temperature, pH, pressure, or salinity

Halorhodopsin a light-driven chloride pump that accumulates Cl^- within the cytoplasm

Hydrothermal vent a deep-sea hot spring emitting warm (~20°C) to superheated (>300°C) water

Hyperthermophile a species of *Bacteria* or *Archaea* whose growth temperature optimum is 80°C or greater

Korarchaeota a phylum of *Archaea* that contains the hyperthermophile *Korarchaeum cryptophilum*

Methanogen a methane-producing species of the *Archaea*

Nanoarchaeota a phylum of *Archaea* that contains the hyperthermophilic parasite *Nanoarchaeum equitans*

Phytanyl a branched-chain hydrocarbon containing 20 carbon atoms and commonly found in the lipids of *Archaea*

Reverse DNA gyrase a protein universally present in hyperthermophiles that introduces positive supercoils into circular DNA

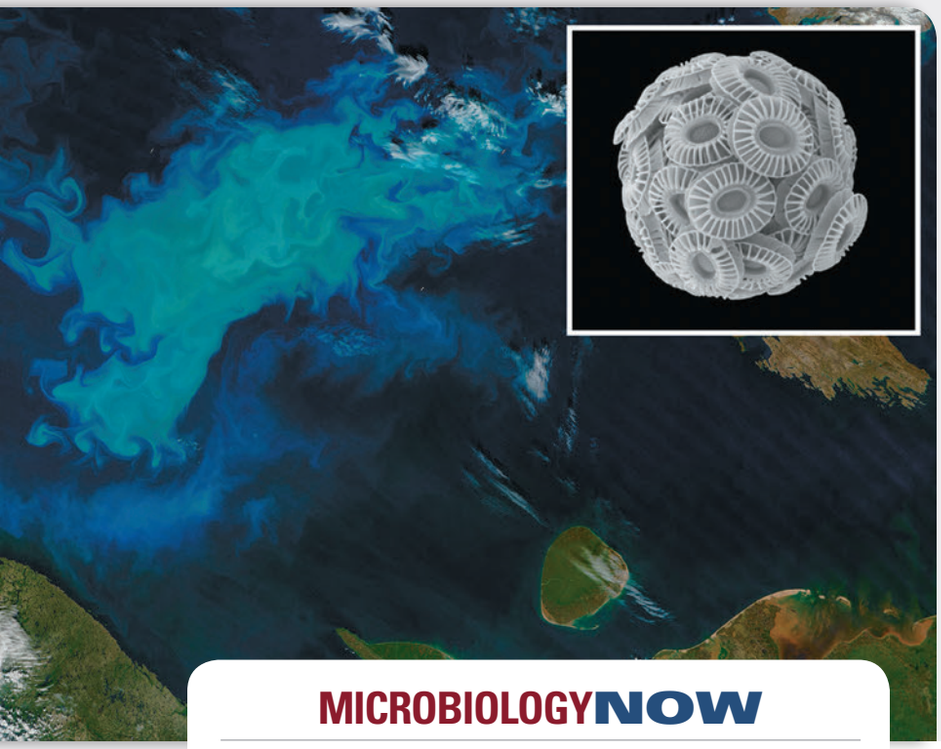
Solfatara a hot, sulfur-rich, generally acidic environment commonly inhabited by hyperthermophilic *Archaea*

TACK superphylum a large collection of related archaeal phyla including the eponymous *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota*

Thaumarchaeota a phylum of *Archaea* that contains widespread species capable of aerobic ammonia oxidization

Thermosome a heat shock (chaperone) protein complex that functions to refold partially heat-denatured proteins in hyperthermophiles

Diversity of Microbial *Eukarya* 18



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MICROBIOLOGYNOW

Coccolithophores, Engineers of Global Climate

Coccolithophores are single-celled phototrophic eukaryotes. They live near the ocean surface worldwide, where they comprise a large portion of the planktonic community. Coccolithophores (photo inset, cell 6 µm in diameter) have armor plates made of calcium carbonate, formed from autotrophic CO_2 fixation, and often develop immense blooms, dense clouds of cells so large they are visible from space (photo, Barents Sea, July 2016). Coccolithophores play an important role in global climate because their carbonate armor is heavy. It sinks to the ocean floor and is stabilized, eventually forming chalk, a type of sedimentary rock. Chalk deposits worldwide reveal the power of coccolithophores to remove carbon from the atmosphere and turn it into rock. Our ability to predict global climate depends, in part, on how much CO_2 coccolithophores remove from the atmosphere.

The more than 200 species of coccolithophores vary tremendously in their distribution, ecology, and morphology (see Figure 18.15). No one knows for certain why they form armor plates, but we do know that forming these carbonate shells requires a very large amount of cellular energy. The shells may provide coccolithophores protection from predators or prevent bacterial or virus attachment. Alternatively, the shells might enhance photosynthetic efficiency by functioning as lenses, or in some way trap and concentrate CO_2. They also might function to protect cells from ultraviolet radiation.

Since coccolithophores vary dramatically in shape, it seems likely that the costs and benefits of shell formation vary from species to species. Climate change is causing elevated CO_2, higher temperatures, and greater ocean acidity. The former two conditions may enhance coccolithophore growth, whereas the latter should inhibit it. The fate of coccolithophores and their role in determining Earth's future likely depends on their diversity and the costs and benefits of forming their carbonate armor in oceans gradually being transformed by climate change.

Source: Monteiro, F.M., et al. 2016. Why marine phytoplankton calcify. *Sci. Adv.* 2: e1501822.

I • Organelles and Phylogeny of Microbial *Eukarya*

Microbial *Eukarya* include protists, fungi, and algae. Primary symbioses caused these microbes to acquire mitochondria and red and green algae to also acquire chloroplasts. Many other protists acquired chloroplasts from the uptake of red or green algae in secondary symbioses.

In this chapter, we consider the phylogeny and diversity of microbial eukaryotes. While plants and animals are the most conspicuous members of the *Eukarya*, microbial eukaryotes are far older and more phylogenetically diverse than are their macroscopic relatives. *Eukarya* exhibit tremendous morphological and ecological complexity but a limited range of metabolic diversity. Metabolically, eukaryotic organisms are almost exclusively chemoorganotrophic or phototrophic and most species are obligate aerobes. Nearly all *Eukarya* depend on their organelles—mitochondria and chloroplasts (◀ Section 2.14)—for generating energy. The nutritional lifestyles of microbial eukaryotes are highly varied and include *phagotrophy* (obtaining energy by consuming other cells or detritus), *saprotrophy* (obtaining energy by absorbing organic matter from the environment), *phototrophy* (generating energy from light), and/or *symbiosis* (a persistent interaction with another organism that can be beneficial, commensal, or parasitic, Chapter 23).

Microbial eukaryotes can be defined by their morphology as *protists*, *algae*, or *fungi*. **Protist** is an informal term referring to any eukaryotic microorganism that is not a plant, animal, or fungus. Protists are found in many different phylogenetic lineages of *Eukarya* and are typically phagotrophic, though some are phototrophic. **Algae** include unicellular and multicellular eukaryotes that perform photosynthesis but are not plants. While some algal groups share an ancestor with plants, as we will see shortly, phototrophic microbes have evolved independently in multiple different lineages of *Eukarya*. **Fungi** are nonphototrophic eukaryotic microorganisms with rigid cell walls composed of chitin. The fungi form a single phylogenetic group, which includes yeasts, molds, and mushrooms. Fungi typically exhibit saprotrophic and/or symbiotic lifestyles and are often associated with (primarily aerobic) decomposition.

One thing that unites all *Eukarya* is that their ancestors had complex cell structure and true organelles. The structural complexity of the eukaryotic cell (◀ Sections 2.13–2.15) has allowed the domain *Eukarya* to evolve tremendous morphological diversity. As a result, complex cellular structures and multicellularity are common traits of eukaryotic microorganisms. We begin our consideration of microbial eukaryotes by examining the evolutionary history of the domain *Eukarya* and the endosymbiotic origins of organelles.

Mastering Microbiology
Art Activity: Figure 18.2 Endosymbioses

18.1 Endosymbioses and the Eukaryotic Cell

The exact evolutionary origin of the eukaryotic cell remains uncertain, but biologists agree that the eukaryotic cell is a genetic chimera containing genes from multiple sources. The last common ancestor of all eukaryotic cells was a single-celled microorganism closely related to *Archaea*. This microorganism had certain features common to all eukaryotic cells, including a nucleus, a cytoskeleton, spliceosomes, and a genome with spliceosomal introns (that is, those introns processed by spliceosomes), but it lacked energy-producing organelles—the mitochondria and chloroplasts. These were obtained by endosymbiosis (◀ Section 13.4).

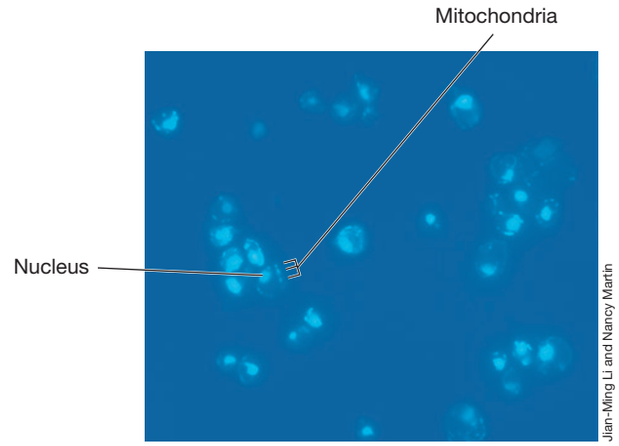


Figure 18.1 Organellar DNA. Cells of the yeast *Saccharomyces cerevisiae* have been stained with the fluorescent dye DAPI that binds to DNA. Each mitochondrion has two to four circular chromosomes that stain blue with the dye.

The acquisition of mitochondria was a foundational event in the origin of *Eukarya*. Mitochondria are derived from *Bacteria* and they contain bacterial DNA (**Figure 18.1**; ◀ Section 2.14). The acquisition of mitochondria and the establishment of eukaryotic cell structure set the stage for the evolution of diverse microbial lineages in addition to complex multicellular organisms such as plants and animals.

Primary Endosymbioses

Endosymbiosis has played a major role in the origin and diversification of *Eukarya*. All *Eukarya* are descended from cells that contained respiratory mitochondria. Mitochondria evolved through endosymbiosis, in which the ancestor of *Eukarya* formed a symbiotic relationship with a bacterial cell capable of respiration (◀ Sections 2.14 and 13.4). This bacterium may have initially invaded the cytoplasm of the eukaryotic cell as an intracellular parasite or it may have been acquired by *phagocytosis*, but for some reason these partners entered into a stable symbiotic relationship. Over time, the bacterial endosymbiont and its eukaryotic partner evolved mutual dependency until they eventually became a single cell, inseparable. This event is considered **primary endosymbiosis** because the bacterial symbiotic partner was acquired directly by the eukaryotic host cell.

All phototrophic eukaryotes—plants and algae—require chloroplasts to perform photosynthesis. Chloroplasts, like mitochondria, were acquired through primary endosymbiosis (**Figure 18.2**). In this case, a eukaryotic cell, which already contained mitochondria, acquired a phototrophic cyanobacterium as an endosymbiont (◀ Section 13.4). As with the mitochondria, this cyanobacterial endosymbiont evolved mutual dependency with its eukaryotic host until they became inseparable. However, primary endosymbiosis is not the end of the story for the evolution of organelles.

Secondary Endosymbioses

Primary endosymbiosis with a cyanobacterium gave rise to the *Archaeplastida*, a lineage that includes green algae, red algae, and plants (Figure 18.2 and see Figure 18.3). However, following this *primary* endosymbiosis event, several different groups of protists acquired chloroplasts through *secondary endosymbiosis*. **Secondary endosymbiosis** occurs when a cell that already contains an endosymbiont is engulfed by a second host cell (Figure 18.2). For example, many protists became phototrophic by acquiring an *algal endosymbiont* that already contained a chloroplast. Over time, many features of the algal endosymbiont were lost but its chloroplasts remained, as did the ability to perform photosynthesis.

Secondary endosymbiosis has occurred multiple times within *Eukarya*, giving rise to a diversity of phototrophic eukaryotes (Figure 18.2). The chloroplasts of euglenids and chlorarachniophytes were acquired through secondary endosymbiosis with green algae, while those of the *Alveolata* (ciliates, apicomplexans, and dinoflagellates) and *Stramenopiles* were obtained through secondary endosymbioses with red algae (Figure 18.2 and see Figure 18.3). The ancestral red algal chloroplasts were apparently lost from some lineages, such as the ciliates, or became greatly reduced in size in others, such as the apicomplexans. In some other organisms, such as the dinoflagellates, the red algal chloroplast was replaced altogether with chloroplasts from different algae, including green algae. The legacy of endosymbiosis can be detected even if an endosymbiont is lost, because some endosymbiont genes are transferred to the host and become part of the host genome (◀ Section 13.4); these genes are then revealed when the genome is sequenced.

These many examples of endosymbiotic events underscore the importance of endosymbiosis in the evolution and diversification of microbial eukaryotes. It is unlikely that primary endosymbiotic events occurred only twice in evolutionary history—after all, trial and error is the essence of evolution—and secondary endosymbioses are known to have occurred multiple times in several lineages

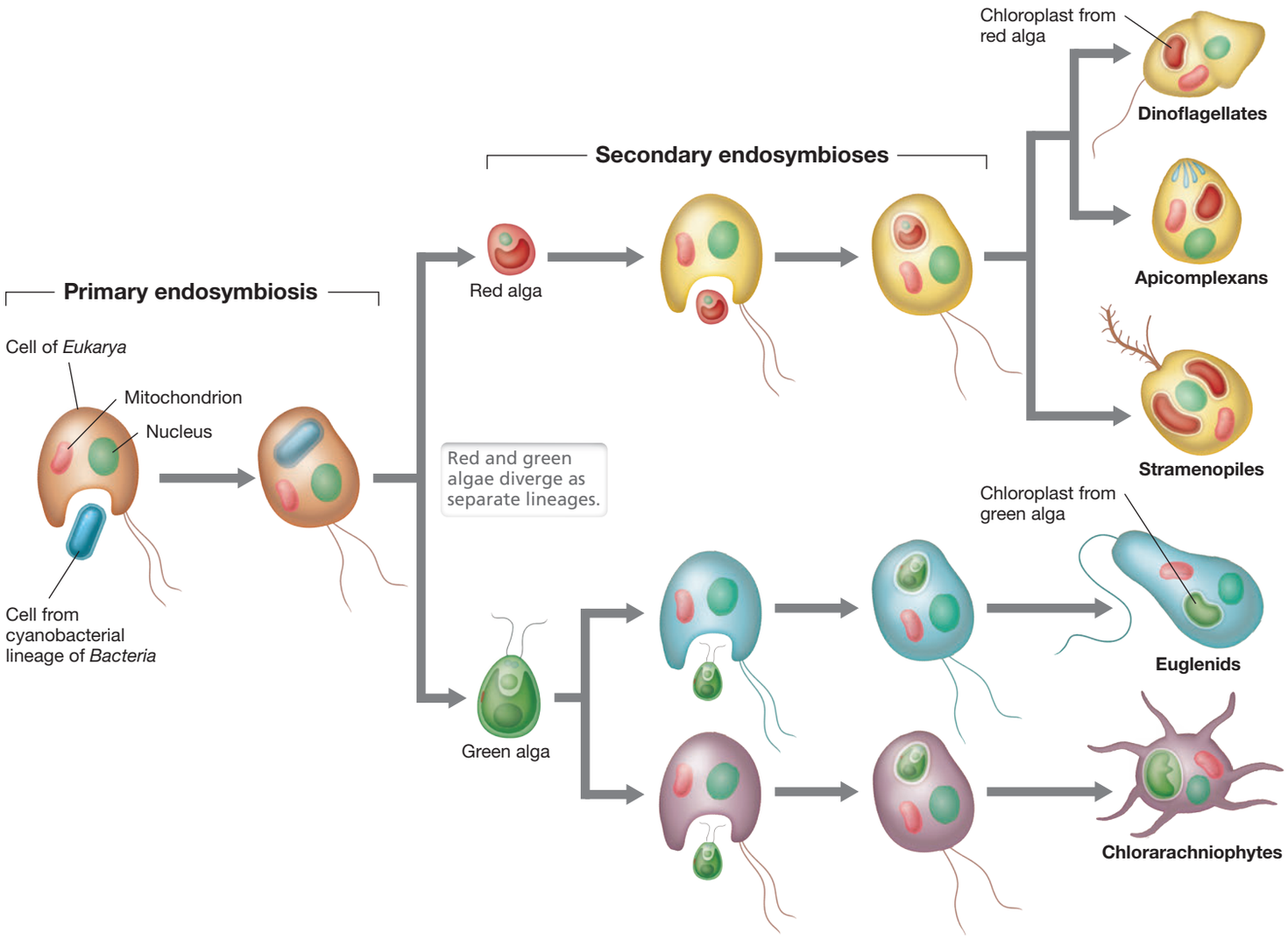


Figure 18.2 Endosymbioses. Following primary endosymbiotic association(s) leading to the mitochondrion, primary endosymbioses with phototrophic *Bacteria* led to the red and green algae. Secondary symbioses of green and red algae spread the property of photosynthesis to many independent lineages of protists.

(Figure 18.2). There are even examples of animals that engulf phototrophic protists to carry out photosynthesis for extended periods (▶ Section 23.13). Indeed, it appears that endosymbioses are a common and ongoing occurrence in the eukaryotic world, conferring obvious metabolic advantages on the eukaryotic partner and perhaps other, not-so-obvious advantages, that remain to be discovered.

Check Your Understanding

- Which was acquired first: chloroplasts or mitochondria?
- Describe how euglenids came to acquire chloroplasts.
- Distinguish between primary and secondary endosymbioses.

18.2 Phylogenetic Lineages of *Eukarya*

Based on conclusions drawn from molecular phylogenetic studies and supported by microfossil discoveries, it appears likely that a major phylogenetic radiation took place rather early in eukaryote evolution. This was likely triggered by the endosymbiotic acquisition of mitochondria, the foundational event in the evolution of the eukaryotic cell. All extant *Eukarya* contain mitochondria, structures homologous to mitochondria, or some genetic trace of these structures. What promoted this primary endosymbiotic event is unknown, but quite possibly it was the accumulation of O_2 in the atmosphere from cyanobacterial photosynthesis (◀ Figure 13.1). Mitochondria would have improved the ability of the early eukaryotic cell to generate energy, providing eukaryotes a considerable advantage over anaerobic competitors. Somewhat later, another primary endosymbiotic event occurred between eukaryotic cells and cyanobacteria, giving rise to the first algae. This event was followed by a series of secondary endosymbioses (Section 18.1 and Figure 18.2) that gave rise to the diversity of phototrophic eukaryotes we see today.

Eukaryotic Evolution: The Big Picture

Although phylogenies based on ribosomal RNA gene sequences (Chapter 13) confirm the three domains of life—*Bacteria*, *Archaea*, and *Eukarya*—our picture of eukaryotic evolution has changed dramatically with the incorporation of data from whole genome sequences. There are currently five *supergroups* of *Eukarya* (a supergroup is not an official taxon but rather a cluster of related phyla): *Archaeplastida*, the *SAR clade*, *Excavates*, *Amoebozoa*, and *Opisthokonta* (**Figure 18.3**). The *Archaeplastida* include the entire plant kingdom as well as all red algae, green algae, and glaucophytes; these phototrophic lineages resulted from the primary endosymbiosis of chloroplasts (Figure 18.2). The *Stramenopiles*, *Alveolata*, and *Rhizaria* include highly diverse protists including both heterotrophic and phototrophic species. These three groups share an ancestor and they

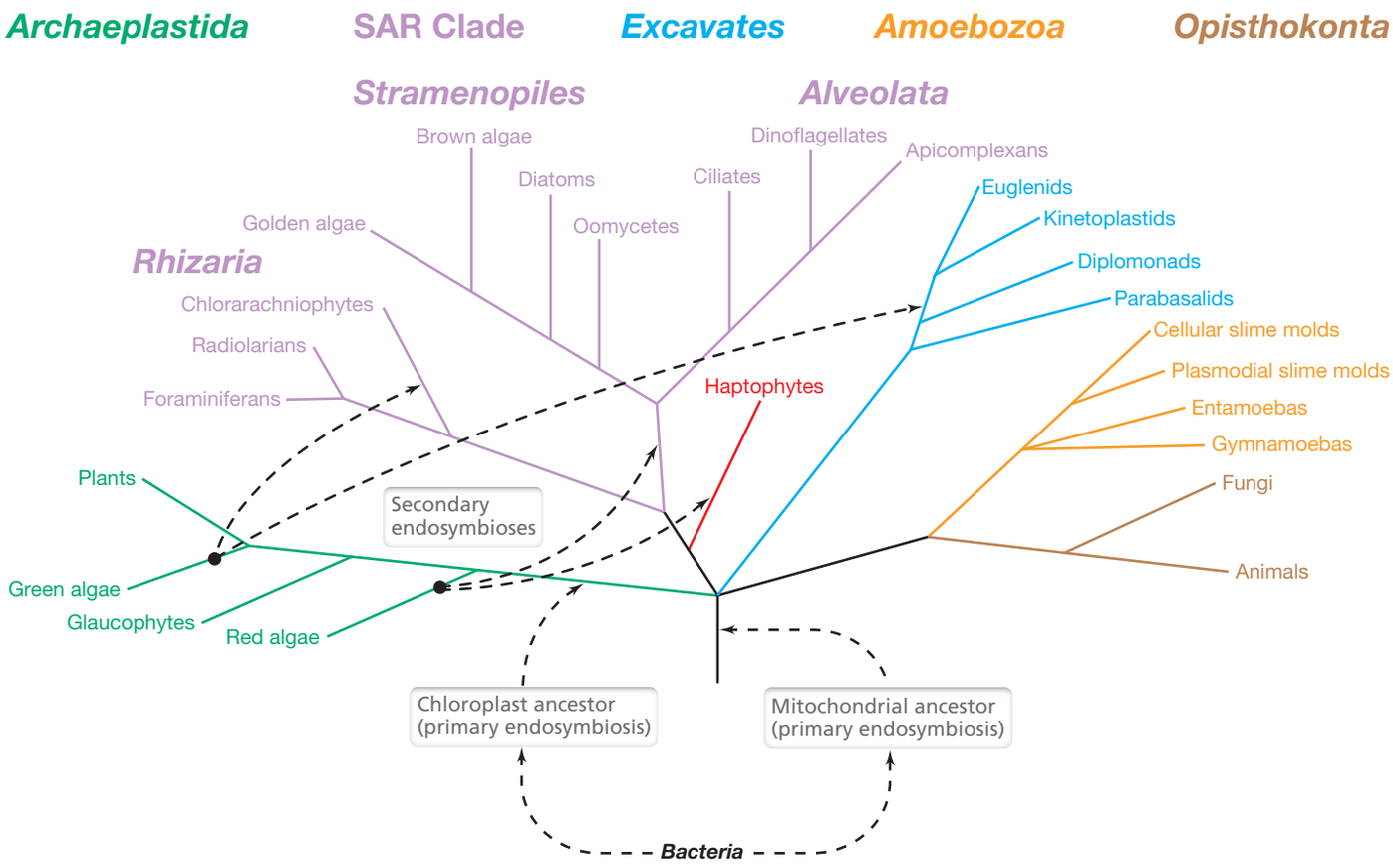


Figure 18.3 Phylogenetic tree of *Eukarya*. The tree diagram provides a schematic view of phylogenetic relationships for five of the best-characterized supergroups within the *Eukarya*. Colors are used to identify the supergroups in the tree. Dashed lines indicate both primary endosymbioses of mitochondria and chloroplasts, and the secondary endosymbioses of red and green algae. Protists are common in all eukaryotic lineages except the *Opisthokonta* (*Fungi* and *Animalia*) and the plants.

are classified together into the SAR clade (an acronym for *Stramenopiles, Alveolata*, and *Rhizaria*). Where it occurs, phototrophy within these lineages was acquired as a result of secondary endosymbiosis (Figure 18.3 and Section 18.1). *Excavates* are diverse protists, many of which are anaerobic, and some of which have acquired phototrophy as a result of secondary endosymbiosis (Figures 18.2 and 18.3). The *Amoebozoa* include many forms of amoebae and slime molds, though cells with an amoeboid morphology occur in many other lineages of *Eukarya* as well. Finally, the *Opisthokonta* include the well-known kingdoms *Fungi* and *Animalia*.

The phylogenetic tree shown in Figure 18.3 should not be considered the final word on eukaryotic evolution. For example, many phyla of *Eukarya* have been discovered that do not fall neatly within these five groups and their position in the tree of life remains uncertain. For example, the haptophytes (Section 18.7) are an unusual and globally important group of microbes that form their own separate branch deep within the eukaryotic phylogenetic tree (Figure 18.3). New genome sequences continue to be unraveled, new organisms continue to be discovered, and new aspects of eukaryotic biology continue to be revealed. With each new discovery, we shed more light on eukaryotic phylogeny, and hence our understanding of eukaryotic phylogeny will continue to improve as our knowledge grows.

Phylogenetic Insights on Endosymbiosis

As we have seen, endosymbiosis has clearly been an important aspect of eukaryotic evolution, and the acquisition of the mitochondrion by primitive *Eukarya* was central to the evolutionary success of this domain. However, some parasitic microbial eukaryotes such as *Giardia* and *Microsporidia* lack mitochondria. The *Microsporidia* were once thought to be ancient members of the *Eukarya*, descended from an ancestor of *Eukarya* that lacked mitochondria. We now know, however, that these amitochondriate eukaryotes are descended from eukaryotic ancestors that once had mitochondria (see the position of *Microsporidia* in Figure 18.24) but then lost them for some reason, perhaps while reverting to an anaerobic lifestyle such as in *Giardia*, *Entamoeba*, and several other anaerobic parasitic eukaryotes. However, the genomes of amitochondriate eukaryotes typically retain a few genes of mitochondrial origin, and these molecular leftovers are strong evidence that the organisms once had mitochondria.

The tree of *Eukarya* (Figure 18.3) also shows how secondary endosymbioses account for the origin of chloroplasts in some unicellular phototrophic eukaryotes. Following primary endosymbiosis of the cyanobacterial ancestor of chloroplasts by early mitochondrion-containing eukaryotes, these now phototrophic eukaryotes diverged into red and green algae. Then, in secondary endosymbioses, ancestors of the euglenids, kinetoplastids, and chlorarachniophytes engulfed green algae while ancestors of the *Alveolata* and *Stramenopiles* engulfed red algae (Section 18.1). These secondary endosymbioses account for the great phylogenetic diversity of phototrophic eukaryotes, which we explore later in this chapter.

With a feeling for the "big picture" of eukaryotic evolution, we now move on to consider microbial eukaryotes themselves. During our tour, a diverse array of nutritional lifestyles, cellular accessories, interesting habitats, and ecological strategies for survival will unfold.

Check Your Understanding

- What are the five major supergroups within the *Eukarya*?
- What supergroup was formed as a result of the primary endosymbiosis of chloroplasts?
- What group of microbial eukaryotes is most closely related to animals?

II • Protists

The term "protist" describes any microbial eukaryote that is not a plant, animal, or fungus. Protists are highly diverse in their phylogeny, morphology, and ecology, and their metabolisms range from chemoorganotrophic to phototrophic.

With the big picture of eukaryotic phylogeny as our guidepost, we begin with the protists, an enormously diverse group defined loosely by their morphology. Protists include both phototrophic and nonphototrophic microbial eukaryotes, and while most are unicellular, some are multicellular. Protists are widely distributed in nature, exhibit a wide range of morphologies, and show great phylogenetic diversity.

UNIT 4

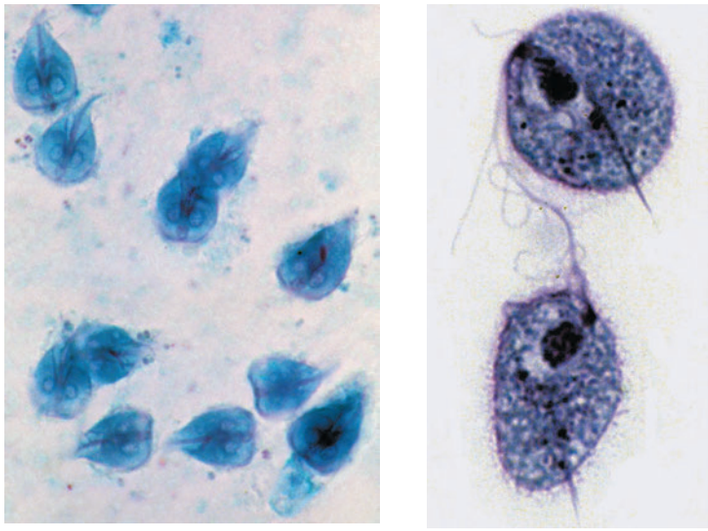
18.3 *Excavates*

KEY GENERA: *Giardia, Trichomonas, Trypanosoma, Euglena*

The *Excavates* are a diverse group of protists including both chemoorganotrophs and phototrophs, with some species being anaerobic. Once considered as a coherent taxonomic group, the *Excavates* include a collection of distantly related organisms whose phylogeny remains controversial. We start with the diplomonads and parabasalids, flagellated protists that *lack* mitochondria and chloroplasts. These microbes live in anoxic habitats, such as animal intestines, either symbiotically or as parasites, and conserve energy from fermentation. Some diplomonads cause serious and common diseases in fish, domestic animals, and humans, and one parabasalid causes a major sexually transmitted disease of humans. Both groups share a relatively recent common ancestor before they diverged to form separate phylogenetic lineages (Figure 18.3).

Diplomonads

Diplomonads (**Figure 18.4*a***) characteristically contain *two nuclei* of equal size, and also contain mitosomes, much reduced mitochondria lacking electron transport proteins and enzymes of the citric acid cycle (◀ Figure 3.12). The diplomonad *Giardia* has a relatively small genome for a eukaryote, about 12 megabase pairs (Mbp). The genome is also quite compact, contains few introns, and lacks genes for many metabolic pathways, including the citric acid cycle. These characteristics likely account for the organism's parasitic and anaerobic lifestyle. *Giardia intestinalis* (Figure 18.4*a*), also known as *Giardia lamblia*, causes giardiasis, one of the most common waterborne diarrheal diseases in the United States.We examine human giardiasis in Section 34.4.



(a) (b)

Figure 18.4 Diplomonads and parabasalids. *(a)* Light photomicrograph of cells of *Giardia intestinalis*, a typical diplomonad. Note the dual nuclei. Cells are about 10 μm wide. *(b)* Light photomicrograph of cells of the parabasalid *Trichomonas vaginalis*. Cells are about 6 μm wide. The spearlike structure (axostyle) is used to attach the cell to urogenital tissues.

Parabasalids

Parabasalids contain a *parabasal body* that, among other functions, gives structural support to the cell's Golgi complex. These anaerobic microbial eukaryotes lack mitochondria but contain *hydrogenosomes*. **Hydrogenosomes** are anaerobic organelles that resemble and evolved from mitochondria and produce ATP through fermentation reactions resulting in the production of H_2 and acetate. Parabasalids live in the intestinal and urogenital tract of vertebrates and invertebrates as parasites or as commensal symbionts. The parabasalid *Trichomonas vaginalis* is motile by a tuft of flagella (Figure 18.4*b*) and causes a widespread sexually transmitted disease in humans (▶ Section 34.4 and Figure 34.9).

The genomes of parabasalids are unique among eukaryotes in that most of them lack introns, the noncoding sequences characteristic of eukaryotic genes (◀ Sections 6.6 and 10.4). In addition, the genome of *T. vaginalis* is surprisingly huge for a parasitic organism, about 160 Mbp, and includes genes acquired from bacteria by horizontal gene transfer. Much of the genome of *T. vaginalis* contains repetitive DNA sequences and transposable elements (◀ Section 13.9), which has made genomic analyses difficult. But *Trichomonas* is still thought to contain nearly 60,000 genes, about twice that of the human genome and near the upper limit observed thus far for eukaryotic genomes.

Kinetoplastids

Kinetoplastids are a well-studied group of *Excavates* and are named for the presence of the *kinetoplast*, a mass of DNA present in their single, large mitochondrion. Kinetoplastids live primarily in aquatic habitats, where they feed on bacteria. Some species, however, are parasites of animals and cause serious diseases in humans and vertebrate animals. Cells of *Trypanosoma*, a genus of organisms that infect humans, are small, about 20 μm long, thin, and crescent-shaped. Trypanosomes have a single flagellum that originates in a basal body and folds back laterally across the cell where it is enclosed by a flap of cytoplasmic membrane (**Figure 18.5**). Both the flagellum and the membrane participate in propelling the organism, making effective movement possible even in viscous liquids, such as blood, where pathogenic trypanosomes are often found.

Trypanosoma brucei (Figure 18.5) causes *African sleeping sickness*, a chronic and usually fatal human disease. The parasite lives and grows primarily in the bloodstream, but in the later stages of the disease it invades the central nervous system, causing an inflammation of the brain and spinal cord that is responsible for the characteristic neurological symptoms of the disease. The parasite is transmitted from host to host by the tsetse fly, *Glossina* spp., a blood-sucking fly found only in certain parts of Africa. After moving from the human to the fly in a blood meal, the parasite proliferates in the intestinal tract of the fly and invades the insect's salivary glands and mouthparts, from which it is transferred to a new human host by a fly bite (▶ Section 34.6).

Other kinetoplastids that are human parasites include *Trypanosoma cruzi*, the causative agent of *Chagas disease*, and *Leishmania* species, the causative agents of cutaneous and systemic *leishmaniasis*. Chagas disease is spread by the bite of a blood-feeding insect called the "kissing bug." The disease is usually self-limiting, but it can become chronic and lead to a fatal infection. Leishmaniasis is a disease of tropical and subtropical regions transmitted to humans and other mammals by a bite from the sand fly. This potentially fatal disease can be localized to the skin surrounding a fly bite or can infect the spleen and liver and cause systemic infection.Both Chagas disease and leishmaniasis are covered in more detail in Section 34.6.

Euglenids

Another well-studied group of excavates are the euglenids (**Figure 18.6**). Unlike the kinetoplastids, these motile microbial eukaryotes are nonpathogenic and are both chemotrophic *and* phototrophic. Most euglenids contain two flagella, dorsal and ventral, and their active motility allows the organisms to access both illuminated and dark habitats in their environment to support their alternate nutritional lifestyles.

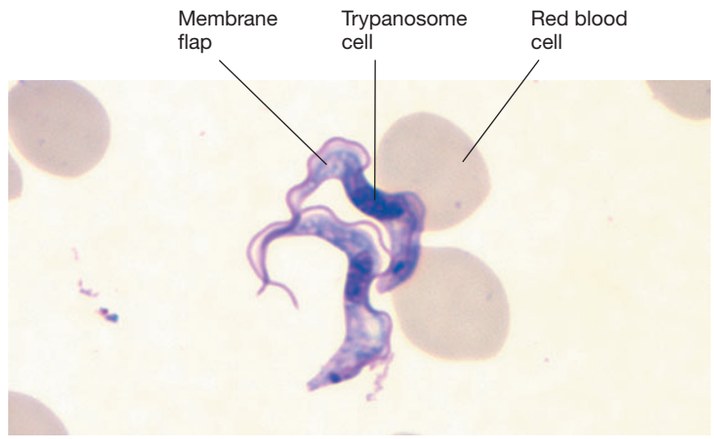
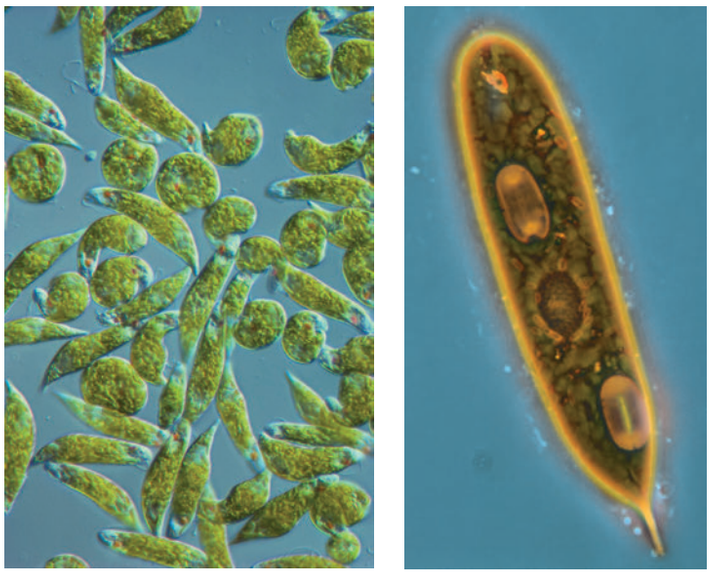


Figure 18.5 Trypanosomes. Photomicrograph of the flagellated kinetoplastids *Trypanosoma brucei*, the causative agent of African sleeping sickness. Blood smear preparation. The cells of *T. brucei* are about 3 μm wide.



(a) (b)

Figure 18.6 ***Euglena*, a euglenid.** *(a)* This phototrophic protist, like other euglenids, is not pathogenic. A cell is about 15 μm wide. *(b)* High-magnification view.

Euglenids live exclusively in aquatic habitats, both freshwater and marine, and contain chloroplasts, which support phototrophic growth (Figure 18.6). In darkness, however, cells of *Euglena*, a typical euglenid, can lose their chloroplasts and exist as chemoorganotrophs. Many euglenids can also feed on bacterial cells via **phagocytosis**, a process of surrounding a particle with a portion of their flexible cytoplasmic membrane to engulf the particle and bring it into the cell. The engulfed particle remains in this structure (called the *phagosome*) where it is digested, and the released nutrients are made available to nourish the cell.

Check Your Understanding

- Contrast the two nutritional options for *Euglena*.
- What diseases are caused by *Trypanosoma cruzi*, *Leishmania*, and *Giardia*?
- How do diplomonads obtain energy?

18.4 *Alveolata*

KEY GENERA: *Gonyaulax, Plasmodium, Paramecium*

The *Alveolata* as a group are characterized by their *alveoli*, cytoplasmic sacs located just under the cytoplasmic membrane. Although the function of alveoli is unknown, they may help the cell maintain osmotic balance by controlling water influx and efflux, and in the dinoflagellates, they may function as armor plates. Three phylogenetically distinct, although related, kinds of *Alveolata* are known: the *ciliates*, which use cilia for motility; the *dinoflagellates*, which are motile by means of a flagellum; and the *apicomplexans*, which are parasites of humans and other animals (Figure 18.3).

Ciliates

Ciliates possess *cilia* (**Figure 18.7**) at some stage of their life cycle. Cilia are structures that function in motility and may cover the cell or form tufts or rows, depending on the species (◀ Section 2.15 and Figure 2.48*a*). Probably the best-known and most widely distributed ciliates are those of the genus *Paramecium* (Figure 18.7). Like many other ciliates, *Paramecium* use cilia both for motility and to obtain food by ingesting particulate materials such as bacterial cells through a distinctive funnel-shaped oral groove. Cilia that line the oral groove move material down the groove to the cell mouth, also called the *gullet* (Figure 18.7*b*). Once in the gullet, the material is enclosed in a vacuole by phagocytosis. Digestive enzymes secreted into the vacuole then break down the material as a source of nutrients.

Ciliates are unique among protists in having two kinds of nuclei, *micronuclei* and *macronuclei*. Genes in the macronucleus regulate basic cellular functions, such as growth and feeding, whereas those of the micronucleus are involved in sexual reproduction, which occurs through a partial fusion of two *Paramecium* cells and exchange of micronuclei. The genome of *Paramecium* is huge, with macronuclear genes numbering about 40,000, nearly twice that of humans.

Many *Paramecium* species (as well as many other protists) are hosts for endosymbiotic *Bacteria*, *Archaea*, or eukaryotes, the latter usually green algae. These organisms may play a nutritional role, synthesizing vitamins or other growth factors used by the host cell. Several anaerobic ciliated protists also contain endosymbionts. For example, ciliated protists in the termite hindgut contain endosymbiotic methanogens (*Archaea*) that consume H_2 plus CO_2 to form methane (CH_4). Ciliates themselves can also be symbiotic: Obligately anaerobic ciliates are present in the rumen, the forestomach of ruminant animals, and play an important role in the digestive and fermentative processes of the animal (▶ Sections 23.15 and 23.16).

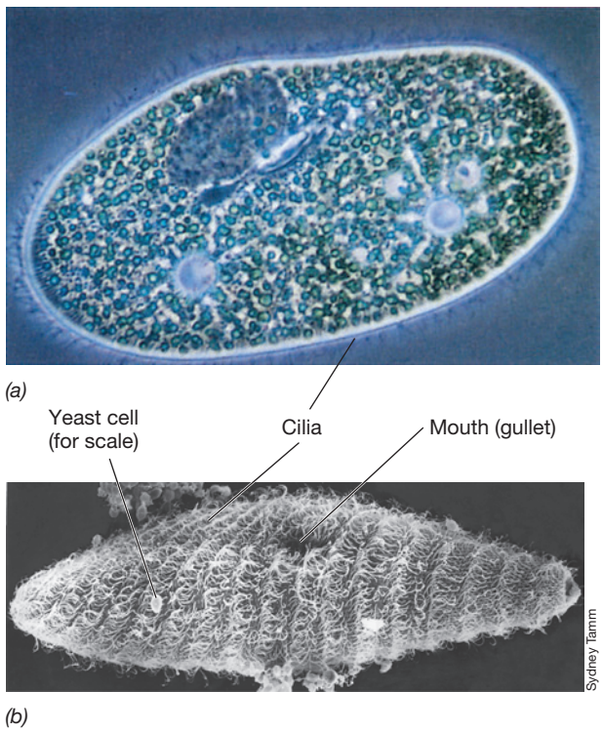


Figure 18.7 ***Paramecium*, a ciliated protist.** *(a)* Phase-contrast photomicrograph. *(b)* Scanning electron micrograph. Note the cilia in both micrographs. A single *Paramecium* cell is about 60 μm in diameter.

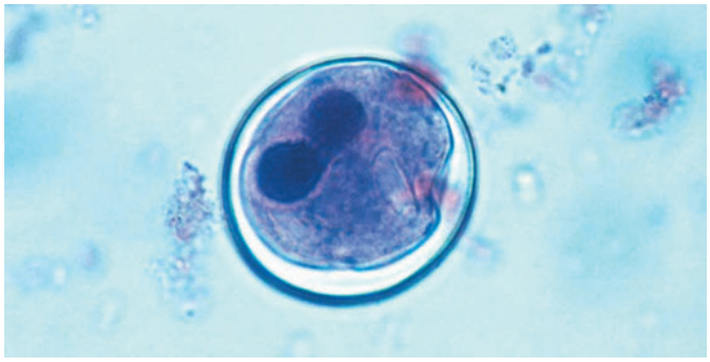
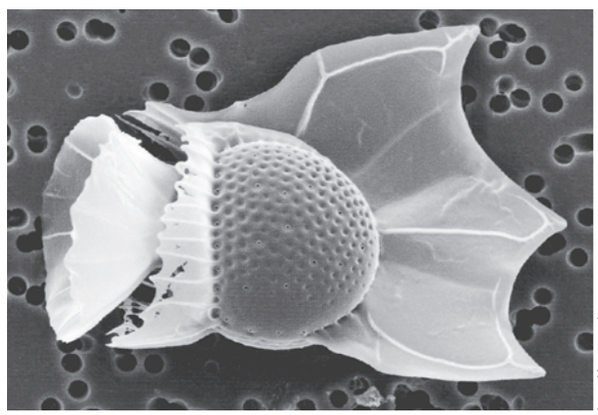


Figure 18.8 ***Balantidium coli*, a ciliated protist that causes a dysentery-like disease in humans.** The dark blue–stained lobed structure in this *B. coli* cyst obtained from swine intestine is a dividing macronucleus. The cell is about 50 μm wide.

Some ciliates are animal parasites, although this lifestyle is less common in ciliates than in some other groups of protists. The species *Balantidium coli* (**Figure 18.8**), for example, is primarily an intestinal parasite of domestic animals but occasionally infects the intestinal tract of humans, producing dysentery-like symptoms. Cells of *B. coli* form cysts (Figure 18.8) that promote their transmission in infected food or water.

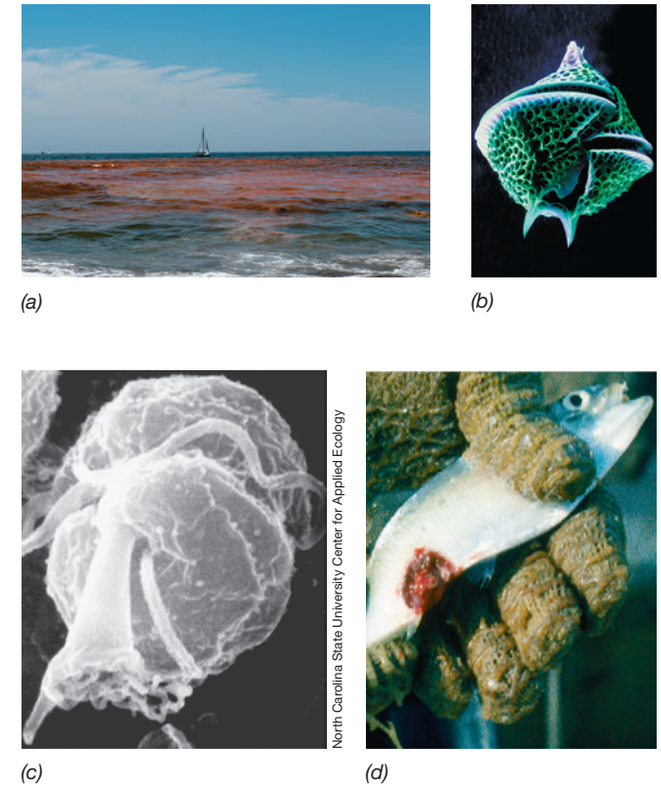
Dinoflagellates

Dinoflagellates are a diverse group of motile marine and freshwater phototrophic organisms (**Figure 18.9**) that acquired the capacity to photosynthesize through secondary endosymbioses (Figures 18.2 and 18.3). Eukaryotic flagella (◀ Section 2.15 and Figure 2.48) encircle the cell and impart spinning movements that give dinoflagellates their name (*dinos* is Greek for "whirling"). Dinoflagellates have two flagella of different lengths and with different points of insertion into the cell, transverse and longitudinal. The transverse flagellum is attached laterally, whereas the longitudinal flagellum originates from the lateral groove of the cell and extends lengthwise (see Figure 18.10*b*). Some dinoflagellates are free-living, whereas others live a symbiotic existence with animals that form coral reefs, obtaining a sheltered and protected habitat in exchange for supplying phototrophically fixed carbon as a food source for the reef. A number of free-living species are capable of bioluminescence and emit light when disturbed at night. This bioluminescence results in a "sparkling" effect that can often be observed in coastal seas and bioluminescent bays.



Irena Kaczmarska

Figure 18.9 **The marine dinoflagellate *Ornithocercus magnificus* (an alveolate).** The cell proper is the globular central structure; the attached ornate structures are called *lists*. A cell is about 30 μm wide.



(a) (b) (c) (d)

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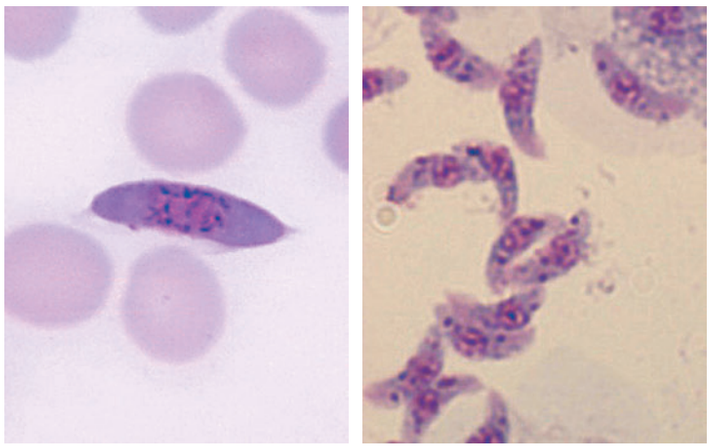
Figure 18.10 **Toxic dinoflagellates (alveolates).** *(a)* Photograph of a "red tide" caused by massive growth of toxin-producing dinoflagellates such as *Gonyaulax*. The toxin is excreted into the water and also accumulates in shellfish that feed on the dinoflagellates. *(b)* Colorized scanning electron micrograph of a cell of the dinoflagellate *Gonyaulax*. The cell is about 30 μm wide. *(c)* Scanning electron micrograph of a toxic spore of *Pfiesteria piscicida*; the structure is about 12 μm wide. *(d)* A fish killed by *P. piscicida*; note the lesions of decaying flesh.

Several species of dinoflagellates are toxic and can cause *harmful algal blooms* (see MicrobiologyNow in the Chapter 15 opener for other toxic microbial blooms). For example, dense suspensions of *Gonyaulax* cells, called "red tides" (**Figure 18.10*a***) because of the red-colored pigments of this organism, can form in warm and typically polluted coastal waters. Such blooms are often associated with fish kills and poisoning in humans following consumption of mussels that have accumulated *Gonyaulax* cells (Figure 18.10*b*) through filter feeding. Toxicity results from a neurotoxin that can cause a condition called *paralytic shellfish poisoning* in humans and some marine animals, such as sea otters. Symptoms include numbness of the lips, dizziness, and difficulty breathing; in severe cases, death can result from respiratory failure. *Pfiesteria* is another toxic dinoflagellate. Toxic spores of *Pfiesteria piscicida* (Figure 18.10*c*) infect fish and eventually kill them by way of neurotoxins that affect movement and destroy skin. Lesions form on areas of the fish, allowing opportunistic bacterial pathogens to grow (Figure 18.10*d*). Symptoms of human toxemia from *Pfiesteria* poisoning include skin rashes and respiratory problems.



(a) (b)

Figure 18.11 Apicomplexans. *(a)* A gametocyte of *Plasmodium falciparum* in a blood smear. The gametocyte is the stage in the malarial parasite life cycle that infects the mosquito vector. *(b)* Sporozoites of *Toxoplasma gondii.*

Apicomplexans

Apicomplexans are nonphototrophic obligate parasites that cause severe human diseases such as malaria (*Plasmodium* species, **Figure 18.11*a***), toxoplasmosis (*Toxoplasma*, Figure 18.11*b*), and coccidiosis (*Eimeria*). These organisms are characterized by nonmotile adult stages, and nutrients are taken up in soluble form across the cytoplasmic membrane as in bacteria and fungi.

Apicomplexans produce structures called *sporozoites* (Figure 18.11*b*; ▶ Section 34.5 and Figure 34.12), which function in transmission of the parasite to a new host, and the name apicomplexan derives from the presence at one apex of the sporozoite of a complex of organelles that penetrate host cells. Apicomplexans also contain *apicoplasts*. These are degenerate chloroplasts that lack pigments and photosynthetic capacity but contain a few of their own genes. Apicoplasts catalyze fatty acid, isoprenoid, and heme biosyntheses, and export their products to the cytoplasm. It is hypothesized that apicoplasts are derived from red algal cells engulfed by apicomplexans in a secondary endosymbiosis (Figures 18.2 and 18.3). Over time, the chloroplast of the red algal cell degenerated to play a nonphototrophic role in the apicomplexan cell.

Both vertebrates and invertebrates can be hosts for apicomplexans. In some cases, an alternation of hosts takes place, with some stages of the life cycle linked to one host and some to another. Important apicomplexans are the coccidia, which are typically parasites of birds, and species of *Plasmodium*, which are malarial parasites (Figure 18.11*a*).We reserve detailed discussion of malaria—a disease that has killed more humans than any other disease—for Section 34.5.

Check Your Understanding

- How does the organism *Paramecium* move?
- What health problem is associated with the organism *Gonyaulax*?
- What are apicoplasts, which organisms have them, and what are their functions?

18.5 *Stramenopiles*

KEY GENERA: *Phytophthora, Nitzschia, Ochromonas, Macrocystis*

The *Stramenopiles* include both chemoorganotrophic and phototrophic microorganisms as well as macroorganisms. Members of this group bear flagella with many short, hairlike extensions (Figure 18.2), and this morphological feature gives the group its name (from Latin *stramen* for "straw" and *pilus* for "hair"). The diatoms, oomycetes, golden algae, and brown algae are the major groups of *Stramenopiles* (Figure 18.3).

Diatoms

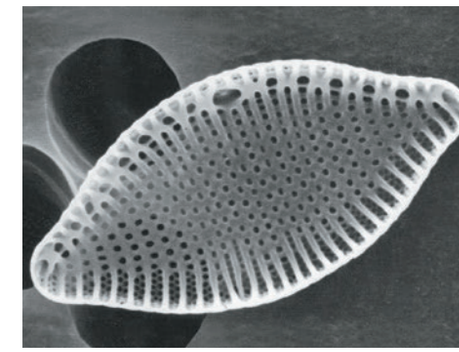
Diatoms include over 200 genera of unicellular, phototrophic, microbial eukaryotes, and are major components of the planktonic (suspended) phytoplankton microbial community in marine and freshwaters. Diatoms characteristically produce an exoskeleton made of silica to which protein and polysaccharide are added. The exoskeleton, which protects the cell against predation, exhibits widely different shapes in different species and can be highly ornate (**Figure 18.12**). This external structure, called a *frustule*, often remains after the cell dies and the organic materials have disappeared. Diatom frustules typically show morphological symmetry, including *pinnate symmetry* (having similar parts arranged on opposite sides of an axis, as in the common diatom *Nitzschia*, Figure 18.12*b*), and *radial symmetry*, as in the marine diatoms *Thalassiosira* and *Asterolampra* (Figure 18.12*c, d*). Because the diatom frustules, which are composed mainly of silica, are resistant to decay, these structures can remain intact for long periods and often sink and remain in the sediments for millions of years. Diatom frustules constitute some of the best-known unicellular eukaryotic fossils, and from dating of frustule samples, it has been shown that diatoms first appeared on Earth relatively recently, about 200 million years ago.

Oomycetes

The oomycetes, also called *water molds*, were previously grouped with fungi based on their filamentous growth and the presence of **coenocytic** (that is, multinucleate) hyphae, morphological traits characteristic of fungi (Section 18.9). Phylogenetically, however, the oomycetes are distant from fungi and are closely related to other *Stramenopiles* (Figure 18.3). Oomycetes differ from fungi in other fundamental ways, as well. For example, the cell walls of oomycetes are typically made of cellulose instead of the chitin cell walls of fungi, and the water molds have flagellated cells, which are lacking in all but a few fungi. Nonetheless, oomycetes are ecologically similar to fungi in that they grow as a mass of hyphae decomposing dead plant and animal material in aquatic habitats.

Oomycetes have had a major impact on human society, as many species are plant pathogens (phytopathogens). The oomycete *Phytophthora infestans*, which causes late blight disease of potatoes, contributed to massive famines in Ireland in the mid-nineteenth century. The famines led to the death of a million Irish and triggered great waves of Irish immigration to North America. Other major phytopathogens include *Pythium*, a common pathogen of greenhouse seedlings, and *Albugo*, which causes "white rusts" on several agricultural crops.

(a)



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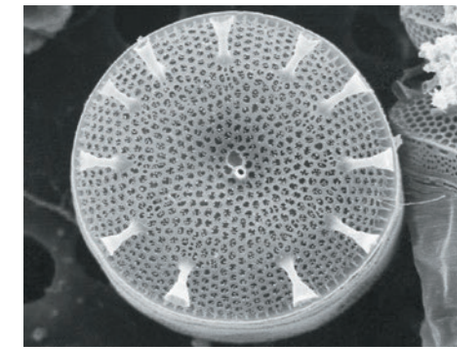

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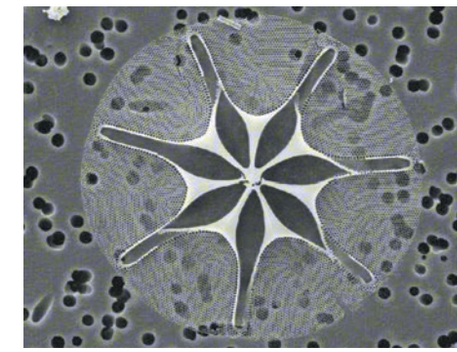


(d)

Figure 18.12 Diatom frustules. *(a)* Dark-field photomicrograph of a collage of frustules from different diatom species showing various forms of symmetry. *(b–d)* Scanning electron micrographs of diatom frustules showing pinnate (part *b*) or radial (parts *c, d*) symmetry. Diatoms vary considerably in size from very small species about 5 μm wide to larger species up to 200 μm wide.

Golden Algae and Brown Algae

Along with the diatoms, golden and brown algae form major lineages of *Stramenopiles*. Golden algae, also called *chrysophytes*, are primarily unicellular marine and freshwater phototrophs. Some species are chemoorganotrophs and feed either by phagocytosis or by transporting soluble organic compounds across the cytoplasmic membrane. Some golden algae, such as *Dinobryon* (**Figure 18.13*a***), found in freshwater, are colonial. However, most golden algae are unicellular and motile by the activity of two flagella of unequal length.

Golden algae are so named because of their golden-brown color (Figure 18.13*a*, *c*). This is due to chloroplast pigments dominated by the brown-colored carotenoid fucoxanthin. The major chlorophyll pigment in golden algae is chlorophyll *c* rather than chlorophyll *a*, and they lack the phycobiliproteins present in red algal chloroplasts (Section 18.15). Cells of the unicellular golden alga *Ochromonas*, the best-studied genus of this group, have only one or two chloroplasts (Figure 18.13*c*).

Brown algae are primarily marine and are multicellular and typically macroscopic. No unicellular brown algae are known. The kelps, such as the giant kelp *Macrocystis* (Figure 18.13*b*), which can grow up to 50 m in length, are perhaps the most widespread of brown algae. *Fucus*, another common seaweed of intertidal regions, can grow up to 2 m. As their name implies, brown algae are brown or green-brown in color depending on how much of the carotenoid pigment fucoxanthin they produce. Most marine "seaweeds" are brown algae and their rapid growth, especially in cold marine waters, can cause nuisance odor problems when they wash ashore and decay.

(a) (b) (c)

Figure 18.13 Golden and brown algae. *(a) Dinobryon*, a golden alga (family *Chrysophyceae*) that forms branched colonies. *(b) Macrocystis*, a marine kelp belonging to the brown algae (family *Phaeophyceae*). *(c) Ochromonas*, a unicellular chrysophyte. The golden or brown color of the chloroplasts of these algae is due to the pigment fucoxanthin.

Check Your Understanding

- What structure of diatoms accounts for their excellent fossil record?
- In what ways do oomycetes differ from and resemble fungi?
- Which chlorophyll pigment is found in golden and brown algae?

18.6 *Rhizaria*

Rhizaria form a diverse group of protists that includes the chlorarachniophytes, radiolarians, and foraminiferans (Figure 18.3). They are distinguished from other protists by their threadlike cytoplasmic extrusions (pseudopodia) by which they move and feed. Some *Rhizaria* have amoeboid morphology and were once mistakenly classified as amoebae because of this and their pseudopodia, but it is now known that many phylogenetically diverse organisms employ pseudopodia for motility and feeding purposes.

Chlorarachniophytes

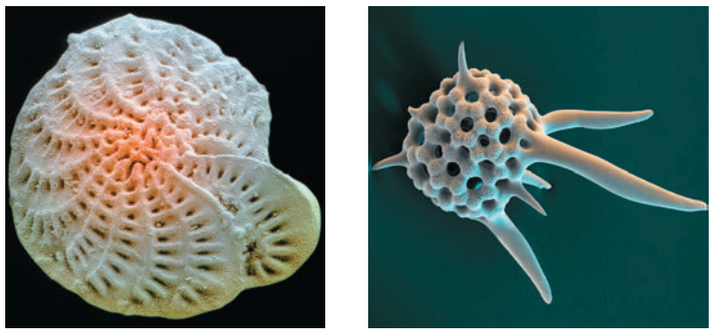
Chlorarachniophytes are freshwater and marine amoeba-like phototrophs that develop a flagellum for dispersal. The group's acquisition of green algal chloroplasts is a prime example of a secondary endosymbiosis (Figure 18.2) and shows how extensively this process has molded several phylogenetically distinct lineages of microbial eukaryotes (Figure 18.3).

Chloroplasts typically have two membranes (◀ Section 2.14; Figures 2.46 and 14.9), derived from the inner and outer membranes of *Cyanobacteria*, which have a gram-negative cell envelope (◀ Sections 2.3 and 2.4). Chloroplasts in chlorarachniophytes, however, have four membranes. In addition, they have **nucleomorphs** tucked in between the two sets of chloroplast membranes. Nucleomorphs are remnant nuclei left over from when the algal endosymbionts were acquired during secondary endosymbiosis (Section 18.1). Both the nucleomorphs and the extra membranes were derived from this algal endosymbiont (Figure 18.2).

It is a testament to endosymbiosis to consider that chlorarachniophytes have merged a minimum of five different genomes: the host genome, the host mitochondrial genome, the algal endosymbiont genome (which has become the nucleomorph), the algal endosymbiont mitochondrial genome, and the algal endosymbiont chloroplast genome! Most nonessential or duplicate genes have been deleted over time, and many genes from the mitochondria and chloroplasts have been transferred to the host genome. As a result, the majority of genes in a chlorarachniophyte are present in the nucleus of the host cell. The nucleomorph itself is greatly reduced in size relative to its ancestor and over time may be lost completely from the chloroplast.

Foraminiferans

In contrast to chlorarachniophytes, foraminiferans are exclusively marine microbes and form shell-like structures called *tests*, which have distinctive characteristics and are often quite ornate (**Figure 18.14*a***). Tests are typically made of organic materials reinforced with calcium carbonate. The test is not firmly attached to the cell, and the amoeba-like cell may extend partway out of the test during feeding. However, because of the weight of the test, the cell usually sinks to the bottom of the water column, and it is thought that the organisms feed on dissolved organic matter and particulate deposits, primarily bacteria, other protists, and the remains of dead organisms near the sediments. Foraminiferan cells can also host a variety of algae that form



(a) (b)

Figure 18.14 Foraminifera and radiolaria. *(a)* A foraminiferan. Note the ornate and multilobed test. The test is about 1 mm wide. *(b)* A spiked radiolarian of the *Nassellaria* group. A test is about 150 μm wide. Both *a* and *b* are colorized scanning electron micrographs.

endosymbiotic relationships with the protist and supply it with organic carbon, probably in exchange for inorganic nutrients derived from the breakdown of dead organisms. Phototrophs are found primarily in planktonic foraminifera that remain suspended in the water column to provide their endosymbionts with sufficient sunlight.

Foraminiferan tests (Figure 18.14*a*) are relatively resistant to decay and are readily fossilized. These buried and preserved tests are quite useful to geologists. Because particular taxa of foraminifera are typically associated with particular strata in the geological record, fossilized foraminiferan tests in samples obtained from exploratory wells are used by oil industry paleontologists as a means to date and assess the petroleum potential of a given drill site.

Radiolarians

Radiolarians are chemoorganotrophic and mostly planktonic marine eukaryotes that reside in the upper 100 m of the ocean where they consume bacteria and particulate organic matter. Some species associate with algae that take on a symbiotic (but not endosymbiotic) role and supply nutrients to the radiolarian.

The name "radiolarian" comes from the radial symmetry of their tests, transparent or translucent mineral skeletons made of silica in one fused piece (Figure 18.14*b*). Along with the accumulation of lipid droplets and large cytoplasmic vacuoles, the needle-like pseudopodia of radiolarians probably help keep the organisms from sinking in their mainly open ocean (planktonic) habitats. However, when cells eventually die, their tests settle to the ocean floor and can build up over time into thick layers of slowly decaying cell material.

Check Your Understanding

- What structure distinguishes rhizaria from all other protists?
- In what way are foraminifera similar to diatoms? In what ways are they different?
- How has endosymbiosis shaped the genomes of chlorarachniophytes?

18.7 Haptophytes

KEY GENUS: *Emiliania*

Haptophytes are a group of unicellular and free-living phototrophic protists found in aquatic environments. Members of this group are widespread and abundant in the oceans, where they have a major impact on the biosphere. All haptophytes share several structural features including the presence of two flagella, two chloroplasts (derived from secondary endosymbiosis of red algae, Figures 18.2 and 18.3), and a unique structure called a *haptonema*. While all haptophytes are phototrophic, some are mixotrophic, obtaining energy both from photosynthesis and from the ingestion of smaller organisms or detritus. The haptonema, for which the group is named, is used when feeding, but it can also facilitate attachment and the avoidance of predators.

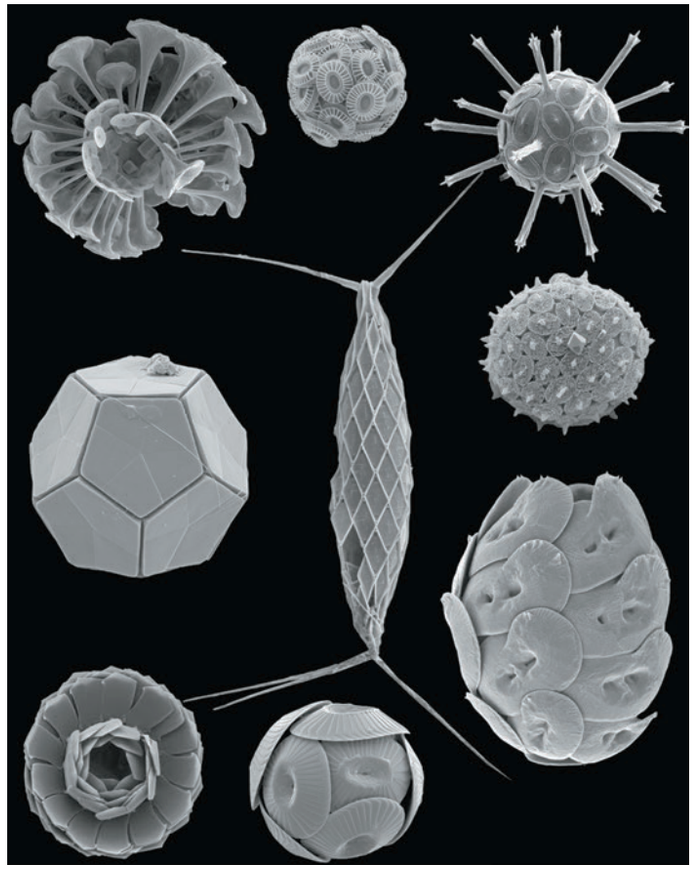
Coccolithophores

Haptophytes, at some point in their life cycle, typically possess rigid polysaccharide plates on their outer surface. The morphology of these plates can vary dramatically between species. One major group of haptophytes, the *coccolithophores*, are distinguished by their ability to form intricate mineralized plates made of calcite, a form of calcium carbonate.

Coccolithophores (**Figure 18.15**) are one of the most abundant and widely distributed unicellular phototrophic eukaryotes in the oceans. One of the most widespread and well characterized of the coccolithophores is *Emiliania huxleyi*. Coccolithophores are found in the photic zone of the ocean from tropical to subpolar latitudes. They can contribute 1 to 10% of the primary productivity in these systems under normal conditions, but they can also form massive blooms in which they account for more than 40% of photosynthetic activity. These blooms can span hundreds of thousands of square kilometers and are visible from space (see MicrobiologyNow in the chapter opener).

Coccolithophores have an exoskeleton composed of *coccoliths*, which are plates made from calcium carbonate (Figure 18.15). The plates could have several purposes. For example, they might be used to enhance photosynthesis by attenuating or focusing sunlight into the cell. Alternatively, the plates may protect the cell from grazing or from attack by bacteria or viruses.

Whatever their purpose, the production of coccolithophore plates has a major impact on Earth's carbon cycle and, by extension, its climate. This is because the plates make coccoliths heavy, and the cells



Dr. Jeremy R. Young

Figure 18.15 Haptophytes. Scanning electron micrographs showing a collection of diploid cells of different species of coccolithophores. These phototrophic microbes have an exoskeleton composed of interlocking coccoliths made from calcium carbonate. *Emiliania huxleyi*, center top, is about 10 μm in diameter.

eventually sink, exporting carbon to the seafloor in the form of calcite. In this way, coccolithophores are a "biological pump" that removes CO_2 from the atmosphere and deposits it on the seafloor where it will eventually form sedimentary rocks (▶ Section 21.7 and Figure 21.20*c*). Indeed, coccoliths are a main component of the chalk deposits found in many regions of the world such as those of the famous White Cliffs of Dover in England. These deposits represent millions of years of carbon deposited on the seafloor, and they are a dramatic illustration of the power of microbes to influence the biosphere.

Life Cycle

Haptophytes have a life cycle that alternates between haploid and diploid cells; only haploid cells contain a haptonema. In the case of *E. huxleyi* (Figure 18.15; ▶ Figure 21.20*a*), diploid cells are observed most commonly and are responsible for the formation of blooms. Diploid cells are nonmotile, reproduce by binary fission to form diploid progeny, and form structurally complex exoskeletons composed of interlocking coccolith scales (Figure 18.15). In response to an environmental trigger, the nature of which remains poorly characterized, diploid cells undergo meiosis resulting in haploid progeny. The haploid cells are motile and do not form interlocking calcified exoskeletons. These noncalcified haploid cells divide by binary fission until some environmental trigger causes them to fuse with another haploid cell, returning the organism to the diploid state.

The two life stages of the coccolithophores are thus so morphologically different that they can easily be mistaken for different species. In nature, however, it is likely that the exoskeleton-containing (diploid) form predominates because this is the form seen in the massive blooms of coccolithophores in oceans worldwide.

Check Your Understanding

- What is a coccolith?
- In what ways are coccolithophores similar to diatoms? How do they differ?
- In what way do coccolithophores influence the global climate?

18.8 *Amoebozoa*

KEY GENERA: *Amoeba, Entamoeba, Physarum, Dictyostelium*

The *Amoebozoa* are a large group of terrestrial and aquatic protists that use lobe-shaped pseudopodia for movement and feeding, in contrast to the threadlike pseudopodia of rhizaria. The major groups of *Amoebozoa* are the *gymnamoebas*, the *entamoebas*, and the *plasmodial* and *cellular slime molds*. Phylogenetically, the *Amoebozoa* diverged from a lineage that eventually led to the fungi and animals (Figure 18.3).

Gymnamoebas and Entamoebas

The gymnamoebas are free-living protists that inhabit aquatic and soil environments. They use pseudopodia to move by a process called *amoeboid movement* (**Figure 18.16**) and feed by phagocytosis on bacteria, other protists, and particulate organic materials. Amoeboid movement results from streaming of the cytoplasm as it flows forward at the less contracted and viscous cell tip, taking the path of least resistance. Cytoplasmic streaming is facilitated by microfilaments (◀ Section 2.15), which exist in a thin layer just beneath the cytoplasmic membrane. *Amoeba* (Figure 18.16) is a common organism in pond waters, with species varying in size from 15 μm in diameter (clearly microscopic) to over 750 μm (visible with the naked eye).

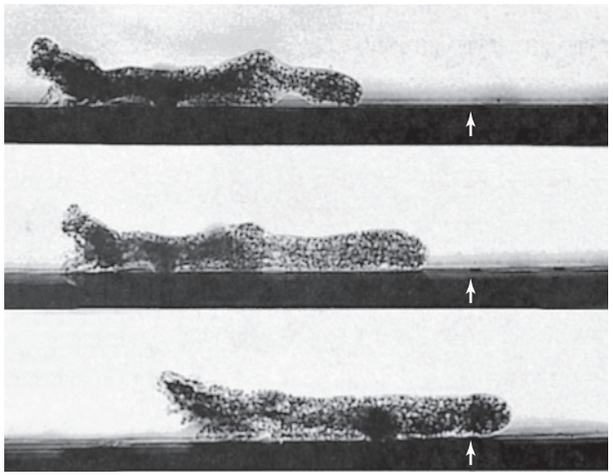


Figure 18.16 Time-lapse view of the amoebozoan *Amoeba proteus*. The time interval from top to bottom is about 6 sec. The arrows point to a fixed spot on the surface. A single cell is about 80 μm wide.

In contrast to gymnamoebas, the entamoebas are parasites of vertebrates and invertebrates. Their usual habitat is the oral cavity or intestinal tract of animals. The anaerobe *Entamoeba histolytica* is pathogenic in humans and causes amebic dysentery, an ulceration of the intestinal tract that results in bloody diarrhea. This parasite forms cysts that are transmitted from person to person by fecal contamination of water, food, and eating utensils. In Section 34.00 we discuss the etiology and pathogenesis of amebic dysentery, an important cause of death from intestinal parasites in humans.

Slime Molds

The **slime molds** were previously grouped with fungi since they undergo similar life cycles and produce fruiting bodies with spores for dispersal. As protists, however, slime molds are motile and can move across a solid surface fairly quickly (see Figures 18.17–18.19). Slime molds are divided into two groups: *plasmodial slime molds* (also called *acellular slime molds*), whose vegetative forms are masses of protoplasm of indefinite size and shape called *plasmodia* (**Figure 18.17**), and *cellular slime molds*, whose vegetative forms are single amoebae. Slime molds live primarily on decaying plant matter, such as leaf litter, logs, and soil, where they consume other microorganisms, especially bacteria. Slime molds can maintain themselves in a vegetative state for long periods but eventually form differentiated spore-like structures that can remain dormant and then germinate later to once again generate the active amoeboid state.

Plasmodial slime molds, such as *Physarum*, exist in the vegetative phase as an expanding single mass of protoplasm called the *plasmodium* that contains many diploid nuclei (Figure 18.17). The plasmodium is actively motile by amoeboid movement, and from this phase, a sporangium containing haploid spores can be produced. When conditions are favorable the spores germinate to yield haploid flagellated swarm cells. The fusion of two swarm cells then regenerates a diploid plasmodium.

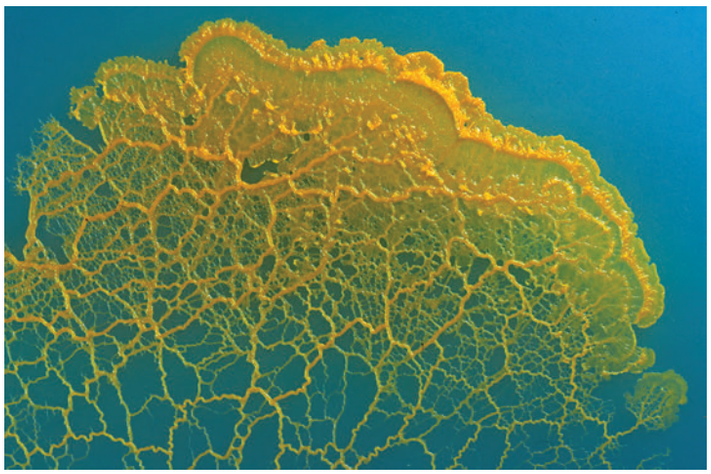


Figure 18.17 Slime mold. The plasmodial slime mold *Physarum* growing on an agar surface. The plasmodium is about 5 cm long and 3.5 cm wide.

In contrast to their plasmodial relatives, cellular slime molds are individual haploid cells and form diploids only under certain conditions. The well-studied cellular slime mold *Dictyostelium discoideum* undergoes an asexual life cycle in which vegetative cells aggregate, migrate as a cell mass, and eventually produce fruiting bodies in which cells differentiate and form spores (**Figures 18.18** and **18.19**). When cells of *Dictyostelium* are starved, they aggregate and form a pseudoplasmodium; in this stage cells lose their individuality, but do not fuse. Aggregation is triggered by the production of cyclic adenosine monophosphate (cAMP). The first cells of *Dictyostelium* that produce this compound attract neighboring cells and eventually aggregate into motile masses of cells called *slugs*. Fruiting body formation begins when the slug becomes stationary and vertically oriented. The emerging structure differentiates into a stalk and a head. The stalk cells form cellulose, which provides the rigidity of the stalk, and the head cells differentiate into spores. Eventually, spores are released and dispersed, with each spore forming a new amoeba (Figures 18.18 and 18.19).

In addition to this asexual process, *Dictyostelium* can produce sexual spores. These form when two amoebae in an aggregate fuse to form a single giant amoeba. A thick cellulose wall develops around this cell to form a structure called the *macrocyst*, and this can remain dormant for long periods. Eventually, the diploid nucleus undergoes meiosis to form haploid nuclei that become integrated into new amoebae that can once again initiate the asexual cycle.

In Part III, we consider the fungi, a huge group of microbial eukaryotes whose service to the ecosystem by decomposing dead organic material cannot be understated.

Check Your Understanding

- How can amoebozoans be distinguished from rhizaria?
- Compare and contrast the lifestyles of gymnamoebas and entamoebas.
- Describe the major steps in the life cycle of *Dictyostelium discoideum*.

UNIT 4

Mastering Microbiology
Art Activity: Figure 18.18 Stages in fruiting body formation in cellular slime mold *Dictyostelium discoideum*

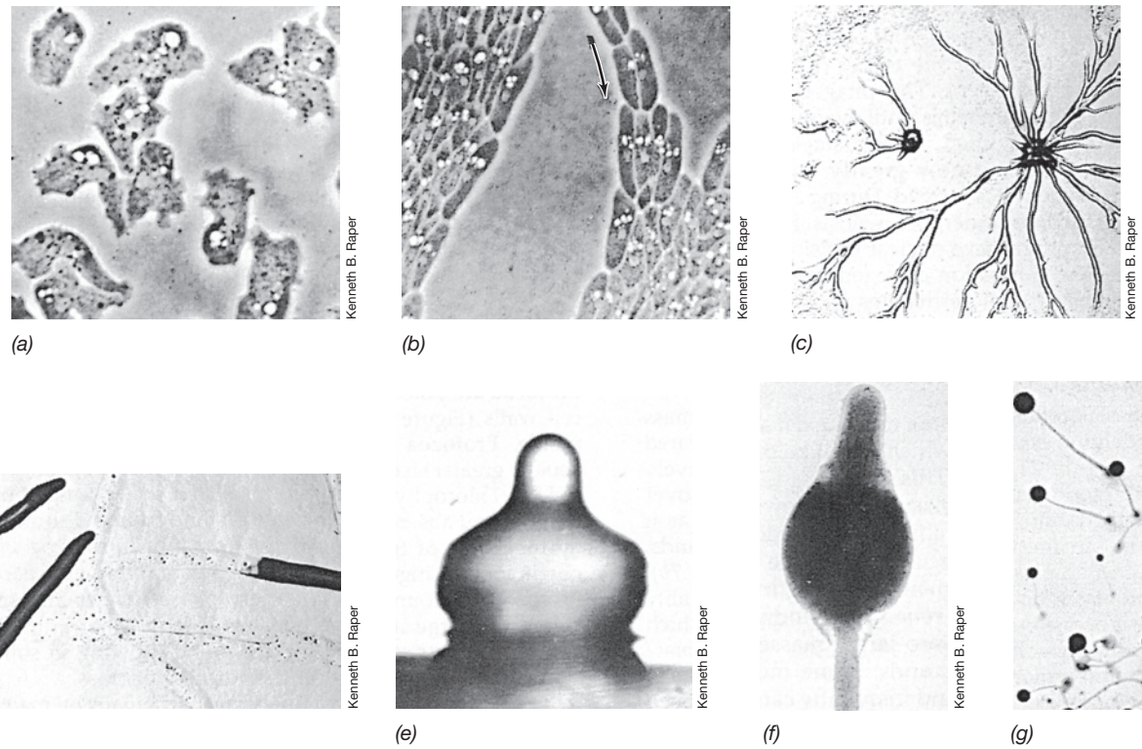


Figure 18.18 Photomicrographs of various stages in the life cycle of the cellular slime mold *Dictyostelium discoideum*. *(a)* Amoebae in pre-aggregation stage. *(b)* Aggregating amoebae. Amoebae are about 300 μm in diameter. *(c)* Low-power view of aggregating amoebae. *(d)* Migrating pseudoplasmodia (slugs) moving on an agar surface and leaving trails of slime behind. *(e, f)* Early stages of fruiting body. *(g)* Mature fruiting bodies. Figure 18.19 shows the sizes of these structures. *Dictyostelium* has long served as a model for development in multicellular organisms, and its genome of 12,500 genes is about half that of the human genome.

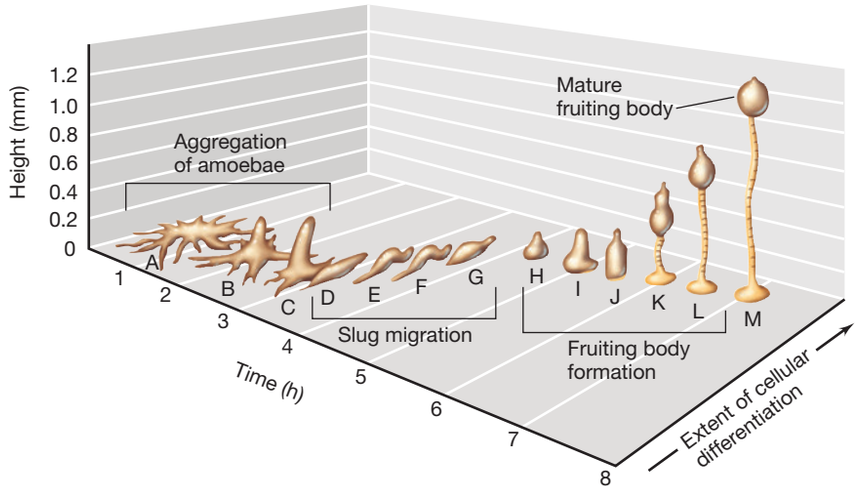


Figure 18.19 Stages in fruiting body formation in the cellular slime mold *Dictyostelium discoideum*. *(A–C)* Aggregation of amoebae. *(D–G)* Migration of the slug formed from aggregated amoebae. *(H–L)* Culmination of migration and formation of the fruiting body. *(M)* Mature fruiting body composed of stalk and head. Cells from the rear of the slug form the head and become spores. *Dictyostelium* also undergoes sexual reproduction (not shown) when two amoebae fuse to form a macrocyst; the fused nuclei in the macrocyst return to the haploid stage when meiosis forms new vegetative amoebae.

III • Fungi

Fungi are nonphototrophic eukaryotic microbes that form rigid cell walls made of chitin. Fungi are major agents of decomposition in soil and other habitats, and some form symbiotic relationships with plants.

The *Fungi* are a large, diverse, and widespread group of mostly nonmotile organisms that includes such well-known groups as the *molds, mushrooms*, and *yeasts*. Approximately 100,000 fungal species have been described, and as many as 1.5 million species could exist. Fungi form a phylogenetic cluster distinct from protists and are the microbial group most closely related to animals (Figure 18.3).

Most fungal species are microscopic and terrestrial. They inhabit soil or dead plant matter and play crucial roles in the decomposition of organic matter. A large number of fungal species are plant pathogens, and a few cause diseases of animals, including humans (Chapter 34). Certain species of fungi also establish symbiotic associations with plants, facilitating the plant's acquisition of minerals from soil, and many fungi benefit humans through fermentation and the synthesis of antibiotics.

18.9 Fungal Physiology, Structure, and Symbioses

In this section we describe some general features of fungi, including their physiology, cell structure, and the symbiotic associations they develop with plants and animals. In the following section, we examine fungal reproduction and phylogeny.

Nutrition, Physiology, and Ecology

Fungi are chemoorganotrophs—typically displaying simple nutritional requirements—and most are aerobic. Fungi nourish themselves by secreting extracellular enzymes that digest polymeric materials, such as polysaccharides or proteins, into monomers that are assimilated as sources of carbon and energy. As decomposers, fungi digest dead animal and plant materials. As parasites of plants or animals, fungi use the same mode of nutrition but take up nutrients from the living cells of the plants and animals they invade rather than from dead organic materials.

A major ecological activity of fungi, especially the basidiomycetes, is the decomposition of wood, paper, cloth, and other products derived from these natural sources. Lignin, a complex polymer in which the building blocks are phenolic compounds, is an important constituent of woody plants, and in association with cellulose, it confers rigidity on them. Lignin is decomposed in nature almost exclusively through the activities of certain basidiomycetes called *wood-rotting fungi*. Two types of wood rot are known: *brown rot*, in which the cellulose is attacked preferentially while the lignin is left unmetabolized, and *white rot*, in which both cellulose and lignin are decomposed. The white rot fungi are of major ecological importance because they play such a key role in decomposing woody materials in forests.

Fungal Morphology, Spores, and Cell Walls

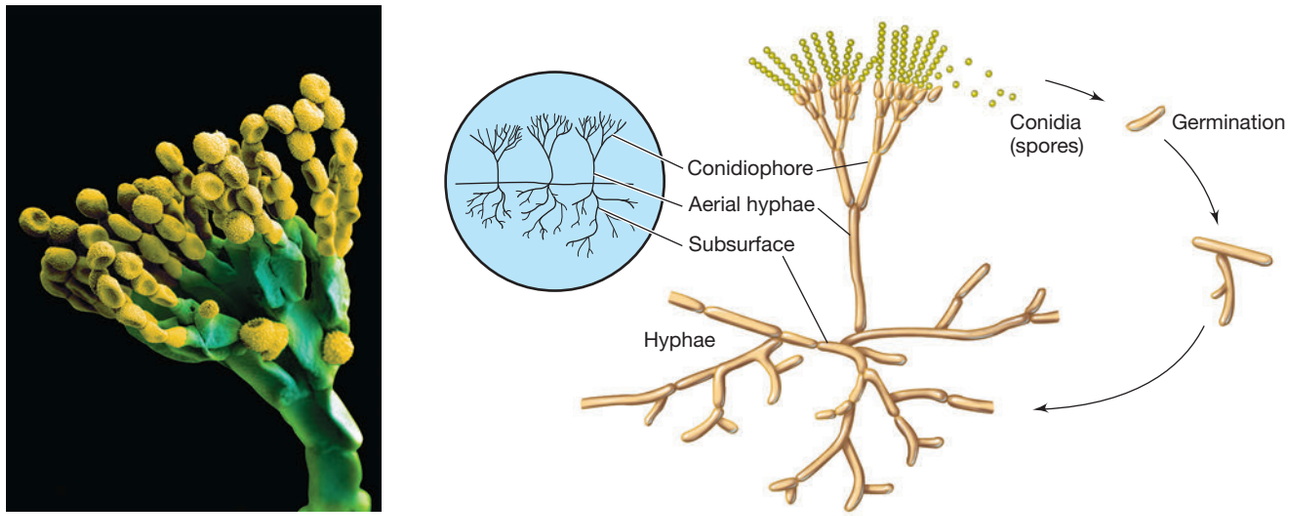
Most fungi are multicellular, forming a network of filaments called *hyphae* (singular, hypha) from which asexual spores are produced (Figure 18.20). Hyphae are tubular cell walls that surround the cytoplasmic membrane. Fungal hyphae are often septate, with cross-walls dividing each hypha into separate cells. In some cases, however, the vegetative cell of a fungal hypha contains more than one nucleus, and hundreds of nuclei can form as a result of repeated nuclear divisions without the formation of cross-walls, a condition called *coenocytic*. Each hyphal filament grows mainly at the tip by extension of the terminal cell (Figure 18.20).

Hyphae typically grow together across and above a surface to form a compact, macroscopically visible tuft called a *mycelium* (Figure 18.21*a*). From the mycelium, aerial hyphae reach up into the air above the surface, and spores called **conidia** are formed on their tips (Figure 18.21*b*). Conidia are asexual spores and they are often pigmented black, green, red, yellow, or brown (Figure 18.21). Conidia give the mycelium a dusty appearance (Figure 18.21*a*) and function to disperse the fungus to new habitats. Some fungi form macroscopic reproductive structures called *fruiting bodies* (**mushrooms** or puffballs, for example), in which millions of spores are produced that can be dispersed by wind, water, or animals (Figure 18.22). In contrast to mycelial fungi, some fungi grow as single cells; these are the **yeasts**.

Most fungal cell walls consist of **chitin**, a polymer of *N*-acetylglucosamine. Chitin is arranged in the walls in microfibrillar bundles, as is cellulose in plant cell walls, to form a thick, tough wall structure. Other polysaccharides such as mannans and galactosans, or even cellulose itself, replace or supplement chitin in some fungal cell walls. Fungal cell walls are typically 80–90% polysaccharide, with only small amounts of proteins, lipids, polyphosphates, and inorganic ions making up the wall-cementing matrix.

UNIT 4

Mastering Microbiology
Art Activity: Figure 18.19 Fungal structure and growth

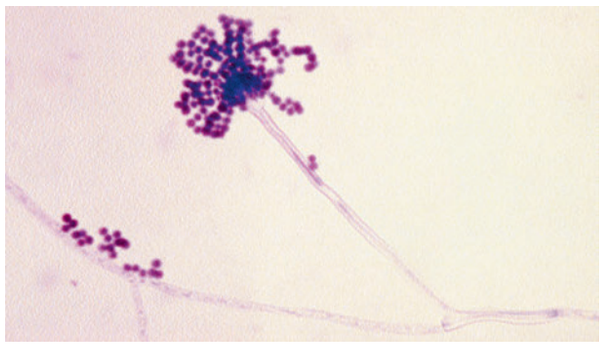


(a) (b)

Figure 18.20 Fungal structure and growth. *(a)* Scanning electron micrograph of asexual spores of a typical mold, *Penicillium chrysogenum*. The conidiophore is shown in green and the conidia are yellow. *(b)* Diagram of a mold life cycle. The conidia can be dispersed by either wind or animals and are about 2 μm wide.

Cheryl L. Broadie

(a)



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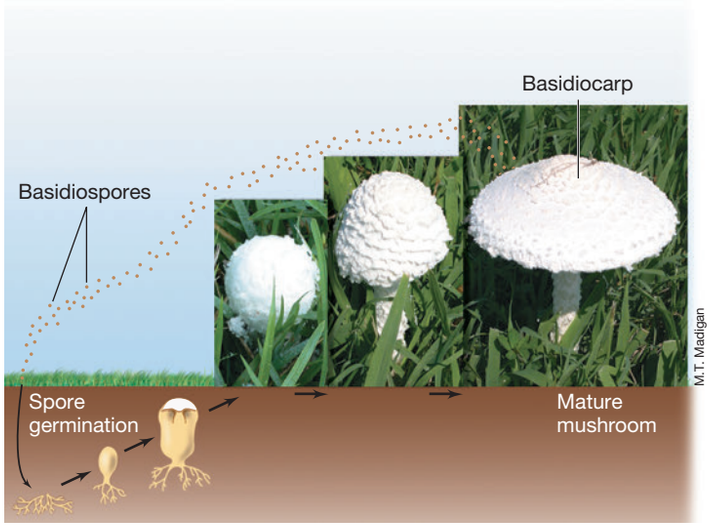
(b)

Figure 18.21 Hyphal fungi (molds). *(a)* Colonies of an *Aspergillus* species (ascomycete), growing on an agar plate. Note the masses of filamentous cells (mycelia) and asexual spores that give the colonies a dusty, matted appearance. *(b)* Conidiophore and conidia of *Aspergillus fumigatus* (see Figure 18.20*b*). The conidiophore is about 300 μm long and the conidia here are about 3 μm wide. These cells were stained to improve contrast. Besides being a common saprophyte, *Aspergillus* can be pathogenic, causing serious pulmonary and occasional systemic infections in humans and some domestic animals. Cancer patients and others with weakened immune systems are particularly susceptible to aspergillosis.

Symbioses and Pathogenesis

Most plants are dependent on certain fungi to facilitate their uptake of minerals from soil. These fungi form symbiotic associations with the plant roots called *mycorrhizae* (the word means, literally, "fungus roots"). Mycorrhizal fungi establish close physical contact with the roots and help the plant obtain phosphate, as well as other minerals, and water from the soil. In return, the fungi obtain nutrients such as sugars from the plant root (▶ Figure 23.22). There are two kinds of mycorrhizal associations. One, *ectomycorrhizae*, typically forms between basidiomycetes (Section 18.14) and the roots of



M.T. Madigan

Figure 18.22 Mushroom life cycle. Mushrooms typically develop underground and then emerge on the surface rather suddenly (usually overnight), triggered by an influx of moisture. Photos of stages in formation of a common lawn mushroom (see also Section 18.14).

woody plants, while the second, *endomycorrhizae*, forms between glomeromycete fungi (Section 18.12) and many nonwoody plants. Some fungi also form associations with cyanobacteria or green algae. These are the *lichens*, the colorful and crusty growths often seen on the surfaces of trees and rocks. We explore the biology of lichens and mycorrhizae in more detail in Sections 23.1 and 23.5, respectively.

Fungi can invade and cause disease in plants and animals. Fungal plant pathogens cause widespread crop and plant damage worldwide, and fruit and grain crops in particular suffer significant yearly losses as a result of fungal infection. Human fungal diseases, called *mycoses*, range from relatively minor and easily cured conditions, such as athlete's foot and jock itch, to serious, life-threatening systemic mycoses, such as histoplasmosis.We consider some major fungal diseases of humans in Chapter 34.

Check Your Understanding

- What are conidia? How does a conidium differ from a hypha? From a mycelium?
- What is chitin and where is it present in fungi?
- Distinguish between mycorrhizae and lichens.

18.10 Fungal Reproduction and Phylogeny

Fungi reproduce by *asexual* means in one of three ways: (1) by the growth and spread of hyphal filaments; (2) by the asexual production of spores (conidia; Figures 18.20 and 18.21); or (3) by simple cell division, as in budding yeasts (**Figure 18.23**). Most fungi also form sexual spores, typically as part of an elaborate life cycle. Some fungi, such as the well-known mold *Penicillium* (the source of the antibiotic penicillin), were long thought to lack a sexual stage and reproduce only by way of conidia. But it has now been shown that the life cycle of *Penicillium* includes a sexual stage.

Sexual Spores of Fungi

Some fungi produce spores as a result of sexual reproduction. The spores develop from the fusion of either unicellular gametes or specialized hyphae called *gametangia*. Alternatively, sexual spores can originate from the fusion of two haploid cells to yield a diploid cell; this then undergoes meiosis and mitosis to yield individual haploid spores.

Depending on the group, different types of sexual spores are produced. Spores formed within an enclosed sac (ascus) are called *ascospores*. Many yeasts produce ascospores, and we consider sporulation in the common baker's yeast *Saccharomyces cerevisiae* in Section 18.13. Sexual spores produced on the ends of a club-shaped structure (basidium) are *basidiospores* (Figure 18.22 and see Figure 18.31*c*). *Zygospores*, produced by zygomycetous fungi such as the common bread mold *Rhizopus* (Section 18.12), are macroscopically visible structures that result from the fusion of hyphae and genetic exchange. Eventually the zygospore matures and produces asexual spores that are dispersed by air and germinate to form new fungal mycelia. Chytrid fungi produce sexual spores, motile by eukaryotic flagella, called *zoospores*.

J. Forsdyke/SPL/Photo Researchers

Figure 18.23 The common baker's and brewer's yeast *Saccharomyces cerevisiae* (*Ascomycota*). In this colorized scanning electron micrograph, note the budding division and scars from previous buds. A single cell is about 6 μm in diameter.

Sexual spores of fungi are typically resistant to drying, heating, freezing, and some chemical agents. However, neither sexual nor asexual spores of fungi are as resistant to heat as bacterial endospores (◀ Section 2.8). Both asexual and sexual spores of fungi can germinate and develop into a new hypha.

The Phylogeny of *Fungi*

Fungi share an ancestor with animals and are more closely related to animals than any other eukaryotic group (Figure 18.3). The last common ancestor of all fungi likely existed sometime between 450 million and 1.5 billion years ago. One of the earliest fungal lineages is thought to be the *Chytridiomycota*, an unusual group of motile fungi that produce zoospores. Thus, the lack of flagella in most fungi indicates that motility is a characteristic that has been lost at various times in different fungal lineages.

Some of the major groups of fungi are shown in the evolutionary tree in **Figure 18.24**. The phylogeny shown in this figure includes several distinct fungal groups: the *Microsporidia*, *Chytridiomycota*, *Mucoromycota*, *Glomeromycota*, *Ascomycota*, and *Basidiomycota* (these groups are considered in detail in the next four sections). The vast majority of described fungal species belong to the *Ascomycota* and *Basidiomycota*. The *Ascomycota* are a large and diverse group of fungi that includes yeasts, such as *Saccharomyces* (Figures 18.23 and 18.24), and molds, such as *Aspergillus* (Figure 18.21). The *Basidiomycota* include fungi that form mushrooms (Figures 18.22, 18.24, and see Figure 18.31), as well as many important plant pathogens such as the rusts and smuts. While a tremendous diversity of fungal species have already been cultured and described, phylogenetic analyses of fungal DNA sequences recovered from environmental samples (▶ Sections 19.6 and 19.8) indicate that more than 90% of fungal species remain uncultured. Clearly, we have much to learn about the biology and phylogeny of the fungi.

Check Your Understanding

- Why is the mold *Penicillium* economically important?
- What are the major differences between ascospores and conidia?
- To what major group of macroorganisms are fungi most closely related?

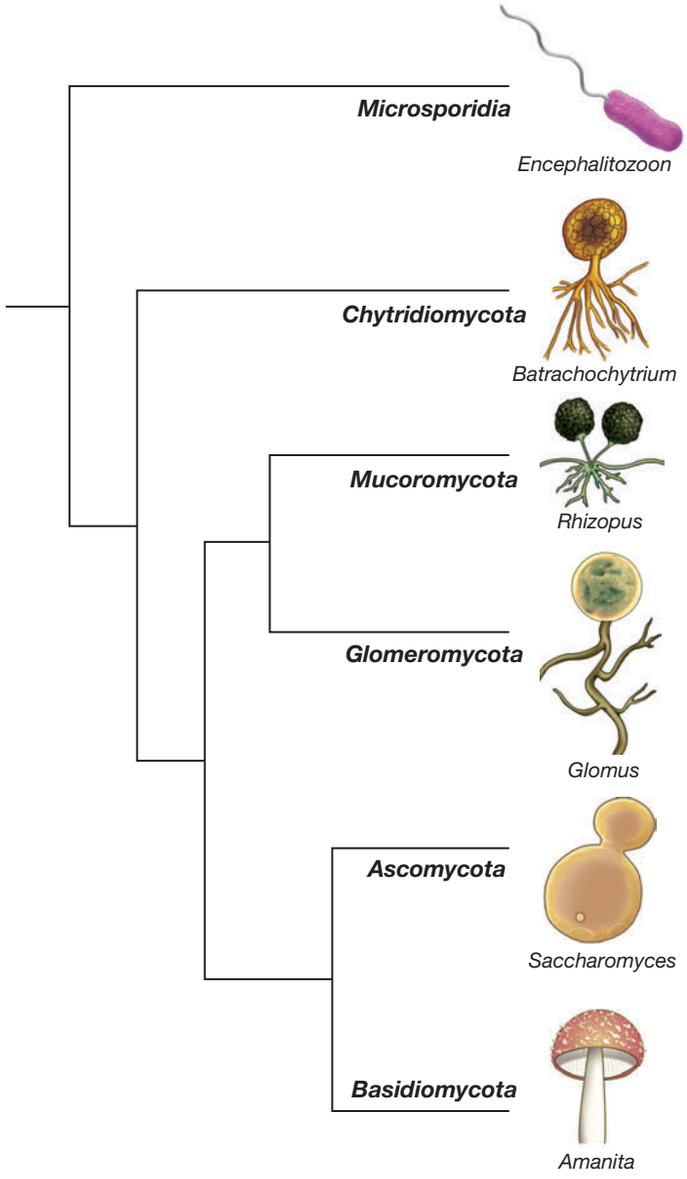


Figure 18.24 **Phylogeny of fungi.** This phylogenetic tree depicts the relationships among the major groups (phyla) of fungi. A typical genus is listed for each group and depicted in the tree.

18.11 *Microsporidia* and *Chytridiomycota*

KEY GENERA: *Allomyces, Batrachochytrium, Encephalitozoon*

The *Microsporidia* and *Chytridiomycota* are ancient phylogenetic groups of parasitic or saprophytic fungi (Figure 18.24). Microsporidia are obligate parasites of a wide variety of animal hosts including humans, whereas the *Chytridiomycota* are primarily aquatic fungi whose species are either parasites or saprophytes.

Microsporidia

Microsporidia are tiny (2–5 μm) unicellular parasites of animals and protists. Based on 18S ribosomal RNA gene sequencing and their lack of mitochondria, *Microsporidia* were once thought to form a very early-branching lineage of *Eukarya*. However, composite gene and protein sequencing has shown the microsporidians are fungi closely related to the *Chytridiomycota* (Figure 18.24). There remains debate as to whether the *Microsporidia* should be included as one of the most deeply divergent lineages within the fungi or whether they should instead be classified as a distinct lineage closely related to fungi.

Microsporidia have adapted to a parasitic lifestyle. They exist as spores when outside of the host. When near a host cell they extend a helical polar tubule that penetrates the host cytoplasmic membrane. The spore then injects its *sporoplasm* into the host cell. The sporoplasm replicates within the cytoplasm of the host cell, forming new spores as it completes its life cycle. Eventually, the cell membrane of the host is disrupted and the spores are released into the surrounding environment, free to infect new cells.

Like most obligate parasites, microsporidia have undergone significant genome reduction, losing many features that would allow them to live outside of a host cell. The microsporidium *Encephalitozoon* (Figure 18.24 and **Figure 18.25*a***), for example, lacks not only mitochondria but also a Golgi complex (another key eukaryotic cell structure, ◀ Section 2.15 and Figure 2.42). Moreover, *Encephalitozoon* cells contain a very small genome of only 2.9 Mbp and only about 2000 genes (this is 1.5 Mbp and 2600 genes *smaller* than that of the bacterium *Escherichia coli*). The *Encephalitozoon* genome lacks genes for major metabolic pathways, such as the citric acid cycle (◀ Section 3.6), meaning that this pathogen must depend on its host for even the most basic of metabolites and metabolic processes.

In humans, *Encephalitozoon* causes chronic debilitating diseases of the intestine, lung, eye, muscle, and some internal organs but is uncommon among healthy adults with normal immune systems. However, microsporidial diseases have appeared with increasing frequency in immune-compromised individuals, such as those with HIV/AIDS or those on long-term administration of immune-suppressing drugs, such as those who have had organ transplants.

Chytridiomycota

Chytridiomycota, or *chytrids*, are one of the earliest diverging lineage of fungi (Figure 18.24), and their name refers to the structure of the fruiting body, which contains their *zoospores* (Section 18.10). These spores are unusual among fungal spores in being flagellated and motile and are ideal for dispersal of these organisms in the aquatic environments, mostly freshwater and moist soils, where they are commonly found.

Many species of chytrids are known and some exist as single cells, whereas others form colonies with hyphae. They include both free-living forms that degrade organic material, such as *Allomyces*, and parasites of animals, plants, and protists. The chytrid *Batrachochytrium dendrobatidis* causes chytridiomycosis of frogs (Figure 18.25*b*), a condition in which the organism infects the frog's epidermal layers, leading to a loss of ions across the membrane and osmotic imbalance. Chytrids have been implicated in the massive die-off of frogs and some other amphibians worldwide, probably in response to increases in global temperatures that have stimulated chytrid proliferation and to increased animal susceptibility due to habitat loss and aquatic pollution.

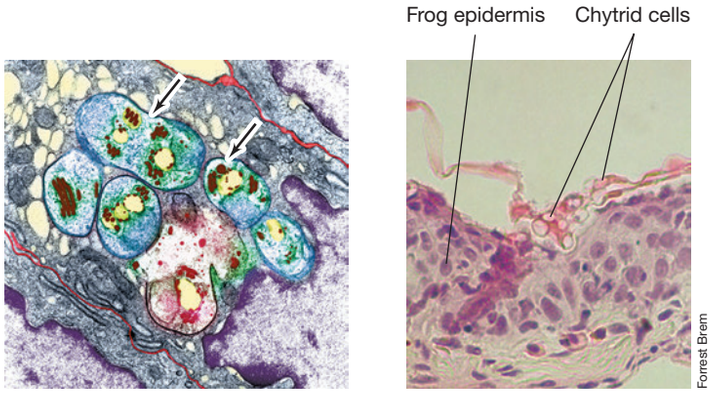


Figure 18.25 ***Microsporidia* and *Chytridiomycota*.** *(a)* Colorized transmission electron micrograph of thin sections of cells of the microsporidium *Encephalitozoon intestinalis* (arrows) growing in human intestinal cells. *(b)* Cells of the chytrid *Batrachochytrium dendrobatidis* stained pink growing on the surface of frog epidermis.

Unresolved aspects of the phylogeny of chytrids suggest that this group is not monophyletic. That is, some organisms currently classified as chytrids may actually be more closely related to species of other fungal groups, such as the *Mucoromycota*, to which we turn next. As is true for the protists, much about the evolution of the chytrids and other groups of fungi remains to be learned.

Check Your Understanding

- What animal group has been most affected by chytrids?
- What are some features of *Microsporidia* that distinguish them from chytrids?

18.12 *Mucoromycota* and *Glomeromycota*

KEY GENERA: *Rhizopus, Glomus*

We consider two groups of fungi here, the *Mucoromycota*, known primarily for their role in food spoilage, and the *Glomeromycota*, important fungi in certain mycorrhizal associations. *Mucoromycota* are commonly found in soil and on decaying plant material, whereas *Glomeromycota* form symbiotic relationships with plant roots. All of these fungi are coenocytic (multinucleate), and while *Glomeromycota* have an exclusively asexual life cycle, *Mucoromycota* often have a life cycle that includes sexual spores called *zygospores* (Section 18.10).

Mucoromycota

The common black bread mold *Rhizopus nigricans* (**Figure 18.26**) is a widespread zygomycete (zygomycetes are fungi that produce zygospores). This organism undergoes a complex life cycle that includes both asexual and sexual reproduction. In the asexual phase the mycelia form sporangia within which haploid spores are produced. Once released, these haploid spores disperse and eventually germinate, giving rise to vegetative mycelia. In the sexual phase, mycelial gametangia of different mating types (analogous to male and female, see Section 18.13) fuse to yield a cell with two nuclei. This diploid cell eventually develops into a *zygospore*, which can remain dormant and resist dryness and other unfavorable conditions. When conditions are favorable, the zygospore can germinate, forming either a diploid mycelium or a sporangium that produces haploid spores for dispersal.

Most species of *Rhizopus* and related zygomycetes are harmless saprophytes whose airborne spores land and form spreading colonies on stale bread (Figure 18.26*a*) and various moist surfaces in the home or on walls and crevices in buildings where moisture is trapped. However, some species are human pathogens. If inhaled in sufficient amounts, spores of pathogenic *Rhizopus* species can cause serious infections of the lungs, sinuses, eyes, nose, and mouth leading to swelling of facial features, asthma-like symptoms, and even fatal systemic fungal infections if the initial infection is not promptly treated.

Glomeromycota

The *Glomeromycota* are a relatively small and unique group of obligately symbiotic fungi in which all known species form associations with plants called *endomycorrhizae* (Section 18.9). As many as 80% or more of land plant species form these associations in which the fungal hyphae enter the plant cell and aid the plant's acquisition of phosphate from the soil in return for fixed carbon from the plant. Most of these *Glomeromycota* are *arbuscular mycorrhizae*, fungi that form structures called *arbuscules* that penetrate cells of their plant host and specialize in nutrient exchange (▶ Section 23.5). As plant symbionts, glomeromycetes are thought to have played a pivotal role in the ability of early vascular plants to colonize land.

As far as is known, glomeromycetes reproduce only asexually and are mostly coenocytic in their hyphal morphology. Asexual spores of *Glomus* (Figure 18.24), a major genus of endomycorrhizae, are collected from the roots of cultivated plants and used as an agricultural inoculant to promote vigorous symbiotic associations between plant and fungus. This natural approach to plant fertilization is a widespread practice in small, sustainable farming operations and has been shown to increase both the growth and nutrient content of tomato, pepper, squash, bean, and several other small fruit and vegetable plants.

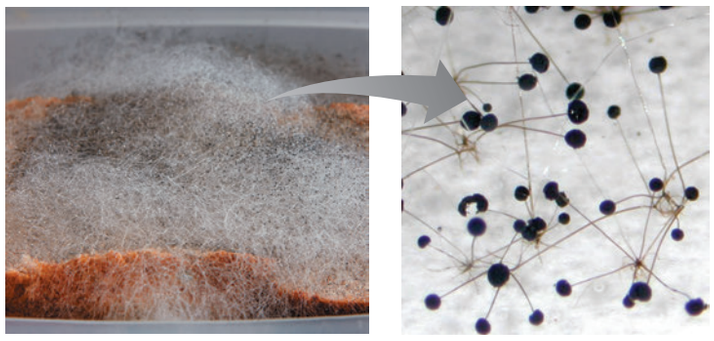


Figure 18.26 ***Mucoromycota*.** *(a)* Moldy bread from growth of the zygomycete *Rhizopus nigricans*. *(b)* Stained mycelium of *Rhizopus* showing the black aerial sporangia containing asexual spores.

Check Your Understanding

- Contrast the habitats of *Mucoromycota* and *Glomeromycota*.
- How does the fungus *Glomus* aid the acquisition of nutrients by plants?

18.13 *Ascomycota*

KEY GENERA: *Saccharomyces, Candida, Aspergillus*

The *Ascomycota*, also called the ascomycetes, are the largest and most diverse group of fungi and they range from single-celled species, such as the baker's yeast *Saccharomyces* (**Figure 18.27** and Figure 18.23), to species that grow as filaments, such as the common mold *Aspergillus* (Figure 18.21). Ascomycetes are found in aquatic and terrestrial environments and take their name from the production of *asci* (singular, ascus), cells in which two haploid nuclei from different mating types fuse to form a diploid nucleus that eventually undergoes meiosis to form haploid ascospores. In addition to ascospores, ascomycetes reproduce asexually by the production of conidia that form at the tips of specialized hyphae called *conidiophores* (Figure 18.21). Both saprophytic and pathogenic yeasts, such as *Candida albicans*, are common in nature. We focus here on the yeast *Saccharomyces* as a model ascomycete.

Saccharomyces cerevisiae

The cells of *Saccharomyces* and other single-celled ascomycetes are spherical, oval, or cylindrical, and cell division typically takes place by budding. In the budding process, a new cell forms as a small outgrowth of the old cell; the bud gradually enlarges and then separates from the parent cell (Figures 18.23 and 18.27).

Yeast cells are typically much larger than bacterial cells and can be distinguished from bacteria microscopically by their larger size and by the obvious presence of internal cell structures, such as the nucleus or cytoplasmic vacuoles (Figure 18.27). Yeasts flourish in sugar-rich habitats such as fruits, flowers, and the bark of trees. Yeasts are typically facultative aerobes, growing aerobically as well as by fermentation. Several yeasts live symbiotically with animals, especially insects, and a few species are pathogenic for animals including humans (▶ Section 34.1 and Figure 34.1*a*,*c*). The most important commercial yeasts are the baker's and brewer's yeasts, which are species of *Saccharomyces*. The yeast *S. cerevisiae* has been studied as a model eukaryote for many years and was the first eukaryote to have its genome completely sequenced (◀ Section 10.4).

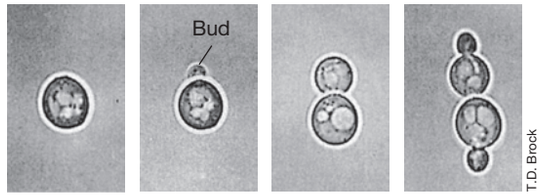


Figure 18.27 Growth by budding division in *Saccharomyces cerevisiae*. A time-lapse series of phase-contrast micrographs shows the budding division process starting from a single cell. Note the pronounced nucleus. A single cell of *S. cerevisiae* is about 6 μm in diameter.

Mating Types and Sexual Reproduction in *Saccharomyces*

The yeast *Saccharomyces* can reproduce by sexual means in which two cells fuse. Within the fused cell, called a *zygote*, meiosis occurs and ascospores are eventually formed. The life cycle of *S. cerevisiae* is depicted in **Figure 18.28**. *S. cerevisiae* can grow vegetatively (nonsexually) as either haploid or diploid cells. *S. cerevisiae* forms two different types of haploid cells called *mating types* designated α (alpha) and *a* (encoded by genes α and *a*); these are analogous to male and female gametes. The genes α and *a* regulate the production of the peptide hormones *α factor* and *a factor*, respectively, which are excreted by yeast cells during mating. The hormones bind to cells of the opposite mating type and bring about changes in their cell surfaces that enable the cells to fuse; once mating has

Mastering Microbiology
Art Activity: Figure 18.27 Life cycle of a typical ascomycete yeast, *Saccharomyces cerevisiae*

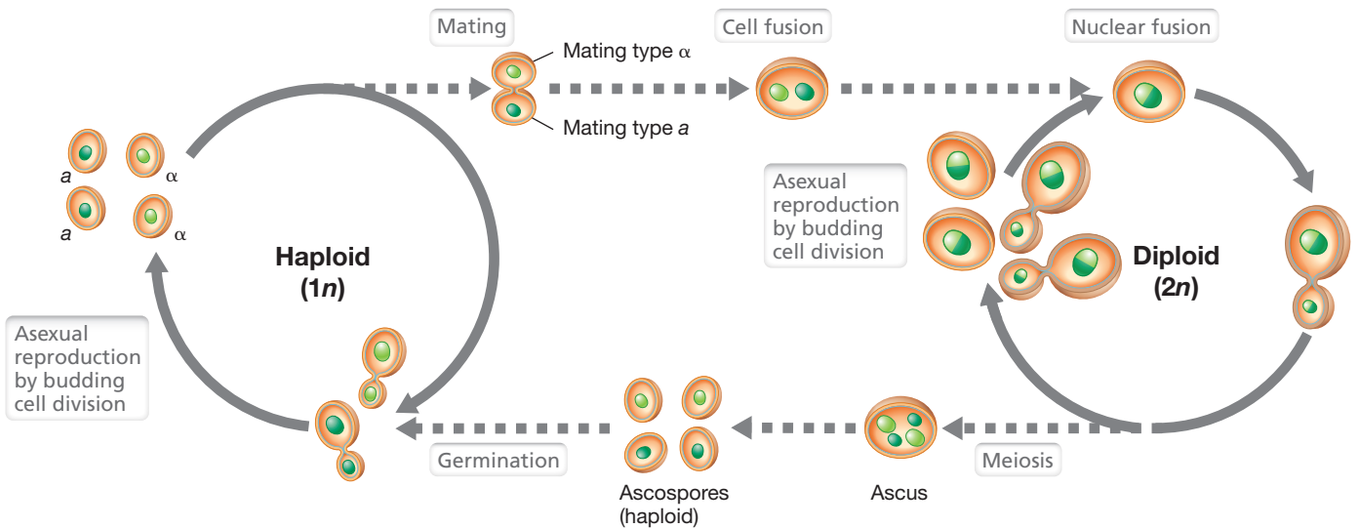
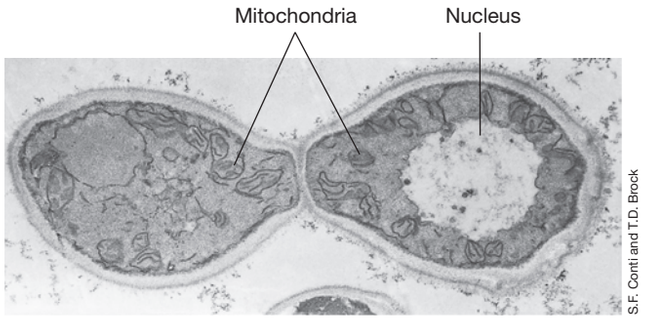
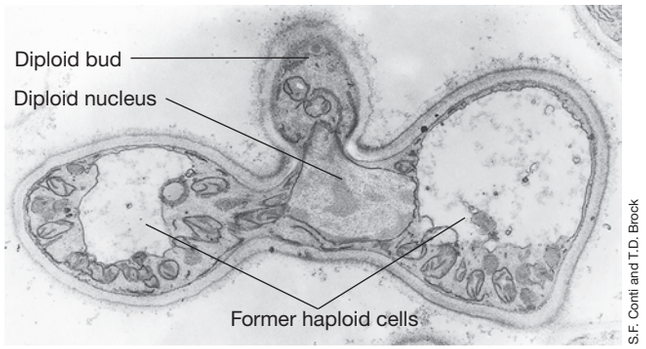


Figure 18.28 Life cycle of a typical ascomycete yeast, *Saccharomyces cerevisiae*. Vegetative growth can occur for long periods as haploid cells or as diploid cells before life cycle events (dashed lines) generate the alternate genetic form.



(a)



(b)

Figure 18.29 Electron micrographs of mating in the ascomycete yeast *Hansenula wingei*. *(a)* Two cells have fused at the point of contact. *(b)* Late stage of mating. The nuclei of the two cells have fused, and a diploid bud has formed at a right angle to the mating cells. This bud becomes the progenitor of a diploid cell line. A cell of *Hansenula* is about 10 μm in diameter.

occurred, the nuclei fuse, forming a diploid zygote (**Figure 18.29**). The zygote undergoes vegetative growth by budding, but under starvation conditions it undergoes meiosis and generates ascospores (Figure 18.28).

Haploid strains of *S. cerevisiae* are genetically predisposed to be either *a* or α but are able to switch their mating type. This switch occurs when the active mating-type gene is replaced with one of two otherwise "silent" genes, as shown in **Figure 18.30**. There is a single location on one of the *S. cerevisiae* chromosomes called the *MAT* (for *mating type*) locus, at which either gene *a* or gene α can be inserted. At this locus, the *MAT* promoter controls transcription of whichever gene is present. If gene *a* is at that locus, then the cell is mating-type *a*, whereas if gene α is at that locus, the cell is mating-type α. Elsewhere in the yeast genome are copies of genes *a* and α that are not expressed, and these are the source of the inserted gene. In the switch (Figure 18.30), the appropriate gene, *a* or α, is copied from its silent site and inserted into the *MAT* location, replacing the gene already present. The old mating-type gene is excised and discarded, and the new gene is inserted.

Whichever gene is inserted in the *MAT* locus is the one that will govern the mating type of the strain. It is thus possible for cells from a pure culture of *S. cerevisiae* derived from a single cell to mate, following a mating-type switch in one or more cells in the culture.

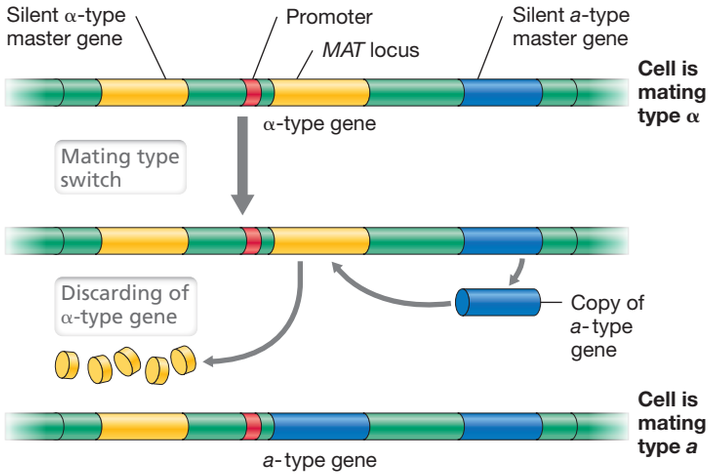


Figure 18.30 The cassette mechanism that switches an ascomycete yeast from mating type α to *a*. The cassette inserted at the *MAT* locus determines the mating type. The process shown is reversible, so type *a* can also revert to type α.

Check Your Understanding

- Are ascospores haploid or diploid cells?
- Explain how a *single* haploid cell of *Saccharomyces* can eventually yield a diploid cell.

UNIT 4

18.14 *Basidiomycota*

KEY GENERA: *Agaricus, Amanita*

Basidiomycota are a large group of fungi, with over 30,000 species described. Many are the commonly recognized mushrooms and toadstools, some of which are edible, such as the commercially grown mushroom *Agaricus*. Others, such as the mushroom *Amanita* (**Figure 18.31*a***), are highly poisonous. Other basidiomycetes include puffballs, smuts, rusts, and an important human fungal pathogen, *Cryptococcus* (▶ Sections 34.1 and 34.2 and Figure 34.1*a*). The defining characteristic of the *Basidiomycota* is the *basidium* (plural, basidia), a structure in which haploid basidiospores are formed by meiosis. The basidium, a word that means "little pedestal" (Figure 18.31*c*), gives the group its name.

Mushroom Development

During most of its existence, a mushroom fungus lives as a simple haploid mycelium, growing vegetatively in soil, leaf litter, or decaying logs. It is the sexual reproductive phase of basidiomycetes that produces the visible mushroom structure (Figures 18.22 and 18.31). In this process, mycelia of different mating types fuse, and the faster growth of the dikaryotic (two nuclei per cell) mycelium formed from that fusion overgrows and crowds out the parental haploid mycelia. Then, when environmental conditions are favorable, usually following periods of wet and cool weather, the dikaryotic mycelium develops rapidly into the fruiting body.

The mushroom fruiting body, called a *basidiocarp*, begins as a mycelium that differentiates into a small button-shaped structure

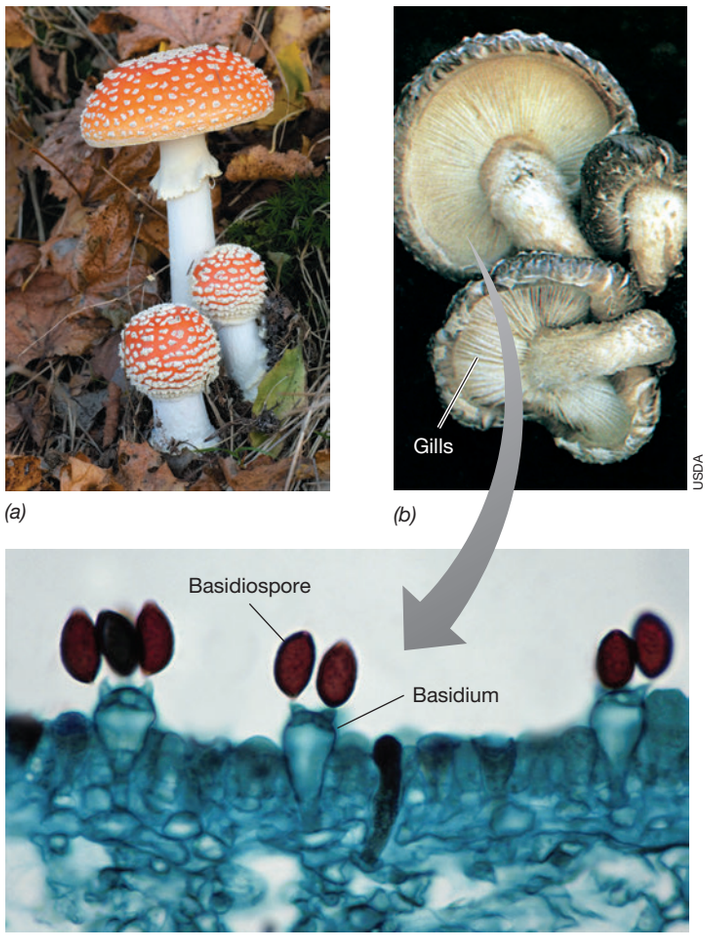


Figure 18.31 Mushrooms. *(a) Amanita*, a highly poisonous mushroom. *(b)* Gills on the underside of the mushroom fruiting body contain the spore-bearing basidia. *(c)* Light micrograph of basidia and basidiospores from the mushroom *Coprinus*.

underground that then expands into the full-grown basidiocarp that we see aboveground, the mushroom (Figures 18.22 and 18.31*a*, *b*). The dikaryotic basidia are borne on the underside of the basidiocarp on flat plates called *gills*, which are attached to the cap of the mushroom (Figure 18.31*b*, *c*). The basidia then undergo a fusion of the two nuclei, forming basidia with diploid nuclei. The two rounds of meiotic division generate four haploid nuclei in the basidia, and each of the nuclei becomes a basidiospore. The genetically distinct basidiospores can then be dispersed by wind to new habitats to begin the cycle again, germinating under favorable conditions and growing as haploid mycelia (Figure 18.22).

Pathogenic Basidiomycetes

The smuts and rusts are plant pathogenic basidiomycetes. Smuts are pathogens of cereal grains and other crops, and the genus *Ustilago* contains several species whose hosts include corn, sugarcane, wheat, and several other grains. The fungus targets the reproductive system of the plant, and in corn, smut causes the developing kernels to form massive tumorlike kernels that give the cob a swollen and burned appearance.

Rust fungi attack rapidly growing plant tissues such as emerging shoots, leaves, and fruits. Rust fungi of the genus *Puccinia* can infect any of a wide variety of plants whereas other rust fungi are more host-restricted. Some common rusts include stem rust of wheat and white pine blister rust, a disease of several pine species that can trigger branch dieback and even death of the entire tree. Pine trees weakened by rust fungi are typically more susceptible to insect attack such as from infestations of the pine bark beetle, a highly destructive pest that has destroyed major stands of various pine species in the western United States and central Europe in recent years. Hence, even if the rust fungus itself is not lethal, the damage it does to the overall vigor of the tree can set the stage for lethal attack by a secondary biological agent or even by strong winds.

We now move on to the final part of this chapter where we will explore the world of algae, phototrophic eukaryotes widespread in nature.

Check Your Understanding

- What are some distinguishing features of basidiospores and of zoospores (Section 18.10)?
- Are basidiospores haploid or diploid?

IV • *Archaeplastida*

The *Archaeplastida* are a diverse phototrophic lineage that includes plants as well as several groups of algae. Species of red and green algae can be microscopic or macroscopic and are found worldwide, primarily in aquatic habitats.

We conclude our tour of eukaryotic microbial diversity with the kingdom *Archaeplastida*, a large group that includes both red and green algae as well as land plants. As we have previously discussed, only the red and green algae originated from primary endosymbiotic events, whereas other protists containing chloroplasts were the result of secondary endosymbioses (Figures 18.2 and 18.3). Here we focus on the red and green algae, a large and diverse group of eukaryotic organisms that contain chlorophylls and carry out oxygenic photosynthesis (◀ Section 14.6).

18.15 Red Algae

KEY GENERA: *Polysiphonia, Cyanidium, Galdiera*

The red algae, also called *rhodophytes*, mainly inhabit the marine environment, but a few species are found in freshwater and terrestrial habitats. Both unicellular and multicellular species are known, and some of the latter are macroscopic.

Major Properties of Red Algae

Red algae are phototrophic and contain chlorophyll *a*; their chloroplasts lack chlorophyll *b* but contain phycobiliproteins, the major light-harvesting pigments of the cyanobacteria (◀ Section 14.4). The reddish color of many red algae (**Figure 18.32**) results from phycoerythrin, an accessory pigment that masks the green color of chlorophyll. This pigment is present along with phycocyanin and

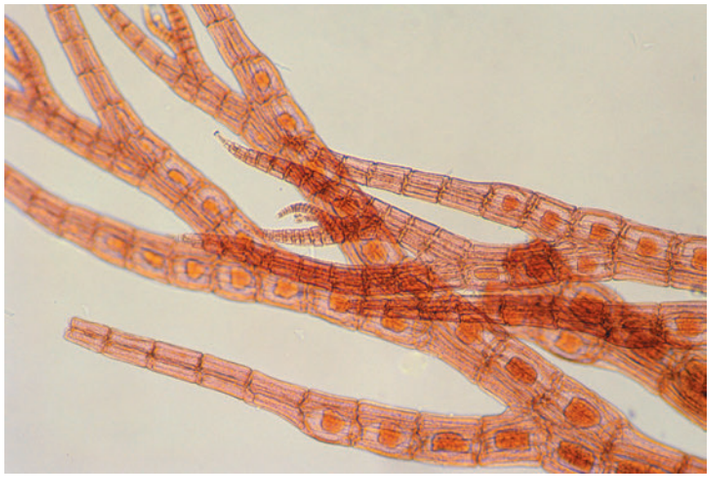
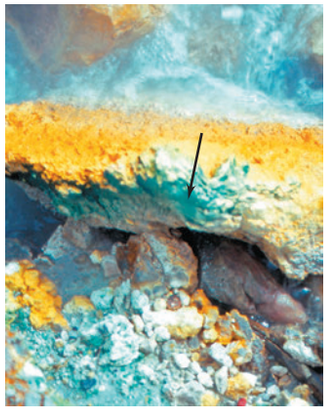
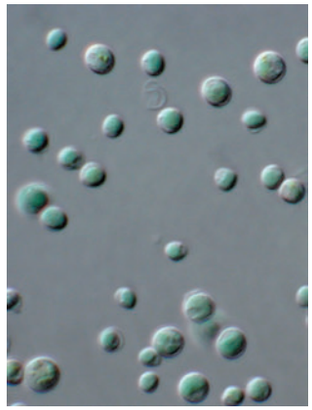


Figure 18.32 ***Polysiphonia*, a filamentous marine red alga.** Light micrograph. *Polysiphonia* grows attached to the surfaces of marine plants. Cells are about 150 μm wide.



Christine Oesterhelt and Gerald Schönknecht

(a)



Richard W. Castenholz

(b)

Figure 18.33 ***Galdieria*, a unicellular red alga.** *(a) Galdieria* inhabits acidic and sulfidic hot springs where it often grows attached to mineral debris as shown here (arrow). *(b)* Cells of *Galdieria* are about 25 μm in diameter and are more blue-green than red in color because the phototroph contains mainly phycocyanin rather than phycoerythrin as its phycobilin (◀ Section 14.4).

allophycocyanin in structures called *phycobilisomes*, the light-harvesting (antenna) components of cyanobacteria. At greater depths in aquatic habitats, where less light penetrates, cells compensate by producing more phycoerythrin and are a darker red, whereas shallow-dwelling species often have less phycoerythrin and can be green in color (see Figure 18.33).

Most species of red algae inhabit marine environments and are multicellular and lack flagella. Some are "seaweeds" and are the source of agar, the solidifying agent used in bacteriological culture media, and carrageenans, thickening and stabilizing agents used in the food industry. Other species of red algae, such as the genus *Porphyra*, are harvested, dried, and used as a wrap in sushi. Different species of red algae are filamentous, leafy, or, if they deposit calcium carbonate, *coralline* (coral-like) in morphology. Coralline red algae play an important role in the development of coral reefs and help strengthen reefs against wave damage (▶ Section 23.13).

Polysiphonia (Figure 18.32) is a genus of filamentous and branched red algae found worldwide in marine environments. The organism is found primarily near shore where the cells grow attached to rocks, other algae, and man-made surfaces such as jetties, concrete abutments, retaining walls, and docks. Nearly 200 species of this genus are recognized, and the organism undergoes a complex life cycle in which an alternation of generations occurs. In this cycle, haploid male and female gametes released from a diploid multicellular organism develop into haploid male and female multicellular organisms. The male alga then releases haploid "sperm" cells that fuse with a specialized reproductive structure on the female alga to yield a diploid zygote; the latter develops a multicellular form and, following meiosis, releases male and female gametes to complete the life cycle.

Cyanidium, Galdieria, and Relatives

In addition to multicellular red algae like *Polysiphonia* (Figure 18.32), unicellular species are also known. One such group, members of the *Cyanidiales* that include the genera *Cyanidium*, *Cyanidioschyzon*, and *Galdieria* (**Figure 18.33**), live in hot, acidic and metal-rich hot springs at temperatures from 30 to 60°C and acidic pH (0.5 to 4.0); under these extreme conditions, no other phototrophic microorganisms (including anoxygenic phototrophs) can exist. The unicellular red algae are unusual in other ways as well. For example, cells of *Cyanidioschyzon merolae* are unusually small (1–2 μm in diameter) for eukaryotes, and the genome of this species, approximately 16.5 Mbp, is one of the smallest genomes known for a phototrophic eukaryote.

Molecular analyses of the *Galdieria* genome have revealed the surprising result that this *eukaryotic* phototroph contains at least 75 genes acquired by horizontal transfer from various *prokaryotic* sources. Some of the key genes transferred encode protections against salt stress and metal toxicity and the construction of a toughened cytoplasmic membrane to withstand the heat and acidity of the *Galdieria* habitat (Figure 18.33*a*). The ability of *Galdieria* to grow in darkness—an unusual feature for an alga—has also been linked to horizontally transferred genes, in particular to genes encoding transport systems for various organic compounds. Although these genetic transfers are unusual in the sense that donor and recipient belong to different phylogenetic domains, they once again underscore the importance of horizontal gene transfer in molding microbial genomes (◀ Section 13.9).

Check Your Understanding

- What traits link cyanobacteria and red algae?
- What physiological properties would be necessary for *Galdieria* to live in its habitat?

18.16 Green Algae

KEY GENERA: *Chlamydomonas, Volvox*

The green algae, also called *chlorophytes*, have chloroplasts containing chlorophylls *a* and *b*, which give them their characteristic green color, but they lack phycobiliproteins and so do not develop the red

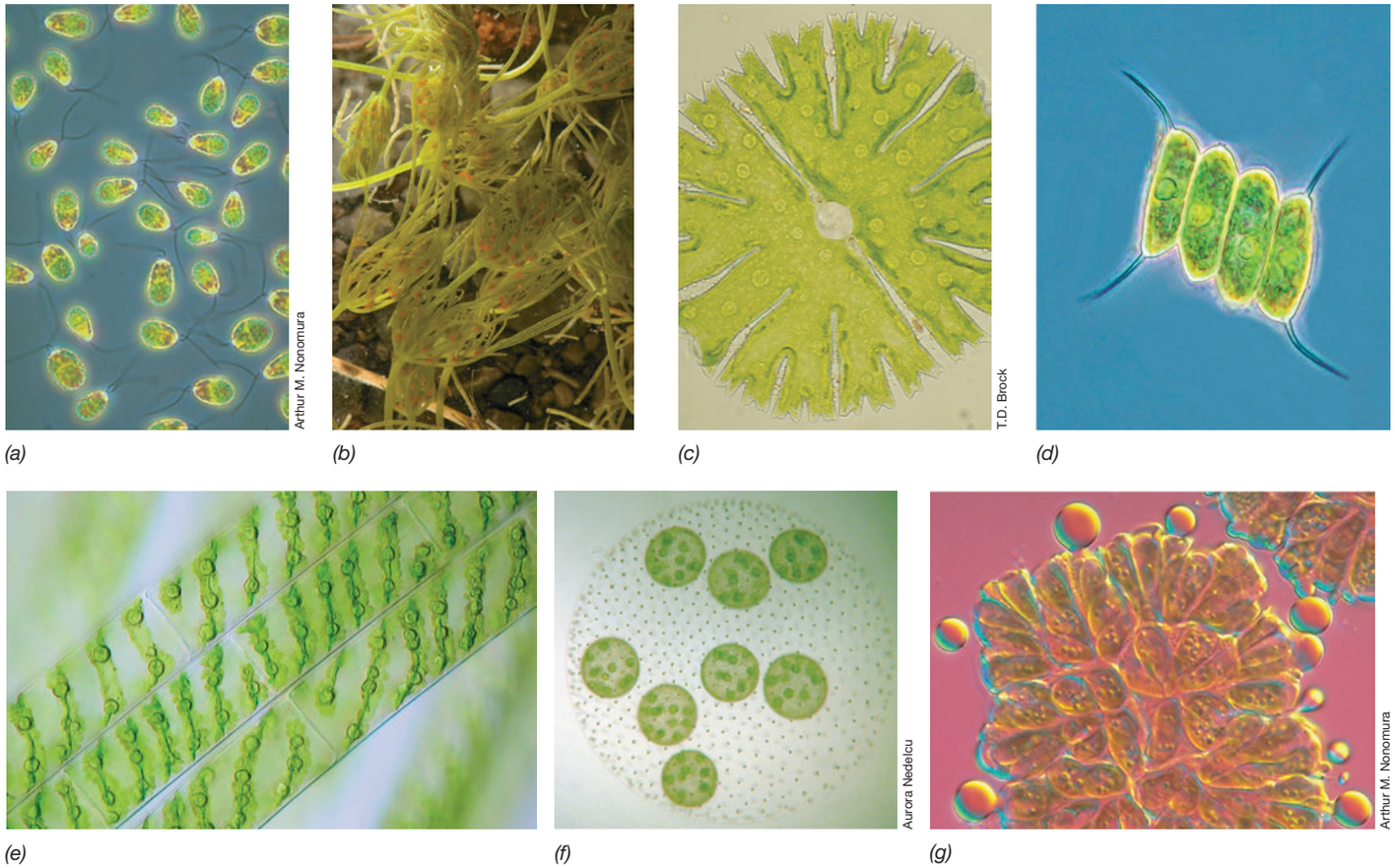


Figure 18.34 **Green algae.** *(a)* A single-celled, flagellated green alga, *Dunaliella*. A cell is about 5 μm wide. *(b)* The plantlike green alga *Chara*. *(c) Micrasterias*. This single multilobed cell is about 100 μm wide. *(d) Scenedesmus*, showing packets of four cells each. *(e) Spirogyra*, a filamentous alga with cells about 20 μm wide. Note the green spiral-shaped chloroplasts. *(f) Volvox carteri* colony with eight daughter colonies. *(g)* The petroleum-producing green alga *Botryococcus braunii*. Note the excreted oil droplets surrounding the cell.

or blue-green colors of red algae (Figures 18.32 and 18.33). In the composition of their photosynthetic pigments, green algae are similar to plants and are closely related to plants phylogenetically. There are two main groups of green algae, the *chlorophytes*, examples of which are the microscopic *Chlamydomonas* and *Dunaliella* (**Figure 18.34*a***), and the charophyceans such as *Chara* (Figure 18.34*b*), macroscopic organisms that often resemble land plants and are actually most closely related to land plants.

Most green algae inhabit freshwater while others are found in moist soil or growing in snow, to which they impart a pink color (◀ Figure 4.23). Other green algae live with fungi in a symbiotic relationship to form lichens (▶ Section 23.1). The morphology of chlorophytes ranges from unicellular (Figure 18.34*a*, *c*), to filamentous (with individual cells arranged end to end, Figure 18.34*e*), to **colonial** (as aggregates of cells, Figure 18.34*f*). Even multicellular species exist, an example of which is the seaweed *Ulva*. Most green algae have a complex life cycle, with both sexual and asexual reproductive stages.

Very Small Green Algae and Colonial Green Algae

One of the smallest eukaryotes known is the green alga *Ostreococcus tauri*, a common unicellular species of marine phytoplankton (▶ Section 20.11 and Figure 20.27*b*). Cells of *O. tauri* have a diameter of approximately 2 μm, and the organism contains the smallest genome of any known phototrophic eukaryote, approximately 12.6 Mbp. *Ostreococcus* has thus provided a model organism for research into the evolution of genome reduction and specialization in eukaryotes.

At the colonial level of organization in green algae is *Volvox* (Figure 18.34*f*). This alga forms colonies composed of several hundred flagellated cells, some of which are motile and primarily carry out photosynthesis, while others specialize in reproduction. Cells in a *Volvox* colony are interconnected by thin strands of cytoplasm that allow the entire colony to swim in a coordinated fashion. *Volvox* has been a long-term model for research on the genetic mechanisms controlling multicellularity and the distribution of functions among cells in multicellular organisms.

Some colonial green algae have potential as sources of biofuels. For example, the colonial green alga *Botryococcus braunii* excretes long-chain (C_{30}–C_{36}) hydrocarbons that have the consistency of crude oil (Figure 18.34*g*). About 30% of the *B. braunii* cell dry weight consists of this petroleum, and there has been heightened interest in using this and other oil-producing algae as renewable sources of petroleum. Evidence from biomarker studies has shown that some

(a)

(b)

Figure 18.35 Endolithic phototrophs. *(a)* Photograph of a limestone rock from the McMurdo Dry Valleys region of Antarctica broken open to show the layer of endolithic green algae. *(b)* Light micrograph of cells of the green alga *Trebouxia*, a widespread endolithic alga in Antarctica.

known petroleum reserves originated from green algae such as *B. braunii* that settled in lake beds in ancient times. Hence, if the scale-up challenges for commercial algal petroleum production could ever be met, it is possible that some fraction of the world's oil supply could someday come from photosynthesis by green algae.

Endolithic Phototrophs

Some green algae grow inside rocks. These *endolithic* (*endo* means "inside") phototrophs inhabit porous rocks, such as those containing quartz, and are typically found in layers near the rock surface (**Figure 18.35*a***). Endolithic phototrophic communities are most common in dry environments such as deserts or cold, dry environments such as Antarctica. For example, in the McMurdo Dry Valleys of Antarctica, where temperatures and humidity are extremely low (◀ Figure 4.22*d*, *e*), life within a rock has its advantages. Rocks in these harsh environments are heated by the sun, and water from snowmelt can be absorbed and retained for relatively long periods, supplying moisture needed for growth. Moreover, water absorbed by a porous rock makes the rock more transparent, thus funneling more light to the algal layers.

A wide variety of phototrophs can form endolithic communities, including cyanobacteria and various green algae (Figure 18.35*b*). In addition to being free-living phototrophs, green algae and cyanobacteria coexist with fungi in endolithic lichen communities (▶ Section 23.1 for discussion of the lichen symbiosis). Metabolism and growth of these internal rock microbial communities slowly weathers the rock, allowing gaps to develop where water can enter, freeze and thaw, and eventually crack the rock, producing new habitats for microbial colonization. The decomposing rock also forms a crude soil that can support development of plant and animal communities in environments where conditions (temperature, moisture, and so on) permit.

Check Your Understanding

- What phototrophic properties link green algae and plants?
- What is unusual about the green algae *Ostreococcus*, *Volvox*, and *Botryococcus*?
- What are endolithic phototrophs?

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CHAPTER REVIEW

I • Organelles and Phylogeny of Microbial *Eukarya*

18.1 Key metabolic organelles of eukaryotes are the chloroplast, which functions in photosynthesis, and the mitochondrion, which functions in respiration. These organelles were originally *Bacteria* that established permanent residence inside other cells (endosymbiosis).

Q Distinguish between a primary and a secondary endosymbiosis. Which groups of protists are derived from which form of endosymbiosis?

18.2 The modern phylogeny of eukaryotes suggests a major radiation of eukaryotic diversity emerging at some time following symbiotic events that led to the mitochondrion. The latter provided eukaryotic cells with a chemotrophic energy-generating powerhouse that is present today in nearly all eukaryotic cells.

Q What are the five major supergroups within the *Eukarya*?

II • Protists

18.3 Diplomonads such as *Giardia* are unicellular, flagellated, nonphototrophic protists. Parabasalids such as *Trichomonas* are human pathogens and contain huge genomes that lack introns. Euglenids and kinetoplastids are unicellular, flagellated protists. Some are phototrophic. This group includes some important human pathogens, such as *Trypanosoma*, and some well-studied nonpathogens, such as *Euglena*.

Q What are hydrogenosomes and what is their function?

18.4 Three groups make up the *Alveolata*: ciliates, dinoflagellates, and apicomplexans. Most ciliates and dinoflagellates are free-living organisms, whereas apicomplexans are obligate parasites of animals.

Q How are alveolates as a group characterized?

18.5 *Stramenopiles* are protists that bear a flagellum with fine, hairlike extensions. They include oomycetes, diatoms, and brown and golden algae.

Q In terms of their photosynthetic pigments, how are brown and golden algae similar?

18.6 The *Rhizaria* include diverse protists such as the phototrophic chlorarachniophytes and foraminiferans, as well as radiolarians, which are chemoorganotrophs.

Q How can *Rhizaria* be distinguished from other protists in their environment?

18.7 Haptophytes include the coccolithophores, unicellular phototrophic microbes that form intricate calcite exoskeletons. Coccolithophores such as *Emiliania huxleyi* are abundant in the oceans and important to global climate because they remove carbon from the atmosphere and deposit it as calcite in deep ocean sediments.

Q Describe the life cycle of *E. huxleyi* and explain why this life cycle can make it difficult to identify species of coccolithophores in the environment.

18.8 *Amoebozoa* are protists that use pseudopodia for movement and feeding. Within *Amoebozoa* are gymnamoebas, entamoebas, and slime molds. Plasmodial slime molds form masses of motile protoplasm, whereas cellular slime molds are individual cells that aggregate to form fruiting bodies from which spores are released.

Q Where are gymnamoebas found in nature? How do they feed?

III • Fungi

18.9 Fungi include the molds, mushrooms, and yeasts. Other than phylogeny, fungi primarily differ from protists by their rigid chitin-containing cell wall, production of spores, and the fact that most fungal species are nonmotile.

Q How can fungi benefit both plants and humans?

18.10 A variety of sexual spores are produced by fungi, including ascospores, basidiospores, and zygospores. From a phylogenetic standpoint, fungi are the closest relatives of animals.

Q List the different types of sexual spores of fungi. Are conidia sexual or asexual spores?

18.11 *Chytridiomycota* and *Microsporidia* are basal to all other known fungal groups in the fungal phylogeny. Some chytrids are amphibian pathogens, while microsporidians are parasites of a variety of animals.

Q In what way do chytrids and microsporidians differ from other fungi?

18.12 *Mucoromycota* form coenocytic hyphae and undergo both asexual and sexual reproduction, and the common bread mold *Rhizopus* is a good example. *Glomeromycota* are fungi that form endomycorrhizal associations with plants.

Q What is the major feature of the ecology of glomeromycetes?

18.13 The *Ascomycota* are a large and diverse group of mostly saprophytic fungi. Some, such as *Candida albicans*, can be pathogenic in humans. There are two mating types in the yeast *Saccharomyces cerevisiae*, and yeast cells can convert from one type to the other by a genetic switch mechanism.

Q How is the mating type of a yeast cell determined?

18.14 *Basidiomycota* include the mushrooms, puffballs, smuts, and rusts. Basidiomycetes undergo both vegetative (asexual) reproduction as haploid mycelia and sexual reproduction via fusion of mating types and formation of haploid basidiospores.

Q What morphological feature unites the *Basidiomycota*, and where is this feature found?

IV • *Archaeplastida*

18.15 Red algae are mostly marine and range from unicellular to multicellular. Their reddish color is due to the pigment phycoerythrin, a key cyanobacterial pigment, present in their chloroplast.

Q In what kinds of habitats would one likely find red algae?

18.16 Green algae are common in aquatic environments and can be unicellular, filamentous, colonial, or multicellular. A unicellular green alga, *Ostreococcus*, has the smallest genome known for a phototrophic eukaryote, while the green alga *Volvox* is a model colonial phototroph.

Q What traits link green algae and plants? What traits link green algae and red algae?

APPLICATION QUESTIONS

1. Explain the differences between mitochondria, chloroplasts, hydrogenosomes, and mitosomes. Explain the evolutionary process that gave rise to hydrogenosomes and mitosomes. Explain why the chloroplasts of red and green algae have two membranes, while those of many other photosynthetic protists have four membranes.
2. Considering all of the groups of microbes covered in this chapter, which of them seem most similar to prokaryotic cells in cell structure? Discuss at least two lines of evidence that show that these microbes are not prokaryotic cells.

CHAPTER GLOSSARY

Algae an informal term for phototrophic eukaryotes other than plants; algae are polyphyletic, being found in diverse groups of *Eukarya* as a result of secondary endosymbiosis

Chitin a polymer of *N*-acetylglucosamine commonly found in the cell walls of fungi

Ciliate any protist characterized in part by rapid motility driven by numerous short appendages called cilia

Coenocytic the presence of multiple nuclei in fungal hyphae without septa

Colonial the growth form of certain protists and green algae in which several cells live together and cooperate for feeding, motility, or reproduction; an early form of multicellularity

Conidia the asexual spores of fungi

Fungi nonphototrophic eukaryotic microorganisms with rigid cell walls made of chitin

Hydrogenosomes organelles that produce ATP through fermentation reactions resulting in the production of H_2 and acetate

Mushroom the aboveground fruiting body, or basidiocarp, of basidiomycete fungi

Nucleomorphs remnant nuclei found in certain algae; derived from an ancestral algal endosymbiont and associated with chloroplasts acquired by secondary endosymbiosis

Phagocytosis a mechanism for ingesting particulate material in which a portion of the cytoplasmic membrane surrounds the particle and brings it into the cell; for phagocytes of the immune system, the process of engulfing and killing foreign particles and cells

Primary endosymbiosis the endosymbiotic acquisition by a eukaryotic host cell of a respiring bacterium or a phototrophic cyanobacterium, followed by evolution of the endosymbiont into mitochondria or chloroplasts

Protist an informal term used to describe eukaryotic microorganisms that are not plants, animals, or fungi; can be heterotrophic or phototrophic, unicellular or multicellular

Secondary endosymbiosis the endosymbiotic acquisition by a mitochondrion-containing eukaryotic cell of a red or green algal cell, which itself contains a chloroplast derived from primary endosymbiosis

Slime mold a nonphototrophic protist that lacks cell walls and that aggregates to form fruiting structures (cellular slime molds) or masses of protoplasm (acellular slime molds)

Yeast the single-celled growth form of various fungi

19 Taking the Measure of Microbial Systems

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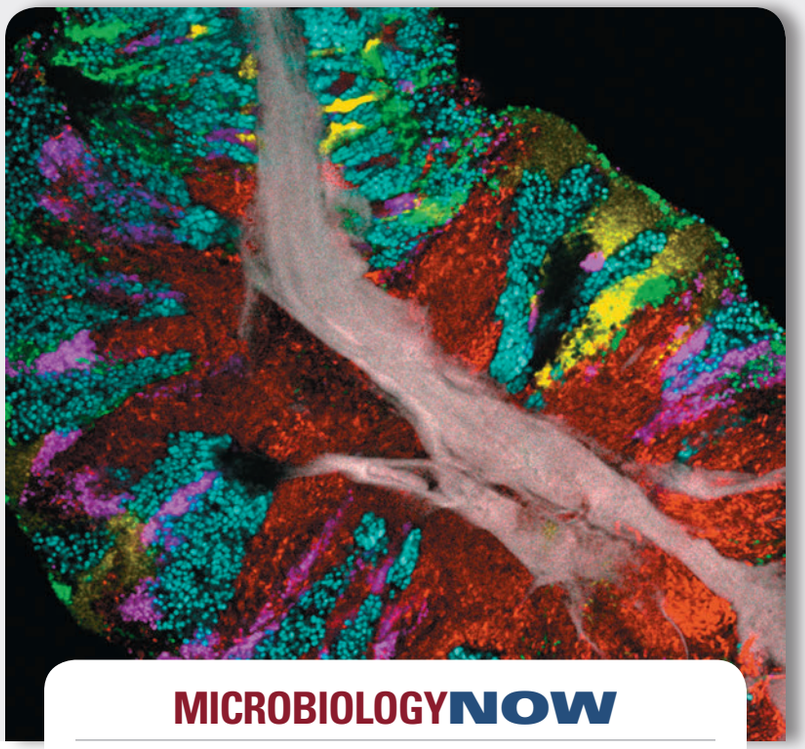


MICROBIOLOGYNOW

Touring Microbial Biogeography Using Combinatorial Imaging

Virtually all microorganisms exist as parts of complex communities that interact through metabolic cooperation. Since complex metabolic interactions are not easily resolved, the microscope is an essential tool for first identifying possible cooperation based on colocalization of different species. However, even the simplest of microbial communities is composed of tens if not hundreds of different species. How it is possible to visualize the distribution of individual species in such a mixture?

As we will see in this chapter, microscopic identification of species commonly uses fluorescence in situ hybridization (FISH), a technique in which cells of individual species are identified by hybridization of fluorescent DNA probes to ribosomal RNA sequences unique to each species. However, the method can visualize only a few different species simultaneously, being limited by the number of dyes that fluoresce in different colors. A variation on standard FISH technology that circumvents this limitation is called CLASI-FISH (*c*ombinatorial *l*abeling *a*nd *s*pectral *i*maging–FISH), which can image more than 100 different species simultaneously. CLASI-FISH hybridizes each cell with a combination of probes specific for that species but labeled with different fluorescent dyes, giving each cell a unique fluorescent spectral signature. Since the resulting fluorescence at each wavelength is a linear combination of emissions from each fluorescent dye, statistical analysis can determine what combination of dyes produced the emission spectrum and therefore identify the contributing species.

The photo shown here was taken from a scraping of the human tongue (the specimen is about 140 µm in diameter, left to right), showing a remarkable organization of microbial species that almost certainly reflects metabolic interactions that can now be further explored using the powerful tools we discuss in this chapter. Brown in the photo is human tissue; the bacteria are: red, *Actinomyces* spp.; green, *Streptococcus* spp.; blue, *Rothia* spp.; yellow, *Neisseria* spp.; and magenta, *Veillonella* spp.

Source: Welch, J.L.M., Dewhirst, F.E., and Borisy, G.G. 2019. Biogeography of the oral microbome: The site-specialist hypothesis. *Annu. Rev. Microbiol.* 73. doi: 10.1146/annurev-micro-090817-062503.

We now begin a new unit devoted to microorganisms in their natural habitats. We learned in Chapter 1 that microbial communities consist of cell populations living in association with other populations in nature. The science of **microbial ecology** is focused on how microbial populations assemble to form communities and how these communities interact with each other and their environments.

The major components of microbial ecology are *biodiversity* and *microbial activity*. To study biodiversity, microbial ecologists must identify and quantify microorganisms in their habitats. Knowing how to do this is often helpful for isolating organisms of interest as well, which is another goal of microbial ecology. To study microbial activity, microbial ecologists must measure the metabolic processes that microorganisms carry out in their habitats. In this chapter we consider modern methods for assessing microbial diversity and activity. Chapter 20 will outline the basic principles of microbial ecology and examine the types of environments that microorganisms inhabit. Chapters 21–24 will complete our coverage of microbial ecology by exploring nutrient cycles, applied microbiology, and the role microbes play in symbiotic associations with other life forms, including humans.

We begin with the microbial ecologist's toolbox, which includes a collection of powerful tools for dissecting the structure and function of microbial communities in relation to their natural habitats.

I • Culture-Dependent Analyses of Microbial Communities

Major advances in molecular, microscopic, and analytical methods have revealed important properties of microorganisms as they exist in nature. However, to fully characterize a microbe, there is no substitute for isolating it in pure culture. In addition to traditional isolation methods, single-cell and high-throughput cultivation methods have greatly advanced success in culturing the "uncultured majority" of microorganisms.

The vast majority of microorganisms, more than 99% of all species by most estimates, have never been grown in laboratory cultures. To date, about 17,000 species of *Archaea* and *Bacteria* have been formally described. By contrast, culture-independent molecular diversity surveys (Sections 19.4–19.8) indicate that many millions—possibly even trillions—of species exist in nature and have yet to be cultured and formally described. This recognition has stimulated the development of new methods for isolating microbes from nature in order to establish pure cultures. Even though a host of sophisticated methods are available for studying microbes in their native environments, culturing a microorganism remains the only way to fully characterize its properties and predict its impact on its environment.

In the first part of this chapter we cover the enrichment approach, a time-honored and useful method for isolating microorganisms from nature, but a method with significant limitations. Enrichment is based on culturing in a selective growth medium, and thus the tools and methods used in this approach are considered *culture-dependent* analyses. As we will see, considerable progress has been made in culturing the more elusive microorganisms in natural populations by using robotics and associated microfabrication technology to establish large numbers of enrichment cultures that can be monitored simultaneously. In the second and third parts of this chapter we consider *culture-independent* analyses, techniques that can tell us much about the structure and function of microbial communities in the absence of actual laboratory cultures. In the final part of this chapter, we consider methods for measuring microbial activities in nature and linking them to specific organisms. Collectively, these methods allow the microbial ecologist to ask both "Who is there?" and "What are they doing?"

19.1 Enrichment Culture Microbiology

For an **enrichment culture**, a medium and a set of incubation conditions are established that are *selective* for the desired organism and *counterselective* for undesired organisms. Effective enrichment cultures duplicate as closely as possible the resources and conditions of a particular ecological niche. Hundreds of different enrichment strategies have been devised, and Tables 19.1 and 19.2 summarize some simple and direct ones.

Inocula

Successful enrichment requires an appropriate inoculum containing the organism of interest. Thus, the making of an enrichment culture

UNIT 5

TABLE 19.1 Some enrichment culture methods for phototrophic bacteria (main C source, CO_2)

Incubation condition	Organisms enriched	Inoculum
Incubation in air		
N_2 as nitrogen source	Cyanobacteria	Pond or lake water; sulfide-rich muds; stagnant water; raw sewage; moist, decomposing leaf litter; moist soil exposed to light
NO_{3-} as nitrogen source, 55°C	Thermophilic cyanobacteria	Hot spring microbial mat
Anoxic incubation		
H_2 or organic acids; N_2 as sole nitrogen source	Purple nonsulfur bacteria, heliobacteria	Same as above plus hypolimnetic lake water (▶ Section 20.9); pasteurized soil (heliobacteria); microbial mats for thermophilic species
H_2S as electron donor	Purple and green sulfur bacteria	
Fe^{2+}, NO_2^- as electron donor	Purple bacteria	

TABLE 19.2 Some enrichment culture methods for nonphototrophic bacteria[a]

Electron acceptor	Electron donor and nitrogen source	Typical organisms enriched	Inoculum source
Chemoorganotrophs			
Aerobes			
O_2	Lactate, benzoate + NH_4^+	*Mycobacterium*, *Nocardia*, *Pseudomonas*	Soil, mud, lake sediments, decaying vegetation
	Hydrocarbons, toluene + NH_4^+	*Mycobacterium*, *Nocardia*, *Pseudomonas*	
	Mannitol or benzoate, N_2 as nitrogen	*Azotobacter*	
	Ethanol + yeast extract, pH 6.0	*Acetobacter*, *Gluconobacter*	
	Cellulose + NH_4^+	*Cytophaga*, *Sporocytophaga*	
	CH_4 + NO_3^-	*Methylobacter*, *Methylomicrobium*	Lake sediments, thermocline of stratified lake
Chemoorganotrophs			
Anaerobic respiration and fermentation			
NO_3^-	Organic acids + NO_3^-	*Pseudomonas* (denitrifying species)	Soil, mud, lake sediments, groundwater seeps, sediments, pasteurized inoculum (80°C for 15 min) for *Bacillus* enrichments
	Yeast extract + NO_3^-	*Bacillus* (denitrifying species)	
ClO_3^-	Acetate	Various (per)chlorate-reducing *Bacteria* and *Archaea*	Contaminated groundwater, rivers, soils, sediments, wastewater treatment systems
PCE	Acetate + H_2 + NH_4^+	*Dehalococcoides* spp.	Tetrachloroethene (PCE)-polluted groundwater
SO_4^{2-}	Lactate, ethanol, organic acids	*Desulfovibrio*, *Desulfotomaculum*	Soil, mud, sediments, sewage sludge
	Acetate, propionate, butyrate	Fatty acid–oxidizing sulfate reducers	
S^0	Acetate, ethanol	*Desulfuromonas*	Soil, mud, sediments, sewage sludge
None	Acetate	*Methanosarcina*, *Methanosaeta*	Mud, lake sediments, rotting plant or animal material, dairy products (lactic and propionic acid bacteria); rumen or intestinal contents (enteric bacteria); sewage sludge; soil, pasteurized inoculum for *Clostridium* enrichments
	Glutamate or histidine	Proteolytic *Clostridium* species	
	Starch + NH_4^+	*Clostridium* spp.	
	Starch + N_2 as nitrogen source	*Clostridium pasteurianum*	
	Lactate + yeast extract	*Veillonella* spp.	
	Glucose + yeast extract (pH 5)	Lactic acid bacteria (*Lactobacillus*)	
	Succinate + NaCl	*Propionigenium*	
	Oxalate	*Oxalobacter*	
Chemolithotrophs			
Aerobes			
O_2	NH_4^+	Ammonia-oxidizing *Bacteria* (*Nitrosomonas*) and *Archaea* (*Nitrosopumilus*), comammox[b] bacteria	Lake sediments, thermocline of stratified lake
	NO_2^-	Nitrite-oxidizing *Bacteria* (*Nitrobacter*, *Nitrospira*)	
	H_2	Hydrogen bacteria	
	H_2S, S^0, $S_2O_3^{2-}$	*Thiobacillus* spp.	
	Fe^{2+}, low pH	*Acidithiobacillus ferrooxidans*	Acid mine drainage
	Fe^{2+}, neutral pH	*Acidovorax*, *Zetaproteobacteria*	Groundwater seeps and geothermal seeps
Chemolithotrophs			
Anaerobes			
Fe^{+3}	Acetate	*Geobacter*, *Geospirillum*	Groundwater
NO_3^-	Fe^{+2} + NO_3^-	Iron-oxidizing chemolithotrophs	Groundwater, groundwater seeps, sediments
	S^0, $S_2O_3^{2-}$	*Thiobacillus denitrificans*	Mud, lake sediments, soil
	H_2	*Paracoccus denitrificans*	
CO_2	H_2 + NH_4^+	Methanogens (chemolithotrophic species only), homoacetogens	Mud, lake sediments, rotting plant or animal material, rumen or intestinal contents, sewage sludge

[a]All media must contain an assortment of mineral salts including N, P, S, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, and other trace elements (◀ Sections 4.1, 4.2). Certain organisms may have requirements for vitamins or other growth factors. This table is a general overview of enrichment methods focused on substrates and atmospheric conditions and has not considered the effects that temperature, pH and salinity can have on enrichment culture outcomes.
[b]Comammox is the complete oxidation of ammonia all the way to nitrate (▶ Section 20.3).

begins with collecting a sample from the appropriate habitat to serve as the inoculum (Tables 19.1 and 19.2). Enrichment cultures are established by placing the inoculum into selective media and incubating under specific conditions. In this way, many common microbes can be isolated. For example, the great Dutch microbiologist Martinus Beijerinck, who conceptualized the enrichment culture technique (◀ Section 1.13), used enrichment cultures to isolate the nitrogen-fixing bacterium *Azotobacter* (**Figure 19.1**). Because *Azotobacter* is a rapidly growing bacterium capable of N_2 fixation in air (◀ Sections 3.12 and 15.9), enrichment using media devoid of fixed nitrogen (for example, ammonia or nitrate) and incubation in air selects strongly for this bacterium and its close relatives. Non-nitrogen-fixing bacteria and anaerobic nitrogen-fixing bacteria are counterselected in this technique.

Enrichment Culture Outcomes

For success with enrichment cultures, attention to both the culture medium and the incubation conditions is important. That is, the *resources* (nutrients) and *conditions* (temperature, pH, osmotic considerations, aerobic or anaerobic, and the like) must closely mimic those of the habitat to offer the best chance of obtaining the organism of interest (▶ Table 20.1).

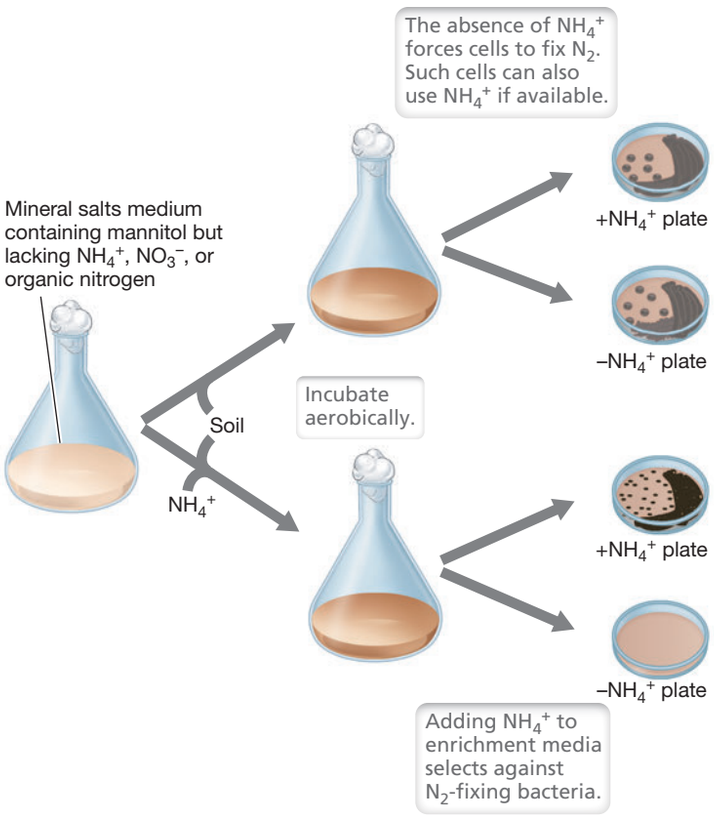


Figure 19.1 The isolation of *Azotobacter*. Selection for aerobic nitrogen-fixing bacteria usually results in the isolation of *Azotobacter* or its relatives. The selective basis of the enrichment is the absence of fixed nitrogen (NH_4^+ in this case) in the culture medium in the upper flask. Thus the medium *selects* from the microbial community those species that can fix N_2 aerobically, of which *Azotobacter* is one of the most rapidly growing. See Section 1.13 and Figure 1.37 for more on the historical importance of *Azotobacter*.

Some enrichment cultures yield nothing. This may be because an organism capable of growing under the enrichment conditions established is absent from the habitat. Alternatively, even though the organism of interest exists in the habitat sampled, the resources and conditions employed in the enrichment may simply be incompatible with its growth. Thus, enrichment cultures can yield a firm *positive* conclusion (that is, that an organism with certain capacities exists in a particular environment because it was enriched) but never a firm *negative* conclusion (that such an organism is not present because the enrichment failed). Moreover, the isolation of the desired organism from an enrichment culture says nothing about the abundance or ecological significance of the organism in its habitat. A positive enrichment proves only that the organism was present in the sample, and in practice, this can result from even a single viable cell.

Mastering Microbiology
Art Activity: Figure 19.1 The isolation of *Azotobacter*

The Winogradsky Column

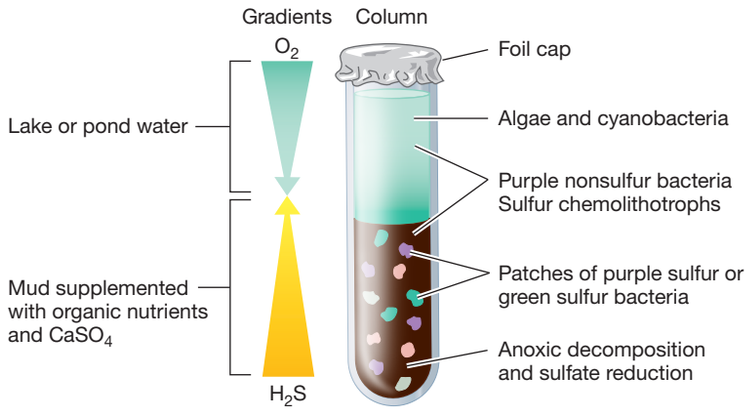
The **Winogradsky column** is an artificial microbial ecosystem and a long-term source of various bacteria for enrichment cultures. Winogradsky columns have been used to isolate phototrophic purple and green bacteria, sulfate-reducing bacteria, and many other anaerobes. Named for the famous Russian microbiologist Sergei Winogradsky (◀ Section 1.13), the column was first used by Winogradsky in the late nineteenth century in his classic studies of soil microorganisms.

Mastering Microbiology
Art Activity: Figure 19.2*a* The Winogradsky column

A Winogradsky column is prepared by filling a glass cylinder about half full with organic-rich, preferably sulfidic mud into which carbon substrates have been mixed. The substrates determine which organisms are enriched. Fermentative substrates, such as glucose, that can lead to acidic conditions and excessive gas formation (which can create gas pockets that disrupt the enrichment culture and let in air) are avoided. The mud is supplemented with small amounts of calcium carbonate ($CaCO_3$) as a buffer and gypsum ($CaSO_4$) as a source of sulfate. The mud is packed tightly in the cylinder, taking care to avoid trapping air, and then covered with lake, pond, or ditch water (or seawater if it is a marine column). The top of the cylinder is covered to prevent evaporation, and the container is placed near a window that receives diffuse sunlight for a period of months.

In a typical Winogradsky column, a diverse community of microbes develops (**Figure 19.2*a***). Algae and cyanobacteria develop quickly in the upper portions of the water column; by producing O_2 these organisms help to keep this zone of the column oxic much as they do in the upper zones of a lake. Fermentative processes in the mud lead to the production of organic acids, alcohols, and H_2, suitable substrates for sulfate-reducing bacteria (◀ Section 15.11). Hydrogen sulfide (H_2S) from the sulfate reducers triggers the development of purple and green sulfur bacteria (anoxygenic phototrophs, ◀ Sections 14.3 and 15.4–15.8) that use sulfide as a photosynthetic electron donor. These organisms typically grow in patches in the mud on the sides of the column but may bloom in the water itself if oxygenic phototrophs are scarce (Figure 19.2*b*). The pigmented cells of the anoxygenic phototrophs can be sampled with a pipette for microscopy, isolation, and characterization (Table 19.1).

Winogradsky columns have been used to enrich both aerobic and anaerobic *Bacteria* and *Archaea*. Besides supplying a ready source of inocula for enrichment cultures, columns can also be supplemented



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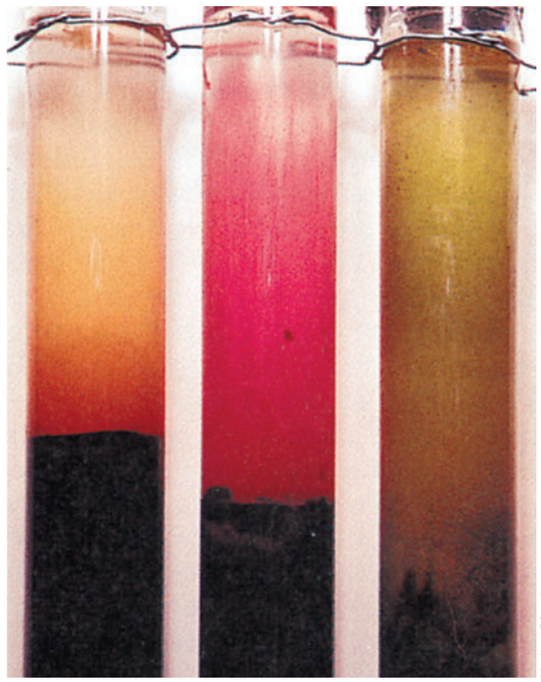


Norbert Pfennig

(b)

Figure 19.2 The Winogradsky column. *(a)* Schematic view of a typical column used to enrich phototrophic bacteria. The column is incubated in a location that receives subdued sunlight. Anoxic decomposition leading to SO_4^{2-} reduction creates the gradient of H_2S. *(b)* Photo of Winogradsky columns that have remained anoxic up to the top; each column had a bloom of a different phototrophic bacterium. Left to right: *Thiospirillum jenense, Chromatium okenii*, both of which are purple sulfur bacteria, and *Chlorobium limicola* (green sulfur bacterium).

with a specific compound to enrich an organism in the inoculum that can degrade it. Once a crude enrichment has been established in the column, culture media can be inoculated for the isolation of pure cultures, as discussed in Section 19.2.

Enrichment Bias

Although the enrichment culture technique is quite useful and still widely practiced, there exists a bias, and sometimes a very severe bias, in the outcome of enrichments. This bias is typically most profound in liquid enrichment cultures where the most rapidly growing organism(s) for the chosen set of conditions dominate. However, using molecular techniques to be described later, we now know that the most rapidly growing organisms in laboratory cultures are often only minor components of the microbial community rather than the most abundant and ecologically relevant organisms carrying out the process of interest. This could be for several reasons including the fact that the levels of resources available in laboratory cultures are typically much higher than those in nature, and the conditions in the natural habitat, including both the types and proportions of different organisms present as well as the physical and chemical conditions, are nearly impossible to reproduce and maintain for long periods in laboratory cultures.

This problem of **enrichment bias** can be demonstrated by comparing the results obtained in dilution cultures (Section 19.2) with classical liquid enrichment. Dilution of an inoculum followed by liquid enrichment or plating often yields different organisms than liquid enrichments established with the same but undiluted inocula. It is thought that dilution of the inoculum eliminates quantitatively insignificant but rapidly growing "weed" species, allowing development of organisms that are more abundant in the community but slower growing. Dilution of the inoculum is thus a common practice in enrichment culture microbiology today. As discussed below, the problem of overgrowth by "weed" species can also be circumvented by physical isolation of the desired organism before introducing it into a growth medium. This can be accomplished by dilution and a variety of classical isolation procedures that we turn to in the next section. However, more recently, sophisticated methods have been developed to physically isolate single cells of interest (or a single type of cells) and place them in a growth medium that is free of undesired cells. We consider these techniques in Section 19.3. Finally, as we will discuss later in this chapter, culture-independent molecular methods have most clearly revealed the limitations of cultivation in capturing the full microbial diversity of most environments.

Check Your Understanding

- Describe the enrichment strategy behind Beijerinck's isolation of *Azotobacter*.
- Why is sulfate (SO_4^{2-}) added to a Winogradsky column?
- What is enrichment bias? How does dilution reduce enrichment bias?

19.2 Classical Procedures for Isolating Microbes

Once a positive enrichment culture has been obtained, the next step is typically to attempt to get the enriched organism in *pure culture*—one containing a single kind of microorganism. Pure cultures are valuable because genomes can be quickly isolated and analyzed and experiments can be done under controlled laboratory conditions to clearly define the physiology of the isolate. Pure cultures have been studied since the days of Robert Koch (◀ Section 1.12), and we considered some of these methods earlier (◀ Section 4.2).

Agar Dilution Tubes and the Most-Probable-Number Technique

Common isolation procedures include the streak plate, agar dilution, and liquid dilution. For organisms that form colonies on agar plates, the streak plate is quick, easy, and the method of choice (**Figure 19.3*a***); if a well-isolated colony is selected and restreaked several successive times, a pure culture is usually obtained. With proper incubation facilities (for example, anoxic jars or anoxic chambers for anaerobes, ◀ Section 4.16), it is possible to purify both aerobes and anaerobes on agar plates by the streak plate method.

In the agar dilution tube method, a mixed culture is diluted in tubes of molten agar medium, resulting in colonies embedded in the agar. This method is useful for purifying anaerobic organisms such as phototrophic sulfur bacteria and sulfate-reducing bacteria from samples taken from Winogradsky columns or other sources. A culture is purified by successive dilutions of cell suspensions in tubes of molten agar medium (Figure 19.3*b*, ◀ Figure 15.28*g*). Repeating this procedure using a colony from the highest-dilution tube as inoculum for a new set of dilutions usually yields pure cultures. A related procedure called the *roll tube method* uses tubes containing a thin layer of agar on their inner surface. The agar can then be streaked for isolated colonies. Because the tubes can be flushed with an oxygen-free gas during streaking, the roll tube method is primarily used for the isolation of anaerobic microbes.

Another purification procedure is the serial dilution of an inoculum in a liquid medium until the final tube in the series shows no growth. When a 10-fold serial dilution is used, for example, the last tube showing growth should have originated from ten or fewer cells. Besides being a method for obtaining pure cultures, serial dilution techniques are widely used to estimate viable cell numbers in the **most-probable-number (MPN) technique** (**Figure 19.4**). MPN methods have been used for estimating the numbers of microorganisms in foods, wastewater, and other samples in which cell numbers need to be assessed routinely. An MPN count of a natural sample can be done using highly selective media and incubation conditions to target one or a small group of organisms or a particular pathogen. Alternatively, a count can be done using complex media to get a general estimate of viable cell numbers (but see Section 4.4 for a caveat that applies to such estimates). Use of several replicate tubes at each dilution improves accuracy of the final MPN obtained.

Criteria for Culture Purity

Regardless of the methods used to purify a culture, once a putative pure culture has been obtained, it is essential to verify its purity. This is typically done through a combination of (1) microscopy, (2) observation of colony characteristics on plates or in dilution tubes, and (3) tests of the culture for growth in other media. In the latter, it is important to test the culture for growth in media and under growth conditions in which the desired organism is predicted to grow poorly or not at all but in which contaminants will grow vigorously. In the final analysis, the microscopic observation of a single morphological type of cell that displays uniform staining characteristics (for example, in a Gram stain) coupled with uniform colony characteristics and the absence of contamination in growth tests with various culture media is strong evidence that a culture is a pure (*axenic*) culture.

Certain molecular methods described in this chapter for characterizing natural microbial communities can also be applied to the verification of culture purity. However, these techniques are complementary and do not substitute for the more fundamental observations of culture characteristics and cellular morphology.

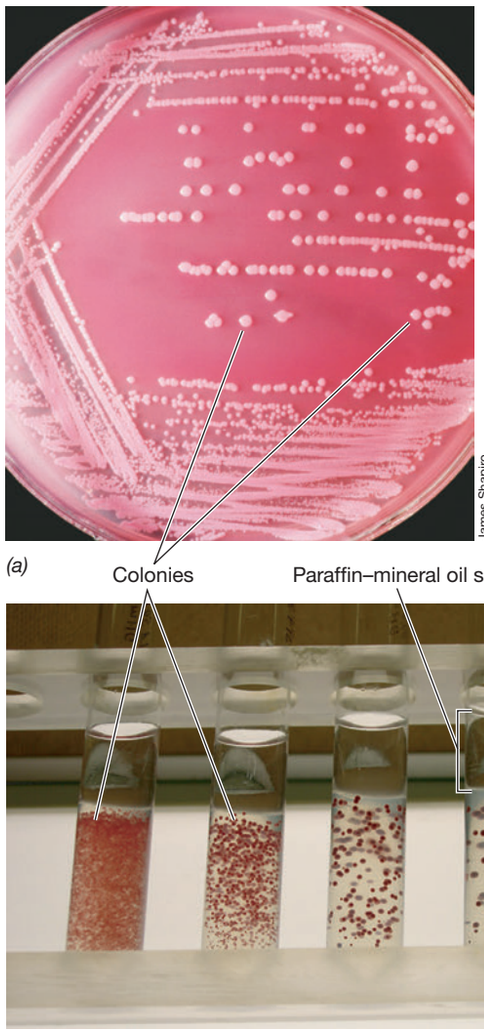


Figure 19.3 Pure culture methods. *(a)* Organisms that form distinct colonies on plates are usually easy to purify. *(b)* Colonies of phototrophic purple bacteria in agar dilution tubes; the molten agar was cooled to approximately 45°C before inoculation. A dilution series was established from left to right, eventually yielding well-isolated colonies. The tubes were sealed with a 1:1 mixture of sterile paraffin and mineral oil to maintain anaerobiosis.

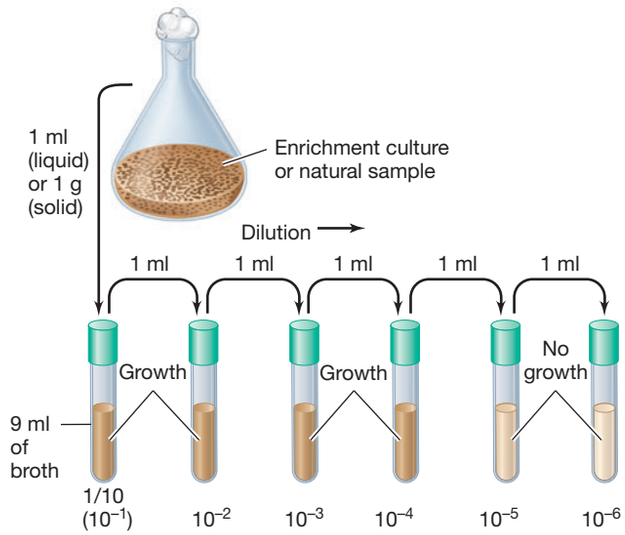


Figure 19.4 Procedure for a most-probable-number (MPN) analysis. Growth in the 10^{-4} but not the 10^{-5} dilution means that cell numbers were at least 10^4 cells/ml in the sample used for inoculation. Because particle-attached microorganisms can skew numbers significantly, gentle methods to disassociate microorganisms from particles are often used prior to dilution. In addition, each dilution tube is mixed thoroughly before removing a sample for the next dilution.

Check Your Understanding

- What is a pure culture and why is obtaining one useful in microbial ecology?
- How does the agar dilution method differ from streaking to obtain isolated colonies?
- Why would microscopic examination alone not be sufficient to establish that a culture is pure?

19.3 Selective Single-Cell Isolation: Laser Tweezers, Flow Cytometry, Microfluidics, and High-Throughput Methods

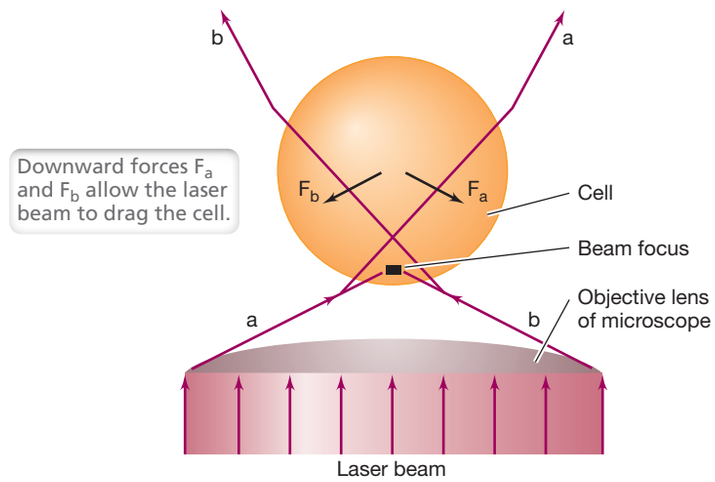
The problem of enrichment bias has fueled the development of new methods for culturing microbes from nature. These advancements have emerged from the understanding that every microbe has a *fundamental niche* and a *realized niche*. The **fundamental niche** refers to the range of environments in which a species will be sustained when it is not resource-limited, such as may result from competition with other species. By contrast, the **realized niche** refers to the range of natural environments supporting a species when it is confronted with factors such as resource limitation, predation, and competition from other species.

Establishing laboratory conditions that fall within the fundamental niche may be sufficient to support an organism once it is in pure culture but may fail to selectively enrich the same organism from a natural sample. Because the realized niche of most microorganisms is unknown, there has been an increasing emphasis on developing methods that physically isolate single cells into separate compartments free from competition with other microbes. These include both manual and robotic methods that function to sort individual cells from an environmental sample, and we consider these methods now.

Laser Tweezers and Flow Cytometry

Laser tweezers consist of an inverted light microscope equipped with a strongly focused infrared laser and a micromanipulation device. Trapping a single cell is possible because the laser beam creates a force that pushes down on a microbial cell (or other small object) and holds it in place (**Figure 19.5*a***). Then when the laser beam is moved, the trapped cell moves along with it. If a mixed sample is in a capillary tube, a single cell can be optically trapped and moved away from contaminating organisms (Figure 19.5*b*). The cell can then be isolated by breaking the tube at a point between the cell and the contaminants and flushing the cell into a small tube of sterile medium. Laser tweezers, when coupled with staining techniques that identify particular organisms (Sections 19.4 and 19.5), can be used to select organisms of interest from a mixture for purification and further laboratory study.

Flow cytometry is a technique for counting and examining a mixture of cells by suspending them in a stream of fluid and passing them through an electronic detector that sorts them according to defined criteria; for example, by cell size, shape, or fluorescent properties. This ability makes cell sorting useful not only for isolating single cells but also for enriching a particular cell type from a mixture. Cell sorters can deposit individual cells into wells of a microtiter plate where each well contains the same growth medium or a slightly different growth medium. Because the growth requirements



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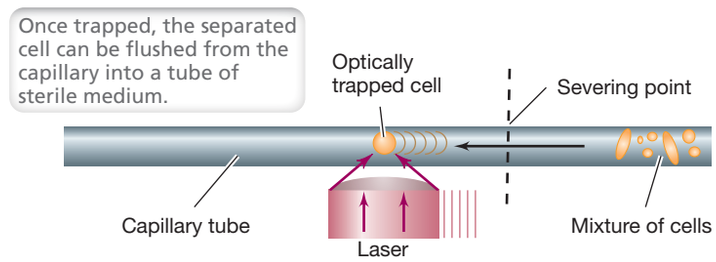


(b)

Figure 19.5 The laser tweezers for the isolation of single cells. *(a)* Mechanism by which individual cells can be isolated. *(b)* Once a cell has been isolated in a capillary tube, it can be tested for subsequent growth in pure culture.

of some organisms include organic compounds and metabolites produced by other organisms that share their environment, addition of filter-sterilized source water (for aquatic organisms) or soil water extract (for soil organisms) can be used to supplement the media tested. Each well in the microtiter plate can then be monitored for growth or some other property either manually or using robotic methods (high-throughput culture, see next subsection). We explore the mechanism and uses of flow cytometry in more detail in Section 19.12 (see Figure 19.42).

High-Throughput Culture and Microfluidic Devices

Continuing innovations in single-cell isolation methodology have spawned **high-throughput culturing methods** and related methods for use on an even smaller scale. High-throughput methods require dilution (or cell sorting) of a sample to yield a single cell in each well of a microtiter plate (**Figure 19.6**). From there, each well is robotically monitored over time for cell growth or a specific target gene. High-throughput methods allow the experimenter to test many alternative sets of resources and growth conditions systematically in an attempt to replicate the realized niche or, alternatively, to allow the organism to occupy its fundamental niche by relieving it from competition. Microtiter wells that are positive for cell growth or a target gene of interest identify the acceptable resources and conditions for growth of a particular microbe and supply valuable clues for the design of laboratory culture media to obtain its growth in pure culture.

High-throughput cultivation has shown increasing success in isolating unique bacteria. For example, high-throughput methods were used for the isolation of one of the most abundant bacteria on Earth, the small marine planktonic bacterium *Pelagibacter ubique* (Figure 19.6). This bacterium thrives on the very dilute pool of dissolved organic matter present in the open oceans and eluded classical enrichment methods for years. But with high-throughput technology, this ecologically important bacterium was brought into laboratory culture where its biology could be studied in more detail.

Microfluidic devices carry the high-throughput concept even further by using microfabrication technology to combine channels and wells for fluid transfer and collection on a miniaturized platform. One such device is less than 10 centimeters long yet holds 3200 nanoliter-sized wells, with each well serving as a small culture vessel (**Figure 19.7**). An environmental sample is introduced into the microfluidic device such that each well receives a single cell. Different medium formulations can be tested, and the media supplemented with a small amount of filter-sterilized water or soil extract collected from the sampled environment (these additions may stimulate growth by providing trace nutrients missing in the culture medium).

Mastering Microbiology
Art Activity: Figure 19.6 Methodological pipeline for high-throughput cultivation of previously uncultured microorganisms

Both cell growth and target genes can be assessed in each well of the microfluidic device; growth is assessed by direct microscopic

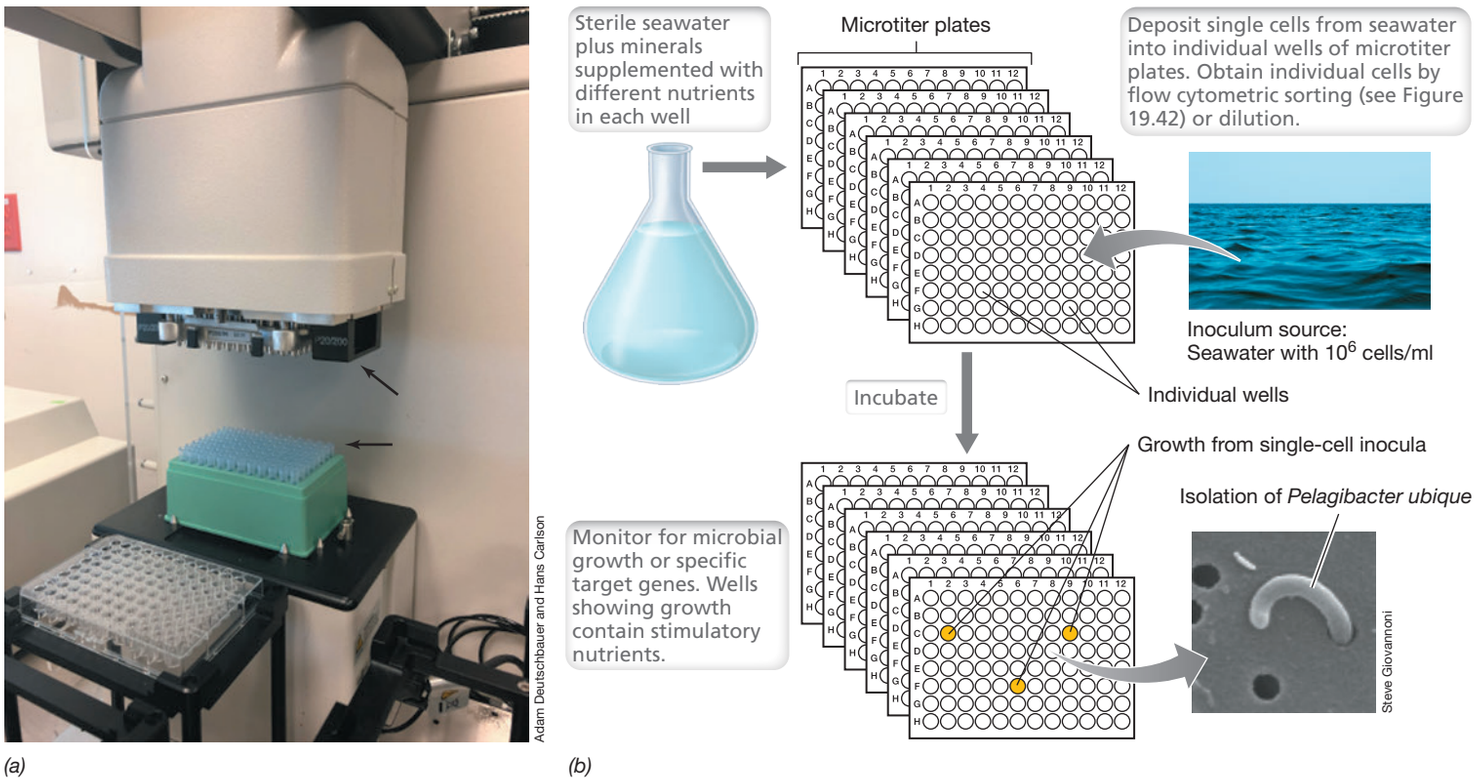


Figure 19.6 Methodological pipeline for high-throughput cultivation of previously uncultured microorganisms. The method shown here was used to isolate the marine bacterium *Pelagibacter ubique*. *(a)* Robotic system for high-throughput multiplexed pipetting of growth medium into microtiter plates (arrows point to pipette tips and robotic pipette holder). *(b)* Following the addition of filter-sterilized seawater and low nutrient concentrations to the individual wells, and deposition of single cells into individual wells of the microtiter plate, pure cultures of *Pelagibacter* and other novel marine *Bacteria* were obtained. *Pelagibacter* is the most abundant bacterium in the open oceans (▶ Section 20.12).

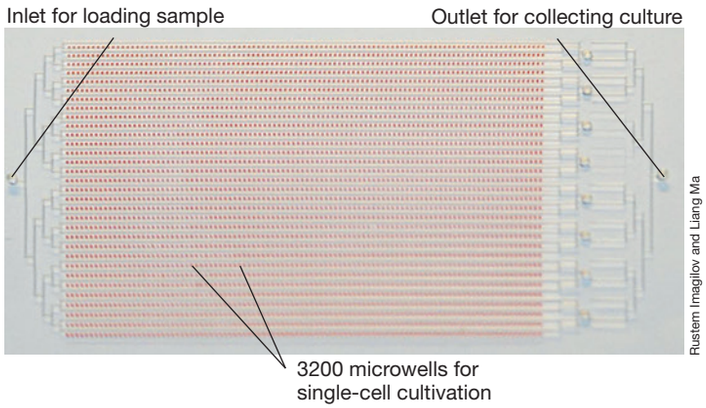


Figure 19.7 Microfluidic platform for cultivation. An environmental inoculum is suspended in a cultivation medium and loaded onto this microfluidic device, enabling confinement of as many as 3200 single cells in nanoliter wells to promote the growth of microcolonies. Following different periods of incubation, cultured populations are collected at the outlet and further grown under conditions demonstrated to support growth on the microfluidic device. The device is about 7 cm wide.

examination of cell numbers in a well under the microscope. If insufficient cellular biomass is available for molecular characterization of specific target genes by methods such as PCR (see Section 19.6), genome amplification as described for single-cell genomics (◀ Section 10.11) can be used prior to further analysis.

A variation on the microfluidics technique employs a microchamber device modified such that each of the tiny chambers is separated from the external environment by a membrane that traps the microbes but allows soluble nutrients to diffuse in and out. Following the introduction of a single cell into each chamber, the device is placed back into the environment from which the inoculum was obtained. Then, after incubation for a month or more, microbes that initiate growth only when incubated under the conditions and resources present in their habitats can often be isolated and subsequently propagated in the laboratory.

Although technology is rapidly advancing the art of isolating new microbes, patience is still needed in any cultivation effort, as the discovery of slow-growing or dormant organisms may require months of incubation. Also, many microbes in nature are likely adapted to extremely low nutrient concentrations and may be inhibited by levels of nutrients used to grow organisms commonly studied in the laboratory. Both high-throughput and microfluidic methods overcome these problems by their ability to separate individual cells from other cells that may release inhibitory materials and by surveying a nearly limitless variety of nutrient conditions. Currently, these methods offer the best opportunity for culturing the most interesting (and likely ecologically relevant) microorganisms from nature.

Check Your Understanding

- How might you isolate a morphologically unique bacterium present in an enrichment culture in relatively low numbers?
- What is meant by "high-throughput" in culturing microorganisms? How has it benefited microbiology?

II • Culture-Independent Microscopic Analyses of Microbial Communities

The microscope has been the microbiologist's foremost tool for studying microbial structure. Today the microscope can also assist in probing microbial diversity and activity, thanks to a suite of fluorescent techniques. These advances have greatly improved our understanding of microbial community structure and provided unprecedented insight into microbial symbiotic relationships with plants, animals, and other microbes.

Microbial ecologists quantify cells in a microbial habitat to estimate abundance of the entire community or, more specifically, relative abundances of the different species in the community. Cell stains are necessary to obtain these types of data, and we detail these methods here. Organisms in natural environments can also be detected by assaying their genes. Genes encoding either ribosomal RNA (rRNA, ◀ Section 13.11) or enzymes that support a specific physiology are the usual targets in these studies. A rapidly developing approach to the study of microbial ecology, called *multi-omics*, combines multiple molecular, analytical, and omics methods (Chapter 10), and is introduced in Section 19.8.

19.4 General Staining Methods

Several general staining methods are suitable for quantifying microorganisms in natural samples. Although these methods do not reveal the physiology or phylogeny of the cells, they are nonetheless reliable and widely used by microbial ecologists for measuring total cell numbers. One method also allows cell viability to be assessed.

Fluorescent Staining with Dyes That Bind Nucleic Acids or Reveal Viability

Fluorescent dyes can be used to stain microorganisms from virtually any microbial habitat. **DAPI** (4′,6-diamidino-2-phenylindole) is a popular stain for this purpose, as is the dye **acridine orange**. There is also increasing use of *SYBR Green I*, a dye that confers very bright fluorescence to all microorganisms, including viruses. These stains bind to DNA and are strongly fluorescent when exposed to ultraviolet (UV) radiation (DAPI absorption maximum, 400 nm; acridine orange absorption maximum, 500 nm; SYBR Green I absorption maximum, 497 nm), making the microbial cells in the sample readily visible and easy to enumerate. Cells stained with DAPI fluoresce blue, cells stained with acridine orange fluoresce orange or greenish-orange, and cells stained with SYBR Green I fluoresce green (Figure 19.8).

Dyes that stain DNA are widely used for the enumeration of microorganisms in environmental, food, and clinical samples. Depending on the sample, background staining is occasionally a problem with fluorescent stains, but because these dyes specifically stain nucleic acids, they are for the most part nonreactive with inert matter. Thus, for many samples, from soil as well as aquatic sources, they can give a reasonable estimate of the cell numbers present. Staining with the brightly fluorescent SYBR Green I also

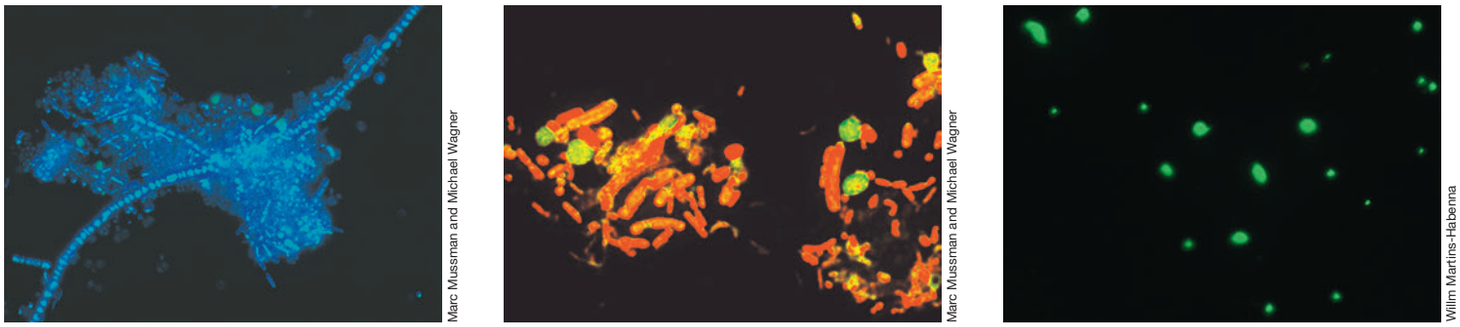


Figure 19.8 Nonspecific fluorescent stains. *(a)* DAPI and *(b)* acridine orange staining showing microbial communities inhabiting activated sludge in a municipal wastewater treatment plant. With acridine orange, cells containing low RNA levels stain green. *(c)* SYBR Green–stained sample of Puget Sound (Washington, USA) surface water showing green-fluorescing bacterial cells. The large cells near the center of the field are 0.8–1.0 μm in diameter.

provides excellent enumeration of aquatic virus populations (▶ Section 20.13). For dilute aquatic samples, cells can be stained following collection on a membrane surface by filtration.

DNA staining is a nonspecific process; *all* microorganisms in a sample are stained. Although this may at first seem desirable, it is not necessarily so. For example, DAPI and acridine orange fail to differentiate between living and dead cells or between different species of microorganisms, so they cannot be used to assess cell viability or to track specific microorganisms in an environment. *Viability staining* solves one of these problems because it differentiates live cells from dead ones. Hence, viability stains yield both abundance and viability data at the same time. The basis of differentiating between live and dead cells lies not with a cell's DNA but whether its cytoplasmic membrane is intact or not. Two dyes that fluoresce green and red are added to a sample; the green-fluorescing dye penetrates all cells, viable or not, whereas the red dye, which contains the chemical propidium iodide, penetrates only those cells whose cytoplasmic membrane is no longer intact and that are therefore dead. Thus, when viewed microscopically, green cells are scored as alive and red cells as dead, yielding an instant assessment of both abundance and viability (**Figure 19.9**).

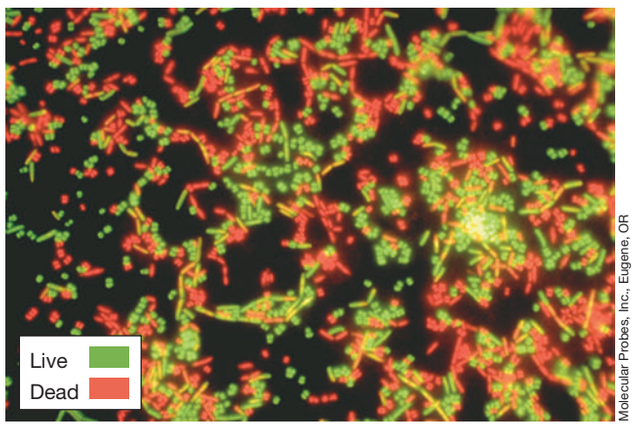


Figure 19.9 Viability staining. Live (green) and dead (red) cells of *Micrococcus luteus* (cocci) and *Bacillus cereus* (rods) stained by the LIVE/DEAD BacLight Bacterial Viability Stain.

Although useful for research that uses laboratory cultures, the live/dead staining method is not suitable for use in the direct microscopic examination of samples from many natural habitats because of problems with nonspecific staining of background materials. However, procedures have been developed to overcome this problem in analyses of aquatic environments; the water sample is filtered, and the filters are stained with the live/dead stain and examined microscopically. Thus in aquatic microbiology, live/dead staining is often used to measure the viability of cell populations in the water column of lakes or oceans, or in the flowing waters of streams, rivers, and other aquatic environments.

Fluorescent Proteins as Cell Tags and Reporter Genes

Bacterial cells can be altered by genetic engineering to make them autofluorescent. As discussed earlier, a gene encoding the **green fluorescent protein (GFP)** can be inserted into the genome of virtually any cultured bacterium (◀ Sections 8.1 and 12.5). When the gene encoding the GFP (*gfp*) is expressed, cells fluoresce green when observed with ultraviolet microscopy (**Figure 19.10**; ◀ Figure 12.17). Although GFP is not useful for the study of natural populations of microorganisms (because these cells lack the GFP gene), GFP-tagged cells can be introduced into an environment, such as plant roots, and then tracked over time by microscopy. Using this method, microbial ecologists can study competition between the native microbiota and a GFP-tagged introduced strain and can assess the effect of perturbations of an environment on the survivability of the introduced strain.

The gene *gfp* and those encoding other **fluorescent proteins** have also been used extensively in laboratory cultures of various bacteria and in controlled environments as *reporter genes*. When the gene is fused with an operon under the control of a specific regulatory protein, transcription can be studied by using fluorescence as the indicator (a "reporter") of activity. That is, when genes containing the fused fluorescent protein gene are transcribed and translated, both the protein of interest and the fluorescent protein are made, and cells fluoresce the characteristic color. For example, expression of *gfp* was used to demonstrate that colonization of alfalfa roots by the nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti* (legume–root nodule symbiosis, ▶ Section 23.4) is promoted by sugars and dicarboxylic acids released by the plant (Figure 19.10*b*, *c*). The

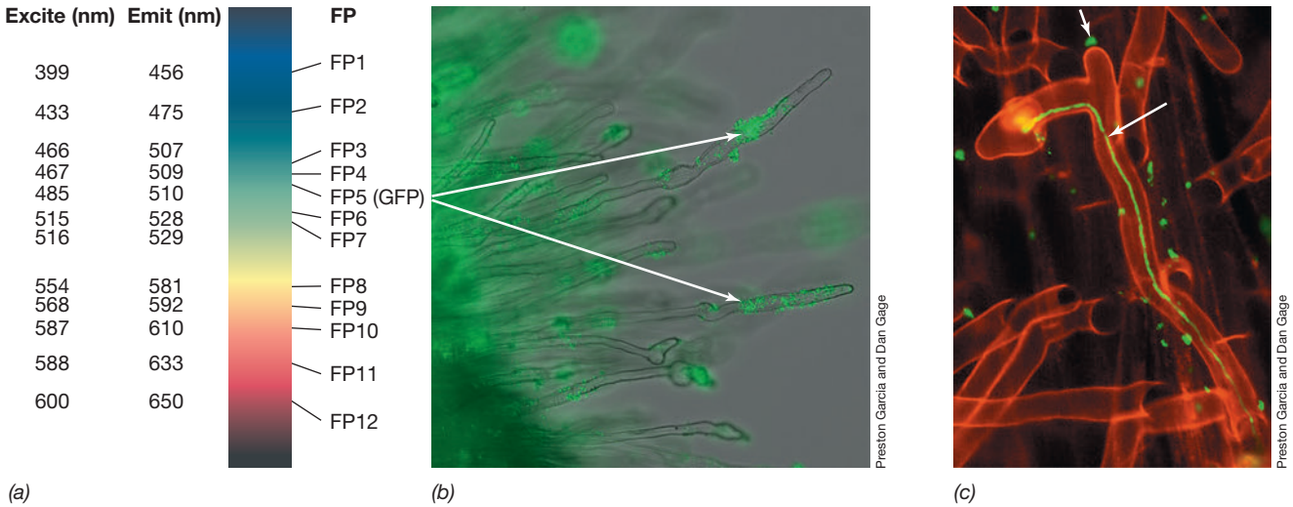


Figure 19.10 **Fluorescent protein reporters.** *(a)* Twelve different fluorescent proteins (FP1–FP12) are known that have distinct excitation (Excite) and emission (Emit) properties. *(b)* Cells of *Sinorhizobium meliloti* (arrows) carrying a plasmid with an α-galactoside-inducible promoter fused to the GFP (FP5); the cells are on clover seedling roots. Green fluorescence indicates that α-galactosides are released and available to support the growth of this bacterium. *(c) S. meliloti* cells (arrows) carrying a plasmid with a succinate-inducible promoter fused to GFP; green fluorescence indicates that succinate or other C_4 dicarboxylic acids have been secreted by the plant root hairs.

emission properties of GFP and other fluorescent proteins isolated from different marine invertebrates (jellyfish, corals, anemones) have since been altered through mutation to yield a broad palette of fluorescent proteins of varying spectral properties (Figure 19.10*a*), offering the experimenter the capability to monitor several species simultaneously.

One drawback to the use of GFP is that to become fluorescent it requires O_2, and thus it is not suitable for tracking cells introduced into strictly anoxic habitats. However, flavin-based fluorescent proteins that do not require O_2 are available to overcome this limitation. These proteins are derived from bacterial and plant photosensory flavoproteins and are more thermally stable than the GFP, making them useful for tracking mildly thermophilic species. In addition to these fluorescent tags for tracking microbes, phylogenetic stains (Section 19.5) are widely used for identifying microbes, and a wide variety of new fluorescent "super-resolution" microscopy techniques are available for tracking individual molecules within a microbial cell (◀ Section 8.1). Thus, fluorescence technology has come a long way since the days when only DAPI and acridine orange were available for visualizing microbial cells in nature.

Check Your Understanding

- How does viability staining differ from stains like DAPI?
- What types of environments limit the application of GFP?
- Why is it incorrect to say that the GFP is a "staining" method?

19.5 Microscopic Specificity: Fluorescence In Situ Hybridization (FISH)

As we have just seen, the microscope is an essential tool for enumerating and assaying microorganisms in a general way—their morphology, their Gram reaction, their viability, and the like. However, the biggest limitation with the methods we have discussed thus far is that none of them reveal the *phylogeny* of the microorganisms observed under the microscope. That is, how many species, or phyla, or even domains, are present in the sample? More specific fluorescent staining methods are required to answer these questions.

When observing unstained or nonspecifically stained natural populations of microorganisms under the microscope (Section 19.4), one should remember that the sample likely contains a phylogenetically diverse community, even if many cells "look" the same. This is because the simple shapes of bacteria can conceal their remarkable diversity, and **Figure 19.11** shows an

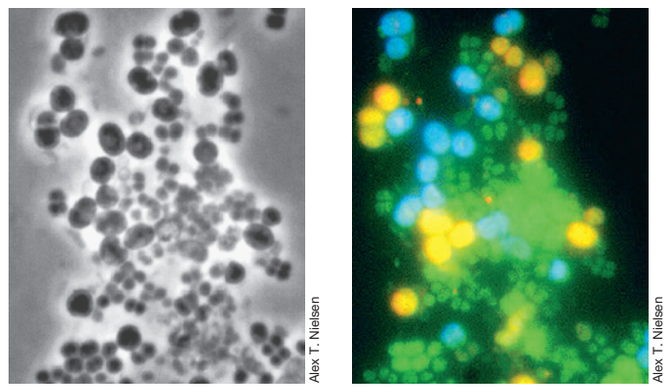
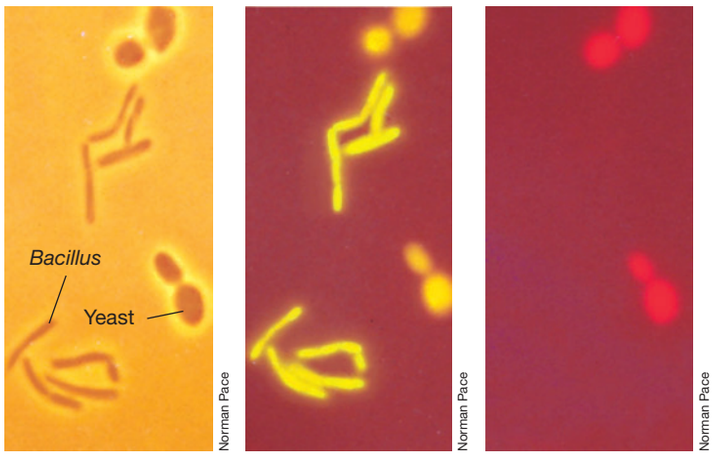


Figure 19.11 **Morphology and genetic diversity.** The photomicrographs shown here, produced by *(a)* phase contrast and *(b)* a technique called phylogenetic FISH (Section 19.5), are of the same field of cells. Although the large oval cells are of a rather unusual size for prokaryotic cells and all look similar by phase-contrast microscopy, the phylogenetic stains reveal that there are two genetically distinct types (one stains yellow and one stains blue). Both cell types are about 2.3 μm in diameter. The smaller, green cells in pairs or clusters are about 1 μm in diameter.



(a) (b) (c)

Figure 19.12 Fluorescently labeled rRNA probes: Phylogenetic stains. *(a)* Phase-contrast photomicrograph of cells of *Bacillus megaterium* (rod, *Bacteria*) and the yeast *Saccharomyces cerevisiae* (oval cells, *Eukarya*). *(b)* Same field; cells stained with a yellow-green universal rRNA probe (this probe hybridizes with rRNA from organisms of any phylogenetic domain). *(c)* Same field; cells stained with a eukaryal probe (only cells of *S. cerevisiae* react). Cells of *B. megaterium* are about 1.5 μm in diameter and cells of *S. cerevisiae* are about 6 μm in diameter.

example of this. The two photos are of the same microscope field, one taken in black and white of cells in a microbial community and the other taken in color of the same cells stained in a way that reveals their phylogeny. If only unstained cells (Figure 19.11*a*) were viewed, it would be easy to conclude that the large ovoid-shaped cells are all of a single species. But staining to reveal phylogeny (Figure 19.11*b*) shows that they are not; the blue and yellow fluorescent stains target different species of large ovoid cells while the green stain reveals yet a third species in the community. Microscopic assessments of phylogenetic diversity such as this can only be made using *phylogenetic stains*, and we consider these here along with some related staining techniques.

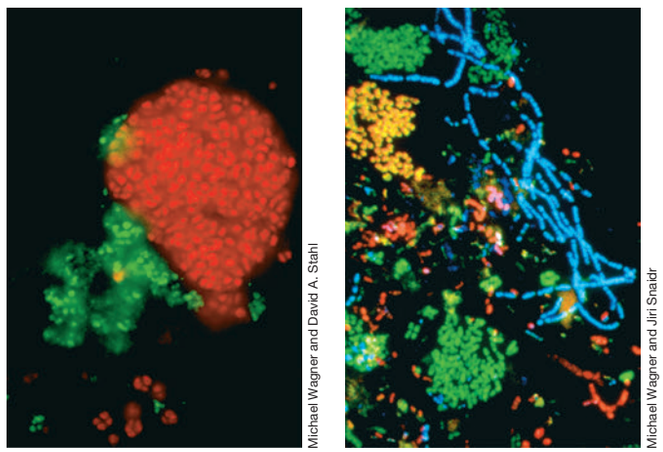
Phylogenetic Identification Using FISH

Because of their great specificity, **nucleic acid probes** can be harnessed as powerful tools for identifying and quantifying microorganisms. Recall that a nucleic acid probe is a DNA or RNA oligonucleotide complementary to a sequence in a target gene or RNA; when the probe and the target come together, they hybridize (◀ Section 12.1). Nucleic acid probes can be made fluorescent by attaching fluorescent dyes to them. The fluorescent probes can then be used to identify organisms that contain a nucleic acid sequence complementary to the probe. This technique is called **fluorescence in situ hybridization (FISH)**, and different applications are described here, including methods that target phylogeny (**Figure 19.12** and see Figure 19.13) or gene expression (see Figure 19.14).

Phylogenetic FISH stains are fluorescing oligonucleotides complementary in base sequence to sequences in ribosomal RNA (16S or 23S rRNA in *Bacteria* and *Archaea* or 18S or 28S rRNA in eukaryotes, Chapter 13). Phylogenetic stains penetrate cells without lysing them and hybridize with rRNA directly in the ribosomes. The number of fluorescent probes bound to a cell reflects the number of its ribosomes. As single microbial cells can contain tens of thousands of ribosomes, strong signals can be achieved. And, because ribosomes are scattered throughout the cell in prokaryotic cells, the entire cell becomes fluorescent (Figures 19.11*b* and 19.12). One limitation of the FISH technique is that slow-growing organisms often have very low numbers of ribosomes and mRNAs, and therefore fluoresce very poorly. However, new staining methods have been developed that measure protein synthesis directly, and provide essential information about the viability and activity of cells in their natural environments, even when growing slowly (see Figure 19.15).

By targeting sites in the rRNA sequence that are variable between different organisms, phylogenetic stains can be designed to be very specific and react with only one species or a handful of related microbial species. Alternatively, by targeting conserved sequences in the rRNA they can be made more general and react with, for example, all cells of a given phylogenetic domain (*Bacteria*, *Archaea*, *Eukarya*) or a given phylum within a domain. Using FISH, an investigator can identify or track a specific organism of interest or an entire domain of interest in a natural sample. For example, if one wished to determine the percentage of a given microbial population that are *Archaea*, an archaeal-specific phylogenetic stain could be used in combination with DAPI (Section 19.4) to assess *Archaea* and total numbers, respectively, and a percentage derived by calculation.

FISH technology can also employ multiple phylogenetic probes at once. With a suite of probes, each designed to react with a particular organism or group and each containing its own fluorescent dye, FISH can image multiple taxa in a habitat in a single experiment (**Figure 19.13** and see MicrobiologyNow on page 648). Similarly, the method easily distinguishes between genetically distinct cells of similar morphology (Figure 19.11*b*). If FISH is combined with confocal microscopy (◀ Section 1.9), it is possible to explore



(a) (b)

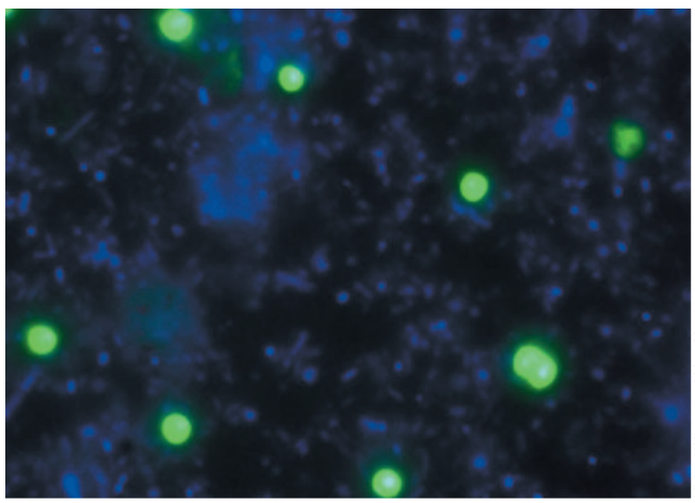
Figure 19.13 FISH analysis of activated sludge from a wastewater treatment plant. *(a)* Nitrifying bacteria. Red, ammonia-oxidizing bacteria; green, nitrite-oxidizing bacteria. *(b)* Confocal laser scanning micrograph of a sewage sludge sample treated with three phylogenetic FISH probes, each containing a fluorescent dye (green, red, or blue) that identifies a particular group of *Proteobacteria* (Chapter 16). Green-, red-, or blue-stained cells reacted with only a single probe; other cells reacted with two (turquoise, yellow, purple) or three (white) probes.

microbial populations with depth, as, for example, in a biofilm (▶ Section 20.4). In addition to microbial ecology, FISH is also an important tool in the food industry and in clinical diagnostics for the microscopic detection of specific pathogens in food products or clinical specimens.

Single-Cell Metabolic Activity Staining Using CARD-FISH and BONCAT-FISH

FISH methods have been developed for measuring the metabolic activity of single cells by using probes for specific messenger RNAs (transcriptional activity) or for protein synthesis (translational activity). Besides characterizing different taxa in a habitat as phylogenetic stains do, these forms of FISH measure *gene expression*. These microscopic methods thus complement phylogenetic staining—techniques that answer the microbial ecologist's important question of "Who is there?"—by answering the other important question in microbial ecology: "What are they doing?"

Measuring transcription requires targeting messenger RNA (mRNA), which is much less abundant in cells than rRNA; because of this, standard FISH techniques cannot be applied. Instead, the signal (fluorescence) must be amplified. A FISH method that enhances the signal is called *catalyzed reporter deposition FISH* (*CARD-FISH*). In CARD-FISH the specific nucleic acid probe contains a molecule of the enzyme peroxidase conjugated to it instead of a fluorescent dye. After there has been time for hybridization between the probe and a specific mRNA, the preparation is treated with a fluorescently labeled compound called *tyramide*, which is a substrate for peroxidase. Within cells containing the nucleic acid probe, the tyramide is converted by the activity of peroxidase into a very reactive intermediate that covalently binds to adjacent proteins; this amplifies the signal sufficiently to be detected by fluorescence microscopy (**Figure 19.14**). Each molecule of peroxidase activates many molecules of tyramide so that even mRNAs present at very low abundance can be visualized. Besides detecting mRNA, CARD-FISH is also useful in phylogenetic studies of microbes that may be growing very slowly, such as organisms inhabiting the open oceans where cold temperatures and low nutrient concentrations limit growth rates (Figure 19.14). Because such cells have few ribosomes compared with more actively growing cells, standard FISH methodology often yields only a weak signal.



Michael Wagner and Marc Mussmann

Figure 19.14 Catalyzed reporter deposition FISH (CARD-FISH) labeling of *Archaea*. Archaeal cells in this preparation fluoresce intensely (green) relative to DAPI-stained cells (blue).

Although cells that fluoresce brightly by FISH are likely actively synthesizing proteins (as indicated by their high number of ribosomes), a direct measure of translational activity can be made using *bioorthogonal noncanonical amino acid tagging* (*BONCAT*) combined with FISH (**BONCAT-FISH**). Bioorthogonal compounds are synthetic molecules that mimic natural metabolites and when assimilated and metabolized by a living cell do not interfere with normal physiological processes. Similarly, bioorthogonal analysis refers to a chemical reaction that can occur inside a living cell without interfering with normal function.

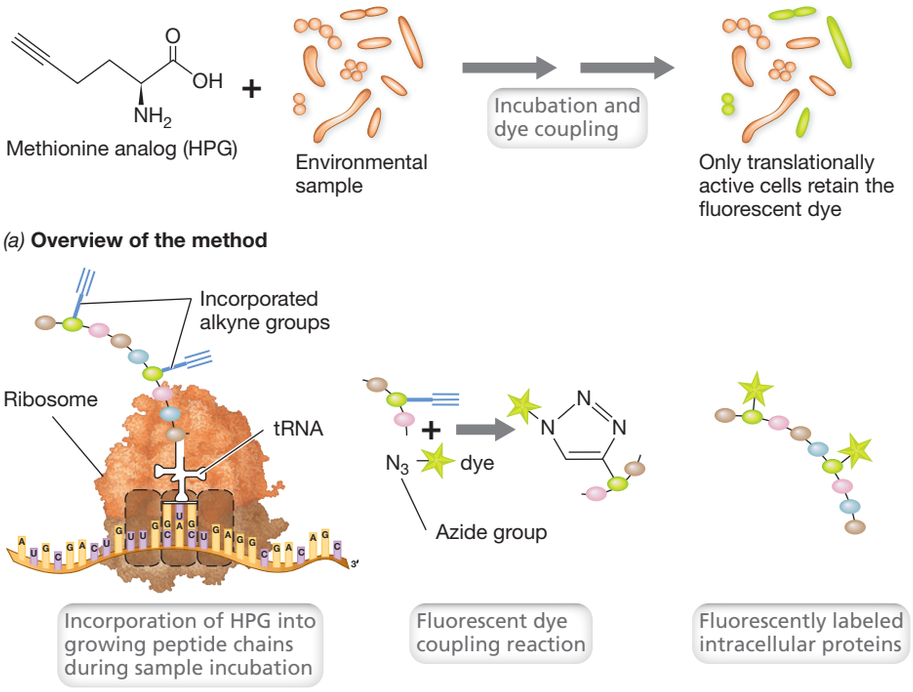
In BONCAT (**Figure 19.15**), cells are first incubated with a modified version of a normal cellular metabolite, such as the amino acid methionine containing a reactive alkyne group (Figure 19.15*a*). This methionine analog is called a noncanonical (meaning nonstandard) amino acid. Following incorporation of this analog into proteins of actively growing cells, the cells are treated with a fluorescent reporter molecule containing a chemically reactive azide group that bonds specifically to the methionine analog by way of its reactive alkyne group (Figure 19.15*b*). The phylogenetic identity of translationally active cells is then determined using standard FISH technology in which the rRNA probe is labeled with a different fluorescent dye from that attached to the azide group. An example of BONCAT-FISH to identify translationally active cells in sediments of a marsh estuary is shown in Figure 19.15*c*. Note that only some of the cell clusters stained green by a bacteria-specific FISH probe are also translationally active, as indicated by BONCAT magenta staining. Thus, both a phylogenetic snapshot and an activity snapshot are obtained in a single experiment.

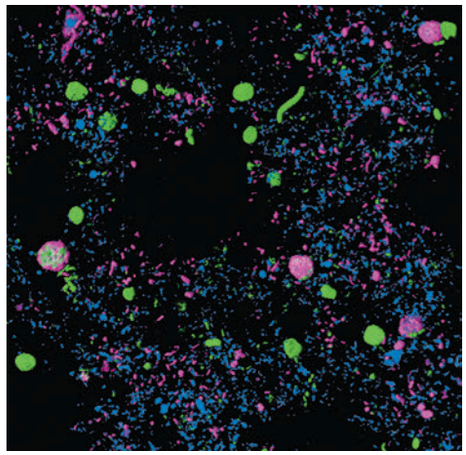
After BONCAT-FISH, further genetic analysis is possible by using cell sorting to recover labeled cells for genomic analysis of sorted populations or of single cells (see Section 19.12). Once cells have been concentrated by cell sorting techniques, sequencing can be done on single genes, specific gene families, or entire genomes to yield an increasingly more detailed picture of the genetic makeup of the targeted cells.

Although the techniques we have just discussed do not require laboratory culture, in Part III we consider what microbial ecologists typically mean when they say their microbial community analyses are "culture-independent."

Check Your Understanding

- What structure in the cell is the target for fluorescent probes in phylogenetic FISH?
- FISH and CARD-FISH can be used to reveal different things about cells in nature. Explain.
- Compare the utility of CARD-FISH versus BONCAT-FISH for evaluating cellular activity.





(c)

Figure 19.15 BONCAT labeling of translationally active microbial cells. *(a)* Overview. Cells in the environmental sample take up the methionine analog L-homopropargylglycine (HPG). Following incubation to allow active cells to take up the HPG and incorporate it into growing peptide chains, cells are treated with a dye that makes translationally active cells fluoresce. *(b)* Chemistry of the process. HPG is incorporated by the cell as methionine in growing polypeptides. When treated with the fluorescent dye-labeled azide, the azide group on the dye binds to the alkyne group on HPG to yield a fluorescent protein. *(c)* BONCAT-FISH analysis of activity and diversity of microbial cells in Little Sippewissett Salt Marsh (Massachusetts, USA). Blue, cells stained with the nonspecific nucleic acid dye DAPI (Section 19.4). Green, cells stained by FISH for the phylogenetic domain *Bacteria*. Magenta, translationally active cells stained using BONCAT. The large green ovoid cells are about 4 μm in diameter, the large magenta cells are about 6 μm in diameter, and the small magenta rod-shaped cells are about 1.5 μm long.

III • Culture-Independent Molecular Analyses of Microbial Communities

Revolutionary changes in DNA sequencing technology have made it possible to rapidly recover vast amounts of gene and genome sequence information from the environment. However, fundamental ecological understanding is possible only when that sequence information is paired with appropriate measures of cellular and community activities.

Microbial biodiversity studies often forgo isolating organisms or even quantifying or identifying them microscopically. Instead, complementary molecular methods are used to measure the biodiversity, potential activities, and realized activities of complex communities. As we now review in this section, gene and genome sequencing serve as an important measure of diversity and potential activity, with the caveat that annotation of all novel gene functions remains a daunting challenge (◀ Section 10.2). However, *detection* of a gene is not the same as gene *expression*. As we will discuss in the following two sections, metabolic activity can be measured at the level of both the community and single cells by combining analyses of gene expression (transcription and translation) with measurements of active processes using the tools of metabolomics (◀ Section 10.10) and isotopic tracers to unravel the food webs sustaining complex microbial communities.

Many surveys of microbial diversity and potential activity are performed at the gene level using the polymerase chain reaction (PCR), DNA fragment analysis by gel electrophoresis (DGGE, T-RFLP, ARISA, each discussed below) or molecular cloning, and DNA sequencing and analysis (Chapters 10 and 12). Increasingly, as a result of changing technologies in DNA sequencing, entire genomes extracted from cells present in an environmental sample are analyzed as a more comprehensive measure of the biodiversity of microbial communities. We begin with an overview of single gene analysis and then introduce **multi-omics,** the integration of data from multiple omics platforms to more fully characterize microbial community structure and function (see Figure 19.21). The term *omics* (Chapter 10) is increasingly used to describe individual or various combinations of genomics, transcriptomics, proteomics, and metabolomics for analysis of organisms and communities of organisms. When these methods are used for the study of complex microbial communities, the terminology is often modified with the prefix "meta" (e.g., *metagenomics*, *metatranscriptomics*, *metaproteomics*) as discussed in Section 19.8.

19.6 PCR Methods of Microbial Community Analysis

We discussed the principle of the polymerase chain reaction (PCR) in Section 12.1. Recall the major steps involved: (1) Two nucleic acid primers are hybridized to a complementary sequence in a target gene; (2) DNA polymerase copies the target gene; and (3) multiple copies of the target gene are made by repeated melting of complementary strands, hybridization of primers, and new synthesis (◀ Figure 12.2). From a single copy of a gene, several million copies can be made for subsequent analyses. PCR finds wide applications in microbial ecology.

Mastering Microbiology
Art Activity: Figure 19.15 Steps in single-gene biodiversity analysis of a microbial community

PCR and Microbial Community Analysis

Which genes are best suited to be target genes for microbial community analyses? Because genes encoding the small subunit ribosomal (SSU) rRNAs are phylogenetically informative and techniques for their analysis are well developed (◀ Section 13.11), they are widely used in community analyses. Moreover, because rRNA genes are universal and contain several regions of high sequence conservation, it is possible to amplify them from all organisms using only a few different PCR primers, even though the organisms may be phylogenetically distantly related. In addition to rRNA genes, genes that encode enzymes for metabolic functions unique to a specific organism or group of related organisms can be the target genes (Table 19.3). Many of the organisms that harbor these genes are described in Chapter 15.

Genes such as those encoding rRNAs that have retained ancestral function while changing in sequence over time as species have diverged are called *orthologs* (◀ Sections 13.8 and 13.11 and Figure 13.16). Assuming absence of horizontal gene transfer (◀ Section 13.9), organisms that share the same or very closely related orthologous genes are called a **phylotype**. In microbial ecology, the phylotype concept is primarily used to provide a natural (phylogenetic) framework for describing the microbial diversity of a given habitat, regardless of whether the identified phylotypes are cultured organisms or not. Thus, the word *phylotype* is widely used to describe the microbial diversity of a habitat based solely on nucleic acid sequences. It is only when additional physiological and genetic information becomes available, typically after the organism is brought into laboratory culture (Sections 19.2 and 19.3), that proposing a genus and species name for a phylotype becomes possible.

In a typical molecular community analysis experiment, total DNA is isolated from a microbial habitat (Figure 19.16). Commercially

TABLE 19.3 Genes commonly used for evaluating specific microbial processes in the environment using PCR

Metabolic process[a]	Target gene	Encoded enzyme
Denitrification	*narG*	Nitrate reductase
	nirK, nirS	Nitrite reductase
	norB	Nitric oxide reductase
	nosZ	Nitrous oxide reductase
Nitrogen fixation	*nifH*	Nitrogenase
Nitrification	*amoA*	Ammonia monooxygenase
Methane oxidation	*pmoA*	Methane monooxygenase
Sulfate reduction	*apsA*	Adenosine phosphosulfate reductase
	dsrAB	Sulfite reductase
Methane production	*mcrA*	Methyl coenzyme M reductase
Degradation of petroleum compounds	*nahA*	Naphthalene dioxygenase
	alkB	Alkane hydroxylase
Anoxygenic photosynthesis	*pufM*	M subunit of photosynthetic reaction center

[a]All of these metabolic processes are discussed in Chapter 14.

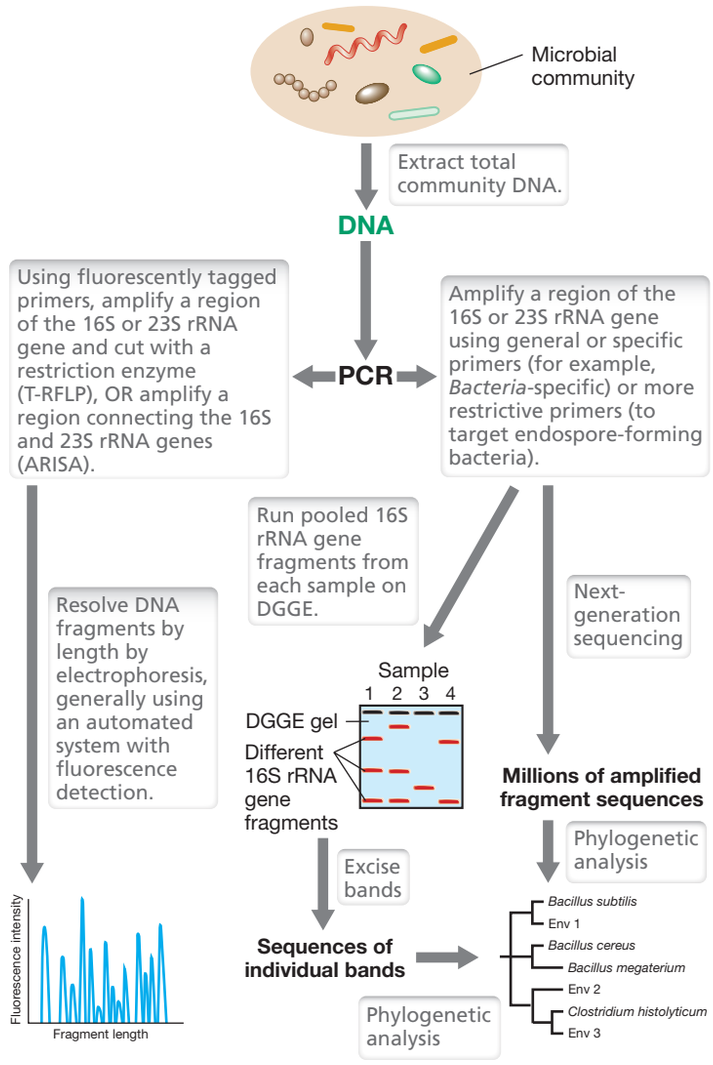


Figure 19.16 Steps in single-gene biodiversity analysis of a microbial community. From total community DNA, 16S rRNA genes are amplified using primers that target only *Firmicutes*, a group of gram-positive *Bacteria* that includes the endospore-forming genera *Bacillus* and *Clostridium*. The 16S rRNA gene products obtained from PCR are then either separated by DGGE or sequenced directly by next-generation sequencing; from the sequence data, a phylogenetic tree is generated. "Env" indicates an environmental sequence (phylotype). Alternatively, in T-RFLP analyses, the PCR-amplified products are labeled with a fluorescent dye and fragmented by cutting with a restriction enzyme before electrophoresis. The number of peaks identified by fluorescence corresponds to the number of phylotypes.

available kits that yield high-purity DNA from soil or other complex habitats are available for this purpose. The DNA obtained is a mixture of genomic DNA from all of the microorganisms that were in the sample from the habitat (see Figure 19.17). From this mixture, PCR is used to amplify the target gene and make multiple copies of each variant (phylotype) of the target gene. If RNA is isolated instead of DNA (to detect those genes being transcribed), the RNA can be converted into complementary DNA (cDNA) by the enzyme reverse transcriptase (◀ Section 11.11) and the cDNA subjected to PCR as for isolated DNA. However, regardless of whether DNA or RNA is originally isolated, the different phylotypes need to be sorted out following the PCR step before they can be sequenced. Sorting can be accomplished using one of three different methods: (1) physical separation by gel electrophoresis (◀ Section 12.2), (2) clone library construction (◀ Sections 12.2 and 12.9), and (3) next-generation sequencing technology (◀ Section 10.2). We consider these methods now.

Denaturing Gradient Gel Electrophoresis: Separating Very Similar Genes

One method to resolve phylotypes is **denaturing gradient gel electrophoresis (DGGE)**, which separates genes of the same *size* that differ in their melting (denaturing) profile because of differences in their *base sequence* (**Figure 19.17*a*, *b***). DGGE employs a gradient of a DNA denaturant, typically a mixture of urea and formamide. When a double-stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to "melt"; at this point, their migration stops (Figure 19.17*b*, *c*). Differences in base sequence cause differences in the melting properties of DNA. Thus, the different bands observed in a DGGE gel are phylotypes that can differ in base sequence significantly or by as little as a single base change.

Once DGGE has been performed, the individual bands are excised and sequenced (Figure 19.17). With 16S rRNA as the target gene, for example, the DGGE pattern immediately reveals the number of phylotypes (distinct 16S rRNA genes) present in a habitat (Figure 19.17*c*). Although not suited for the analysis of highly complex communities, since individual bands may not be fully resolved, the method provides an excellent mechanism to quickly evaluate temporal and spatial shifts in microbial community structure (Figure 19.17*c*). If PCR primers specific for genes other than 16S rRNA are used, such as a metabolic gene (Table 19.3), the variants of this specific gene that exist in the sample can also be assessed. Thus, although the number of bands on a DGGE gel is an overview of the biodiversity in a habitat (Figure 19.17*c*), sequence analysis is still required for identification and to infer phylogenetic relationship.

T-RFLP and ARISA

A rapid method of microbial community analysis is *terminal restriction fragment length polymorphism* (*T-RFLP*). In this method a target gene (usually an rRNA gene) is amplified by PCR from community DNA using a primer set in which one of the primers is end-labeled with a fluorescent dye. The PCR products are then treated with a restriction enzyme (◀ Section 12.2) that cuts the DNA at specific sequences. This generates a series of DNA fragments of varying length, the number of which depends on how many restriction cut sites exist in the DNA. The fluorescently labeled terminal fragments are then separated by size on an automated DNA sequencing instrument that detects fluorescent fragments (thus, only the terminal dye-labeled fragments are detected). The pattern obtained shows the

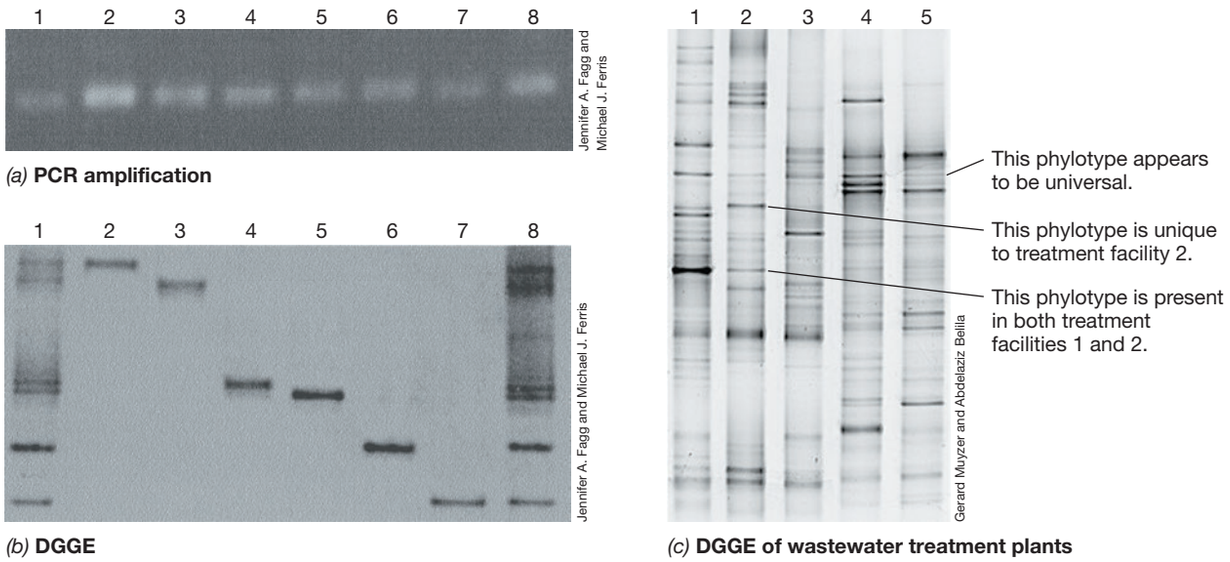


Figure 19.17 PCR and DGGE gels. Bulk DNA was isolated from a microbial community and amplified by PCR using primers for 16S rRNA genes of *Bacteria* (*a*, lanes 1 and 8). Six bands later resolved by DGGE (*b*, lanes 2–7) were excised and reamplified and each gave a single band at the same location on the PCR gel (*a*, lanes 2–7). However, by DGGE analysis, each band migrated to a different location on the gel (*b*, lanes 2–7). Note that all bands migrate to the same location in the nondenaturing PCR gel because they are all of the same size, but they migrate to different locations on the DGGE gel because they have different sequences. *(c)* DGGE profiles of microbial communities from different wastewater treatment facilities amplified using primers for the 16S rRNA genes of *Bacteria*.

rRNA sequence variation and general abundance of different sequence types (fragment fluorescence intensity) in the microbial community sampled (Figure 19.16).

DGGE and T-RFLP both measure *single-gene diversity*, but in different ways. The pattern of bands on a DGGE gel reflects the number of same-length sequence variants of a single gene (Figure 19.17), whereas the pattern of bands on a T-RFLP gel reflects variants differing in DNA sequence of a single gene as measured by differences in restriction enzyme cut sites. The information obtained from a T-RFLP analysis, in addition to providing insight into the diversity and population abundances of a microbial community, can also be used to infer phylogeny. Diagnostic information for each fragment includes knowledge of sequences near both ends (primer sequence and restriction enzyme cut site), knowledge that a second restriction site does not exist within the fragment, and fragment length. Using specialized software, this information can be used to search for matching 16S rRNA sequences in public databases. Although this is of some predictive value, closely related sequences are often not differentiated by these criteria. Thus, T-RFLP generally underestimates the diversity within a microbial community.

A technique related to T-RFLP that provides more detailed analysis of microbial communities is *automated ribosomal intergenic spacer analysis* (*ARISA*), which exploits the typical proximity of 16S and 23S rRNA genes in the genomes of *Bacteria* and *Archaea*. The DNA separating these two genes, called the *internal transcribed spacer* (*ITS*) region, differs in length among species and often also differs in length among the multiple rRNA operons of a single species (**Figure 19.18*a***). The PCR primers for ARISA are complementary to conserved sequences in the 16S and 23S rRNA genes that flank the spacer region. Amplification (Figure 19.18*b*) and analysis (Figure 19.18*c*) are conducted as described for T-RFLP, resulting in a complex pattern of bands that can be used for community analysis. However, ARISA differs from T-RFLP in that ARISA does not require a restriction enzyme digestion following PCR amplification. The word "automated" in the ARISA acronym refers to the use of a DNA sequencing instrument that automatically identifies and assigns sizes to each dye-labeled fragment (Figure 19.18*c*), as can also be done in T-RFLP analyses. ARISA has received greatest application in the study of microbial community dynamics by monitoring, for example, changes in the presence and relative abundance of a specific community member through time and space.

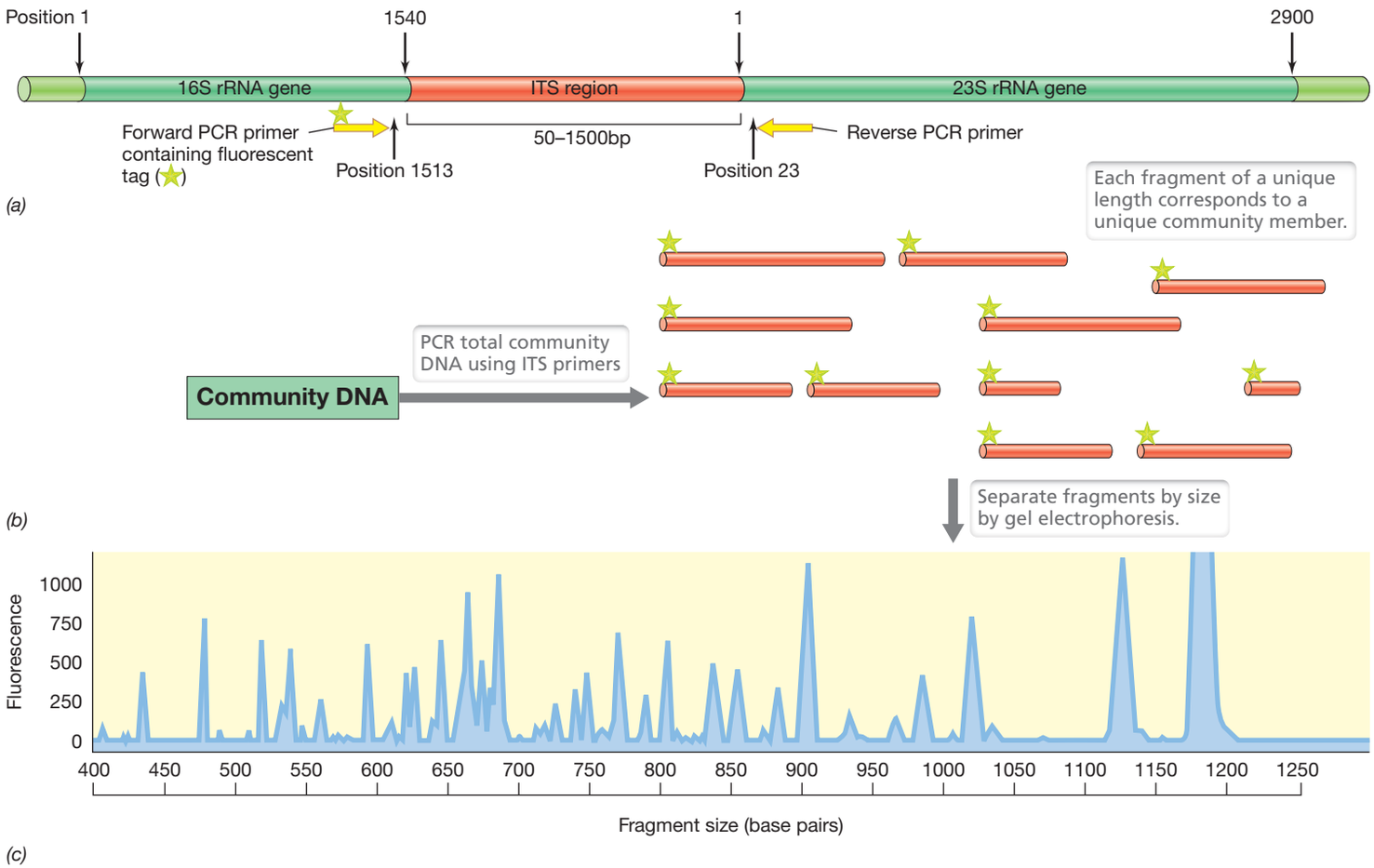


Figure 19.18 Automated ribosomal intergenic spacer analysis (ARISA). *(a)* Structure of rRNA operon spanning the 16S rRNA gene (positions 1–1540), an internal transcribed spacer (ITS) region of variable length, and the 23S rRNA gene (positions 1–2900). The PCR primers, one labeled with a fluorescent dye, are complementary to conserved sequences near the ITS region. *(b)* Amplified DNA fragments of different lengths, each corresponding to a community member. *(c)* Fragment analysis determined by an automated DNA sequencer. The peaks, which correspond to different ITS regions, can be identified by cloning and sequencing the amplified products.

Diversity Studies Using Clone Libraries or Next-Generation Sequencing

Early molecular microbial diversity research relied on the construction of clone libraries to separate individual amplified DNA molecules (*amplicons*); each clone in the library contained a unique sequence that was then used as a template for sequence determination (◀ Sections 10.2 and 12.2). Figure 19.17*a* shows that a 16S rRNA gene amplicon mixture appears as a single band when run on a nondenaturing gel. However, because the amplified target gene came from a *mixture* of different cells, the different phylotypes in the single band have different sequences and need to be sorted out before they are sequenced. Today this is almost exclusively done by next-generation sequencing systems (◀ Section 10.2) rather than by DGGE or by cloning. There are many such sequencing platforms available (**Figure 19.19**), and each has advantages and disadvantages. Long sequence reads are particularly useful for environmental metagenomic studies where the goal is to reconstruct genomes from members of the microbial community (Section 19.8). The MinION (nanopore technology, ◀ Explore the Microbial World, "DNA Sequencing in the Palm of Your Hand," Chapter 10) sequencing system is widely useful for this purpose because it can generate the long sequence reads necessary for genome assembly purposes. Once a genome is assembled, sequence accuracy can be improved using other platforms better capable of sequencing short DNA fragments at high fidelity (Figure 19.19*a*).

MinION and these other next-generation sequencing systems do not require a cloning step, as individual DNA fragments are separated and amplified on the sequencing device itself; thus, PCR products can be used directly for sequencing. Since millions of amplification reactions are then conducted simultaneously, the total number of sequencing reads vastly exceeds what is possible by sequencing individual clones obtained in a clone library one at a time (Figure 19.19). This tremendous volume of sequence data allows for what has been called *deep sequence analyses*, meaning that minor phylotypes that were possibly missed by the more limited and expensive clone library method are now revealed (Figure 19.19*b*). For example, if a particular phylotype were present at only 0.01% in a library of cloned sequences, sequencing of a thousand clones or more would be needed to ever detect it. By contrast, next-generation sequencing would detect this low-abundance phylotype along with its more abundant neighbors. This collection of minor phylotypes, which represent a substantial fraction of total diversity but only a minor component of total organism abundance in most environments, has been called the *rare biosphere* (Figure 19.19). Once next-generation sequencing reveals these rare but potentially very important components of a microbial community, various tools in the microbial ecologist's toolbox can be deployed to learn more about their activities and overall role in their ecosystem.

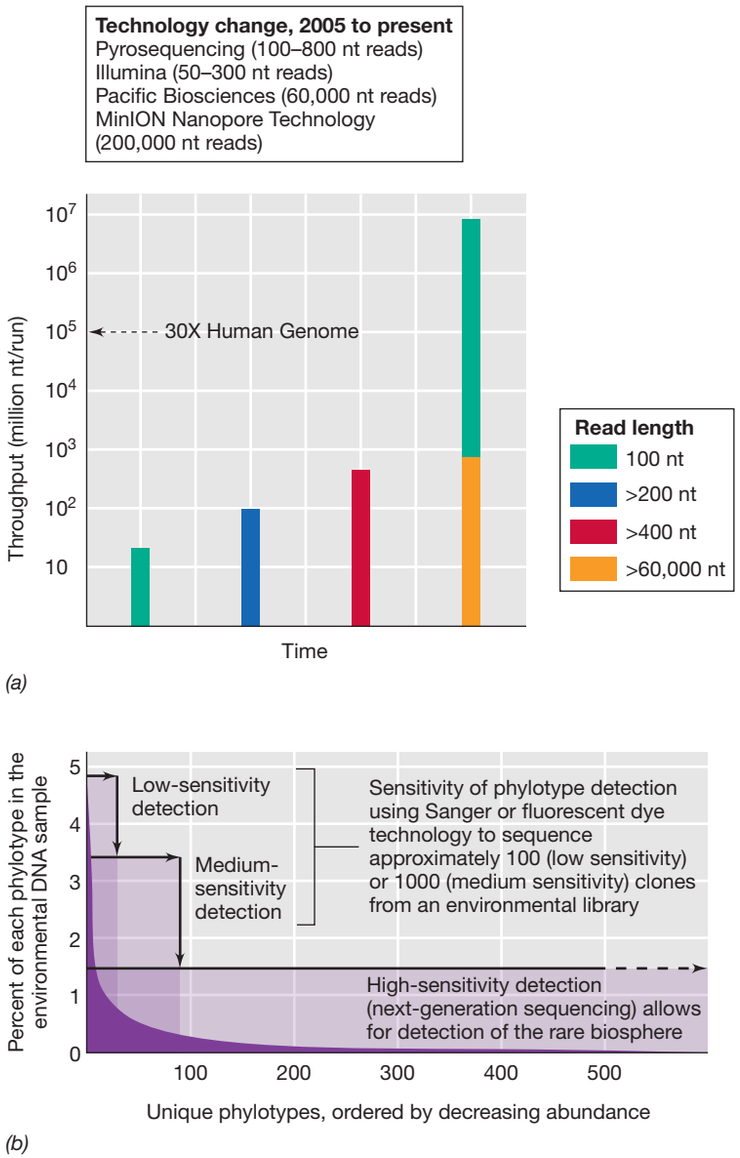


Figure 19.19 Community diversity analyses using next-generation sequencing technology. *(a)* Current sequencing platforms (◀ Section 10.2) have the capacity to generate 10^{12} nucleotides (nt) of sequence in a single sequencing run (requiring a week or less), with individual read lengths varying from 100 to 800 nucleotides. The two segments in the rightmost bar show that technologies generating longer reads have lower throughput per sequencing run. *(b)* This enormous sequencing capacity revealed many unique phylotypes that were not detected using DGGE or clone library sequencing. Fewer than 100 unique phylotypes would be detected by Sanger (first-generation) sequencing of 1000 clones in a library of 16S rRNA gene PCR amplicons. Jed Fuhrman is acknowledged for input to part *b*.

Results of PCR Phylogenetic Analyses

Phylogenetic analyses of microbial communities have yielded surprising results. For example, using the gene encoding 16S rRNA as the target, analyses of natural microbial communities typically show that many phylogenetically distinct *Bacteria* and *Archaea* (phylotypes) are present whose rRNA gene sequences differ from those of all known laboratory cultures. Moreover, using quantitative PCR (qPCR, a variant of PCR that allows each phylotype to be quantified as well as amplified, ◀ Section 12.1), it has often been observed that the most abundant phylotypes in a natural microbial community are ones that have thus far defied laboratory culture. These sobering results make it clear that our knowledge of microbial diversity from enrichment cultures is far from complete and that enrichment bias (Section 19.1) is a serious problem in culture-dependent biodiversity studies. Obviously, much work remains to put our abilities to culture microbes on a par with our existing abilities to detect and identify them in nature.

Check Your Understanding

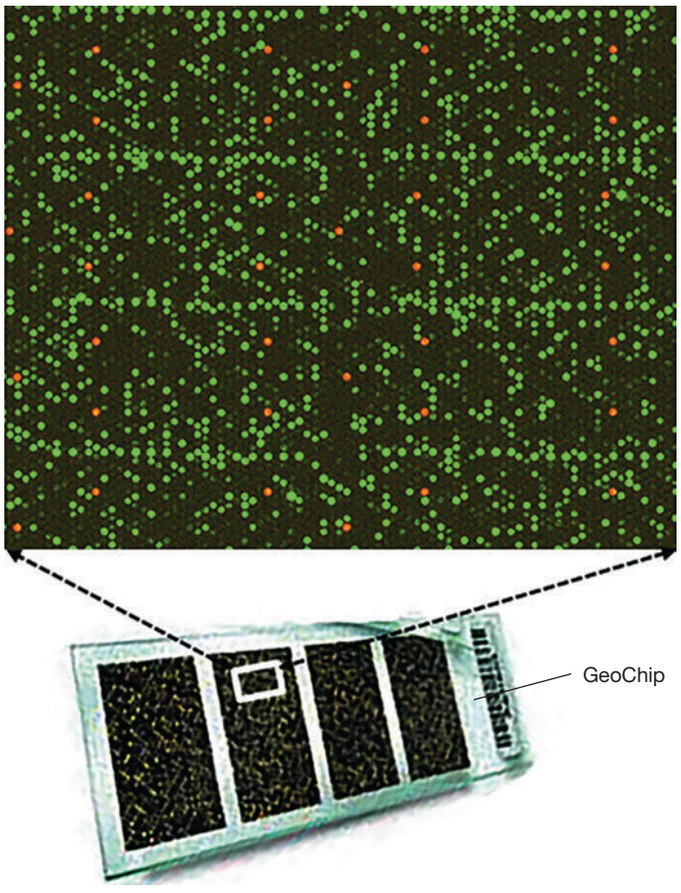
- What could you conclude from PCR/DGGE analysis of a sample that yielded one size band by PCR and one band by DGGE? One size band by PCR and four bands by DGGE?
- What surprising finding has come out of many molecular studies of natural habitats using 16S rRNA as the target gene?
- How has next-generation sequencing technology altered our understanding of microbial community diversity?

19.7 Microarrays for Analysis of Microbial Phylogenetic and Functional Diversity

We previously considered the use of DNA chips—a type of *microarray*—for assessing overall gene expression in a microbial pure culture (◀ Section 10.8). Specific microarrays can also be constructed for rapid gene-based analyses of biodiversity and the functional potential of natural microbial communities. Microarrays designed to exclusively target different 16S rRNA sequence types (phylotypes), called *PhyloChips*, have been used in the past, but microarrays employed for environmental studies now focus on metabolic genes of biogeochemical significance. This type of microarray, the *GeoChip*, detects genes required for selected metabolic processes, including sulfate reduction, ammonia oxidation, denitrification, and nitrogen fixation (**Figure 19.20** and Table 19.3).

The GeoChip—also called a *functional gene microarray*—targets genes that function in a measurable environmental process. However, because genes encoding enzymes of similar function can vary significantly in sequence, the arrays must contain many thousands of probes in order to achieve reasonable coverage of natural diversity. Even then, such arrays may only sample a fraction of the actual functional diversity in a habitat. Successful use of a GeoChip requires several steps: (1) isolation of total community DNA from the sample, (2) random sequence amplification (as is also used for amplification of DNA from a single cell, ◀ Section 10.11), (3) fluorescence labeling of the environmental DNA, and (4) hybridizing the environmental DNA to probes on the GeoChip. The GeoChip functional gene microarray contains over 160,000 probes covering more than 1400 gene families encoding proteins that participate in carbon, nitrogen, and sulfur cycling processes. By measuring the hybridization signal conferred by binding of environmentally derived DNA to each probe on the microarray (Figure 19.20), a rapid appraisal of the potential metabolisms operating in a particular habitat can be obtained.

GeoChips circumvent many of the time-consuming steps of molecular microbial ecology—PCR, DGGE, cloning, and sequencing (Figure 19.16). In addition, the GeoChip is useful for detecting low-abundance genes that would be difficult to identify by sequencing and also offers some information on actual gene abundance. However, an important caveat to the use of microarrays is the possibility



(a)

Functional category	Gene families	Total probes	Database gene coverage (%)
Carbon cycling	149	26922	49
Nitrogen cycling	32	6493	52
Sulfur cycling	27	4739	64
Phosphorus	7	3260	52
Metal homeostasis	121	43432	47
Viruses	115	2857	55
Other	81	10380	42
Organic remediation	104	11591	41
Virulence	639	21152	45
Secondary metabolism	68	4032	56
Electron transfer	15	797	65
Stress response	89	26306	33
TOTAL	**1447**	**161,961**	**44**

(b)

Figure 19.20 GeoChip analysis of functional gene diversity. The GeoChip contains over 161,000 probes covering more than 365,000 gene sequences in public databases, encompassing most major biogeochemical processes. The image shows green fluorescence of varying intensity (approximating gene abundance) following hybridization of fluorescent dye–labeled environmental DNA to the individual probes in one region of the high-density array of probes. The red spots correspond to repeated applications of a known amount of a reference DNA standard. A red-dye-labeled probe complementary to the reference standard was added to the environmental DNA prior to hybridization. Red fluorescence of equal intensity among the reference standard spots confirms that hybridization was uniform throughout the array.

of *nonspecific hybridization*. That is, gene variants that are closely related in sequence may not be resolved in a microarray because of overlapping hybridization patterns. Moreover, totally unrelated genes may yield false positive results if they are sufficiently complementary in base sequence to hybridize to the probe. And finally, unlike nucleic acid sequencing, whose costs keep plummeting yearly, designing and manufacturing gene chips are not yet inexpensive endeavors. Combined with culture-independent phylogenetic diversity studies (Section 19.6), functional gene arrays offer the microbial ecologist a detailed picture of both the diversity of organisms and the diversity of metabolisms in any given environment. However, what the GeoChip and related functional gene arrays *do not* reveal is actual metabolic activity; they show only the *genetic potential* for metabolic activity. We got a taste of how metabolic activity can be measured using BONCAT, a microscopic method of detecting metabolic activity (Figure 19.15). In the next section, we explore other methods of assessing microbial activity that use omics, the most powerful tools in the microbial ecologist's toolbox.

Check Your Understanding

- What are some caveats of interpreting a hybridization signal on the GeoChip?
- What are the advantages and disadvantages of microarray technology compared to sequencing PCR products?

19.8 Environmental Multi-omics: Integration of Genomics, Transcriptomics, Proteomics, and Metabolomics

As was presented in Chapter 10, a more complete understanding of how a microorganism functions requires an integrated accounting of all central cellular processes. These include gene expression, functional knowledge of all gene products and product activities, and all metabolites produced during growth. The analytical tools used to characterize those processes in pure cultures (◀ Sections 10.11–10.13) are now being applied to the study of complex microbial communities to yield an unprecedented view of microbial ecology.

Characterization of a natural system is a much more complex problem than the analysis of a single organism. Not only must processes of each organism be measured, but the many changing biotic and abiotic interactions that modify those processes must also be resolved. Here we consider the methods of genomic, transcriptomic, proteomic, and metabolomic analysis required to unveil patterns of microbial diversity that will ultimately lead to a more predictive understanding of microbial community function and response to environmental change. Collectively called omics or, when used in combination, *multi-omics*, these rapidly evolving methods are having a dramatic impact on our understanding of the structure and function of natural microbial communities. We begin with an overview of individual omics (**Figure 19.21**), and then present exemplar case studies of their application.

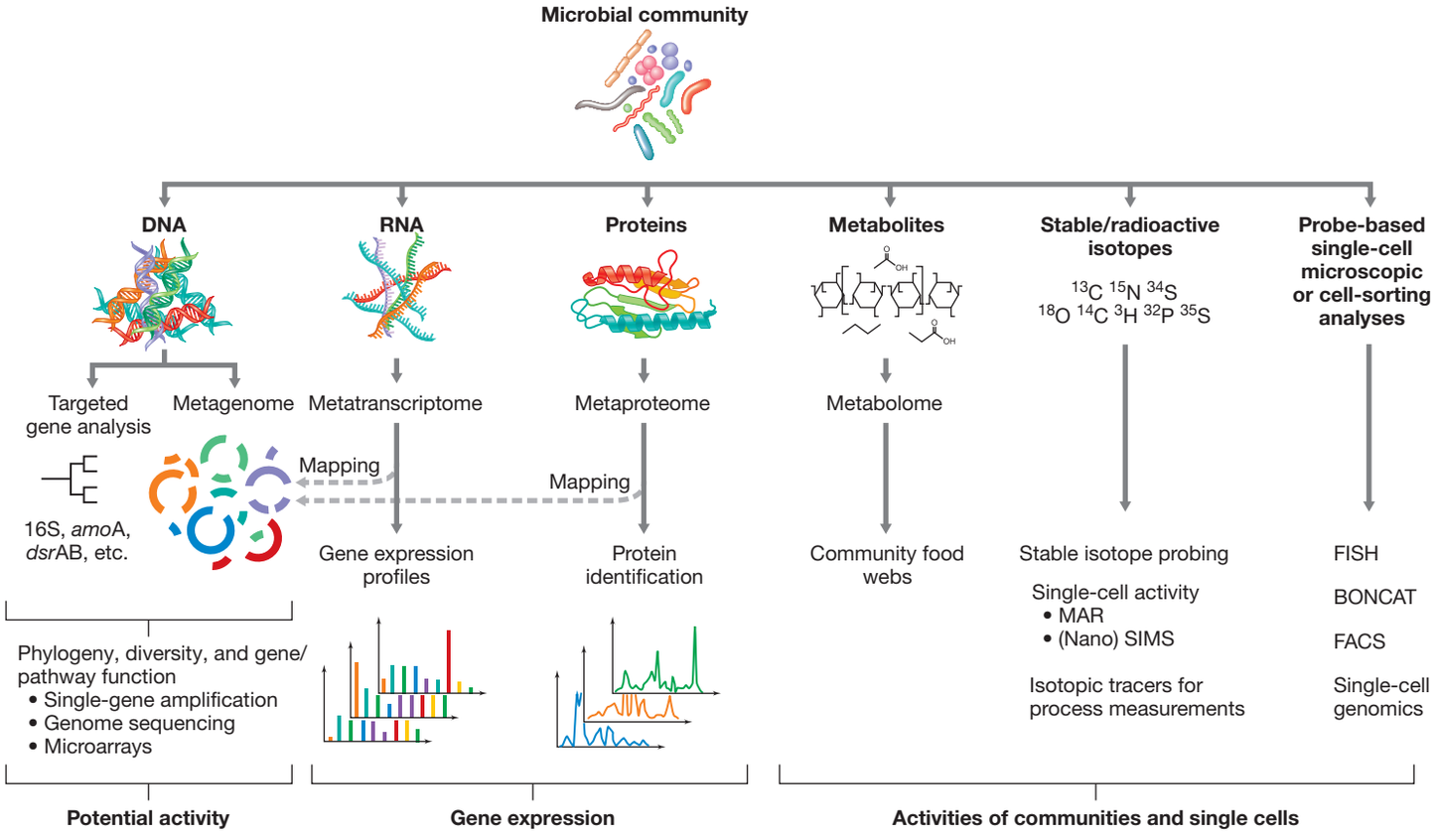
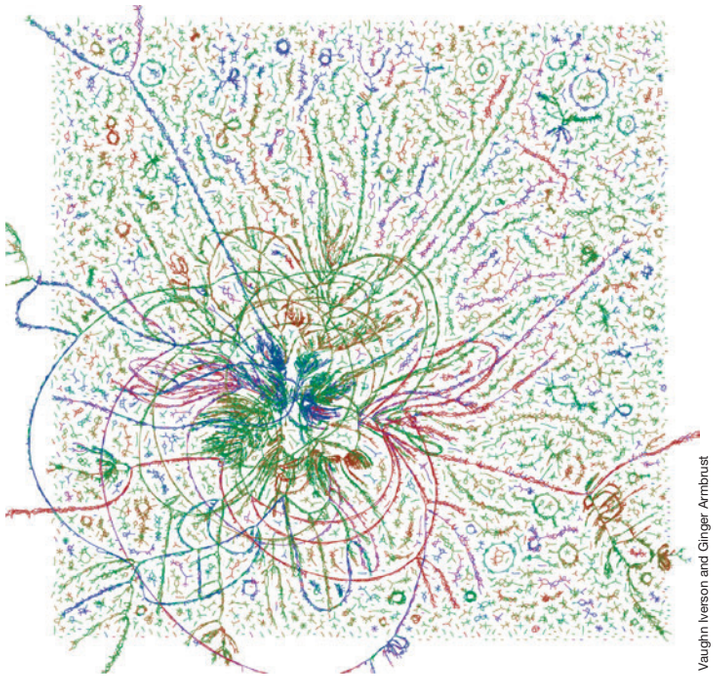


Figure 19.21 Multi-omics methods overview. The combined use of advanced genetic, molecular, microscopic, and isotopic methods is revolutionizing the study of natural microbial communities. Assignment of protein and transcript sequences to specific community members is accomplished by mapping their sequences on unique genomes assembled from metagenomic sequences. Abbreviations: FISH, fluorescence in situ hybridization (Figures 19.11–19.14); BONCAT, bioorthogonal noncanonical amino acid tagging (Figure 19.15); FACS, fluorescence-activated cell sorting (see Figure 19.42); MAR, microautoradiography (see Figure 19.41); SIMS, secondary ion mass spectrometry (see Figure 19.39).

Metagenomics and Reconstructing Environmental Genomes

An encompassing approach to the genetic characterization of microbial communities is **environmental genomics**, also called **metagenomics**. Before the metagenomics era, microbial community analyses typically focused on the diversity of a *single* gene in an environmental sample. By contrast, in environmental genomics, *all* genes in a given microbial community can be sampled and, if the experiment is properly designed, the information obtained can support a much deeper understanding of the structure and function of the community than can single-gene analyses.

The goal of a metagenomics study today is to use next-generation DNA sequencing (◀ Section 10.2) to identify as many genes as possible from an environmental DNA sample and determine the phylogeny of the organism(s) to which the genes belong. Although complete and finished genomes are often not the goal of metagenomics, there is increasing interest in assembling individual genomes from large metagenomic datasets to at least the draft stage. Rather than simply generating a list of all genes present in an environment, nearly complete genomes can better connect functional and phylogenetic aspects of a microbial habitat. An example of this is shown in the "connection graph" of **Figure 19.22** that depicts an assembly of genomes from a coastal marine water sample. A total of 58.5 *billion* nucleotides in the metagenome were used to stitch together these complete and near-complete genomes. Such massive metagenomic undertakings can be highly complex enterprises but often reveal links between physiologies and phylogenies not obtainable in the absence of reconstructed genomes.



Vaughn Iverson and Ginger Armbrust

Figure 19.22 Genome assembly from a coastal marine metagenome consisting of 58.5 billion nucleotides of sequence. This "connection graph" is intended as a visual representation of the complexity and abundance of partial and complete genomes assembled from the water sample. The long strands, colored by differences in the percentage of guanine plus cytosine content, correspond to prokaryotic genomes and the small circular strands are most likely from viruses or plasmids.

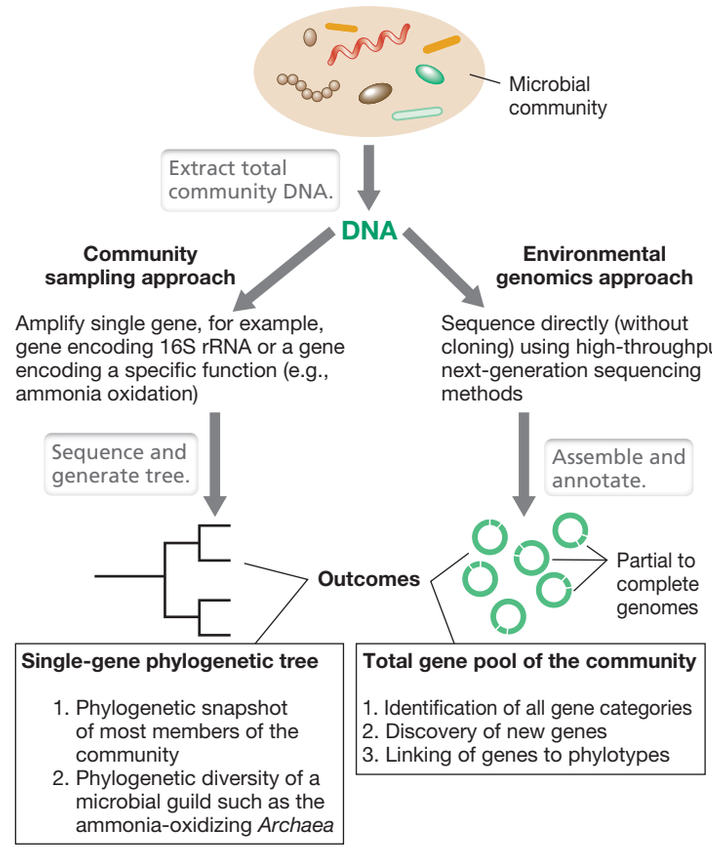


Figure 19.23 Single-gene versus environmental genomic approaches to microbial community analysis. In the environmental genomic approach, all community DNA is sequenced, but the assembled genomes may not all be complete. Total gene recovery is variable and depends on several factors including the complexity of the habitat and the amount of sequence determined. Recovery is typically better when diversity is low and sequence redundancy is high.

A problem with genome assembly from a mixture of environmental DNA sequence reads, however, is that the genomes obtained are unlikely to be either complete or clonal, instead being composed of fragments of DNA from closely related strains of a species (**Figure 19.23**). This has proven to be a major problem in the assembly of soil microbial genomes, for example, from large pools of metagenomic data. A single gram of fertile soil contains about 10^{12} bacterial and archaeal genes and 10^{9} genomes; complete coverage of these genes is not yet feasible with available technology, even with the sequencing of 300 billion nucleotides in one soil study! Single-cell genomics (◀ Section 10.11 and Figure 10.30) may ultimately overcome this problem (see also Section 19.12), but by definition, this method only provides information from a single microbial cell.

Of critical importance in evaluating a genome reconstructed from metagenomic DNA is to assess whether it contains all of the genes required by a cell (for example, all necessary tRNA and rRNA genes and genes encoding essential proteins such as DNA and RNA polymerases) and is therefore a legitimate genome candidate. In addition, an assessment of the relative abundance of genes encoding specific functions is equally valuable, since abundance changes suggest interactions among species or a common response to a particular environmental variable. For example, if a high number of genes were recovered in the pathway for nitrogen fixation, this would

suggest that the environment sampled was limited in NH_4^+, NO_3^-, and other forms of fixed nitrogen, thus selecting for nitrogen-fixing bacteria. Figure 19.23 contrasts the environmental genomic approach with single-gene analysis of microbial communities.

Although metagenomics can reveal much about a microbial habitat, there are many things metagenomics cannot tell us about environmental microbial communities. Currently no methods are available for translating metagenomic sequence data into fundamental physiological information about the microbial community, such as the maximum specific growth rate of different species, their affinities for nutrients, their optimum, minimum, and maximum pH or temperature for growth, or their speed of recovery from starvation. Moreover, any metabolisms never before encountered are unlikely to be deduced from nucleotide sequence data alone. These realities once again underscore why it is important to culture new microbes from nature; at present, there is simply no substitute for culture-based characterization to define many critical aspects of a microbe's functional biology.

Some Examples of Environmental Genomics

Environmental genomics can detect both new genes in known organisms and known genes in new organisms. A large number of interesting microbial communities have been probed using early metagenomic tools. In an early study of bacterial and archaeal diversity in the Sargasso Sea (a low-nutrient region of the Atlantic Ocean near Bermuda), over one billion nucleotides were sequenced and from this over 1800 bacterial and archaeal species were detected, including 148 previously unknown phylotypes and many novel genes. Many of these species had previously been missed using rRNA community analyses that employed PCR and cloning or PCR and DGGE (Section 19.6). Genes that fail to amplify, of course, remain undetected in community analyses, and cloning efficiency is far from 100%. Metagenomics sidesteps these problems by sequencing DNA directly without the need to amplify it or resolve different phylotypes before sequencing.

The Sargasso Sea metagenome study revealed several novel findings such as the presence of candidate ammonia-oxidizing genes in archaeal genomes, a result that was later confirmed with the isolation and description of a new group of *Archaea*, the *Thaumarchaeota* (◀ Section 17.5). Moreover, genes encoding *proteorhodopsin*, a light-sensitive proton pump present in certain *Proteobacteria* and related to bacteriorhodopsin of extreme halophiles (◀ Section 17.1), were found in the genomes of several new phylogenetic lineages of *Bacteria*. However, despite this major sequencing undertaking, much was missed. This is because 1 milliliter of seawater contains approximately 5 *trillion* base pairs (bp) of bacterial genomic DNA and would therefore require 5000 times the sequencing effort just to cover each base pair once on average! Hence, even with current technology (see Table 10.2)—which can generate over a trillion bp of sequence in a few days (Figure 19.19)—no one natural environment has yet been sequenced completely.

Genomic/metagenomic approaches have also revealed variations in genes associated with a single phylotype; that is, in strains that contain identical, or nearly identical, rRNA genes. For example, in studies of *Prochlorococcus*, the most abundant cyanobacterium (oxygenic phototroph) in the ocean (▶ Section 20.11), comparison of the genome sequences of cultured strains with *Prochlorococcus* genes obtained from metagenomic analyses of ocean water identified extensive regions shared between the cultured and environmental populations (**Figure 19.24**). This high level of gene conservation confirms that the organisms in culture are typical of environmental populations. In addition, however, these analyses also identified several highly variable regions in which the genomes of cultured strains differed significantly from those of environmental populations. These variable regions were clustered in the genome as *genomic islands* (chromosomal islands, ◀ Section 13.10), and likely encode

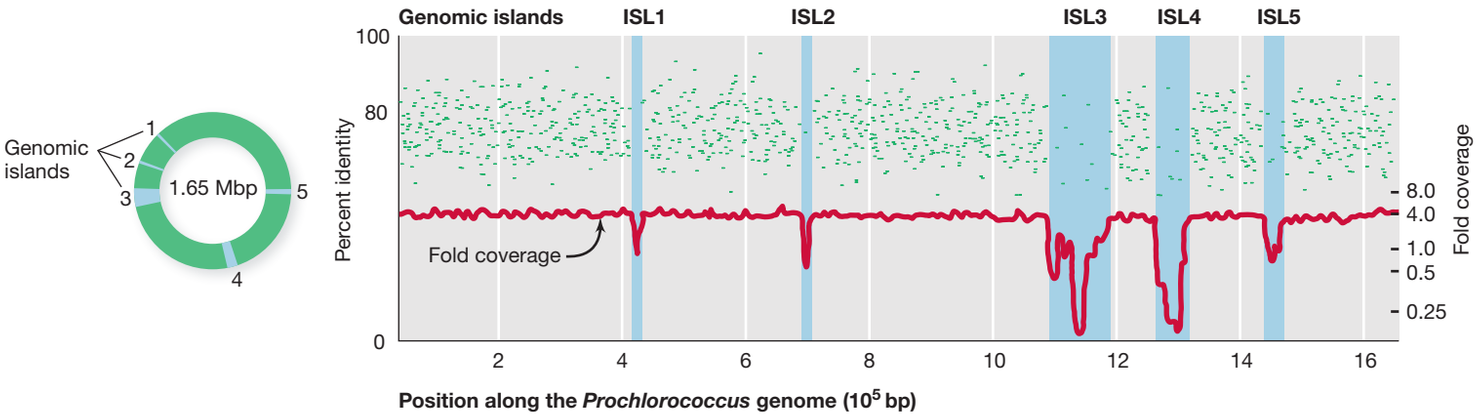


Figure 19.24 Metagenomic analysis. Sequences (represented as green dashes) from the Sargasso Sea metagenome that align to the genome sequence of a cultured *Prochlorococcus*, showing regions where the cultured strain has genes of high similarity (high percent identity) with sequences in the metagenome, and other regions (shaded) where it lacks genes in common (genomic islands, ISL1–ISL5). Since the DNA sequence contained within the genomic islands is thought to encode niche-specific functions, the cultured strain would likely not exhibit the same environmental distribution as strains containing all the island genes. Fold coverage is a measure of how completely the various regions in the *Prochlorococcus* genome are accounted for by similar sequences in the metagenome.

functions that control the growth response of particular *Prochlorococcus* populations to environmental variables unique to their habitat, such as temperature or light quality and intensity (▶ Section 20.11 and Figure 20.26).

Metatranscriptomics, Metaproteomics, and Metabolomics

As we discussed in Chapter 10, the genomics era has spawned several additional "omics," in particular, *metatranscriptomics*, *metaproteomics*, and *metabolomics*. **Metatranscriptomics** is analogous to metagenomics but analyzes the sequences of community *RNA* rather than community *DNA*. The isolated RNA is converted into cDNA by reverse transcription (◀ Section 11.11) before sequencing and analysis as for DNA. Whereas metagenomics describes the functional capacities of the community (for example, the relative abundance of specific genes), metatranscriptomics reveals which genes in the community *are actually being expressed* and the relative level of that expression, at a specific time and place (Figure 19.21). Because the expression of most genes in *Bacteria* and *Archaea* is controlled at the level of transcription (Chapter 7), mRNA abundance can be taken as a census of individual gene expression levels. Thus, gene transcript abundance determined for an entire community can be used to infer the operation of major metabolic processes catalyzed by that community at the time of sampling (**Figure 19.25**).

Metaproteomics, the measure of the diversity and abundance of different *proteins* in a community, is an even more direct measure of cell function than is metatranscriptomics. This is because different mRNAs have different half-lives and efficiencies of translation, and thus will not all yield the same number of protein copies. However, metaproteomics is a greater technical challenge than either metagenomics or metatranscriptomics. Protein identification, usually by

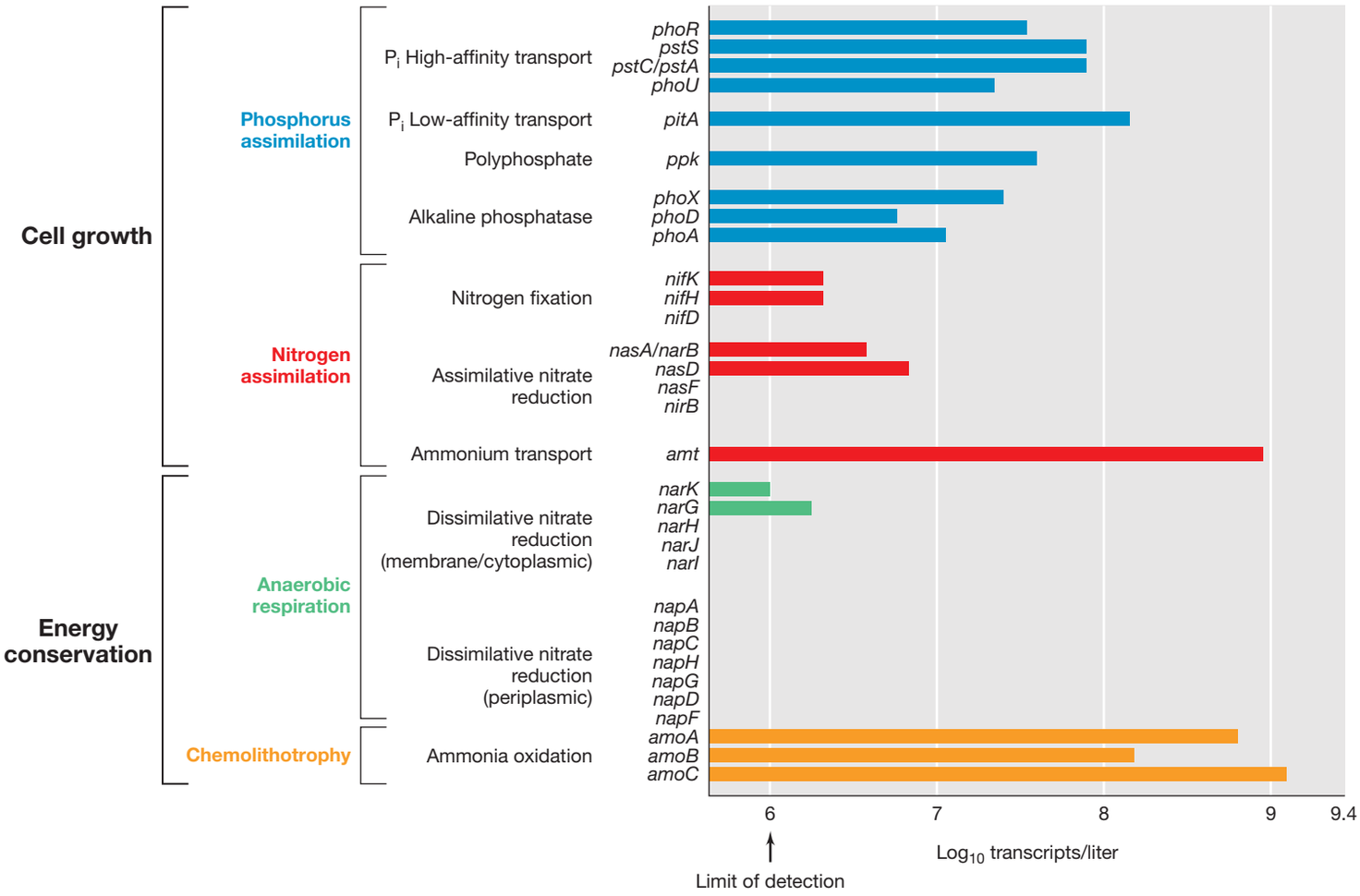


Figure 19.25 Metatranscriptomic analysis of coastal marine surface waters. Expression of genes for key steps in the N and P cycle in a seawater sample determined by sequencing environmental mRNA. These data showed that the microbial community was using both inorganic (high expression of P transporters) and organic (alkaline phosphatase) forms of phosphate (PO_4^{3-}). Low levels of transcripts for genes required for nitrate (NO_3^-) assimilation contrasted with the high expression of genes for ammonia (NH_3) transport and chemolithotrophic NH_3 oxidation. Also, as expected for oxic marine surface waters, there was little expression of genes for NO_3^- respiration. Data courtesy of Mary Ann Moran, University of Georgia Marine Sciences.

mass spectrometric characterization of peptides released from enzymatic digestion of the total protein pool by specific proteases (◀ Section 10.9), relies on naturally available material since it is not possible to amplify protein sequences as one does using PCR to amplify nucleic acids for sequencing. Protein identification also requires at least partial separation of the individual peptides in order to reduce complexity of the sample and a reference genome or metagenome to identify potential coding sequences (see Figure 19.21). **Metabolomics** is the comprehensive analysis of cellular and extracellular metabolites (also called **exometabolites**) of a microbial community and is the most direct measure of the food web structure of a community. As discussed in Chapter 10 (◀ Section 10.10), advanced methods in mass spectrometry are now providing the analytical power required to resolve and quantify the many hundreds of metabolites that form the food webs of natural microbial communities.

The utility and power of integrating omics methods for the study of natural microbial communities has been on display in many ecological studies published in the scientific literature in recent years and was evident in the metatranscriptomic study shown in Figure 19.25. We continue this theme here with two case studies, one employing metaproteomics and the other metabolomics.

Utility of Metaproteomics: An Environmental Case Study of Permafrost

Permafrost covers an estimated 20% of the land on Earth and sequesters large amounts of organic carbon. Because climate change will likely lead to the release of two major "greenhouse gases"—CO_2 and CH_4—from permafrost, identifying the microbes inhabiting actively melting permafrost along with their metabolic potential is essential for climate predictions. Using a combination of metagenomics (◀ Section 10.7) and metaproteomics (◀ Section 10.9), a revealing snapshot of the genes and proteins of the microbial community present in a permafrost meltwater bog has been obtained (**Figure 19.26**).

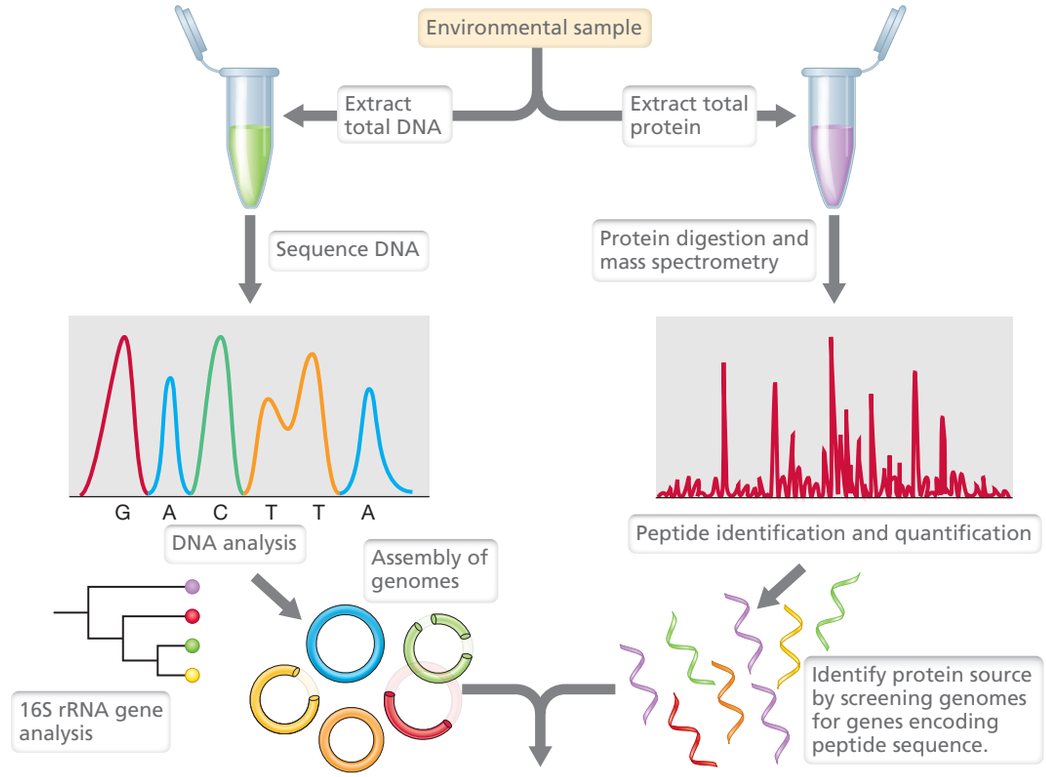
During the metagenomic analysis of the bog, DNA sequences were assembled and annotated into complete and partial genomes (Figure 19.26*a*). This analysis indicated that *Bacteria* of the phyla *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* predominated (Chapters 15 and 16), while methanogens (*Euryarchaeota*) were the dominant *Archaea* (Chapter 17). From the large amount of sequence data obtained, the genomes of three novel (and as yet uncultured) methanogens could be assembled without any cultures of the organisms having been obtained. This indicated that unique methanogens resided in the permafrost—most likely species that function well in the cold (psychrophiles)—and therefore that the potential for increasing rates of methanogenesis as the permafrost melted was high. Using the annotated DNA sequences and the output from the mass spectrometry detection of peptides, the identity of proteins extracted from the bog sample was determined along with their microbial sources (Figure 19.26*b*). The proteomics detected an abundance of functionally diverse proteins including those that participate in cellular housekeeping, transport, and organic carbon respiration. But importantly, and in agreement with the metagenomics data, several proteins associated with C_1 metabolism, including those necessary for both making methane (methanogenesis, ◀ Section 14.15) and oxidizing methane (methanotrophy, ◀ Section 14.16) as well as oxidizing and reducing carbon monoxide (CO dehydrogenases), were also detected in the bog microbial community.

This combined metagenomic and metaproteomic snapshot of a major microbial community highlights the power of omics for resolving the "who" and "how" of complex microbial communities and for using the results to predict the response(s) of these communities to environmental changes. In the case of permafrost melting, the potential for the release of large amounts of CO_2 (due to increased respiration of organic carbon) and CH_4 (due to increased activities of methanogens) from permafrost as climate change progresses (see Section 21.9) was clearly apparent (Figure 19.26).

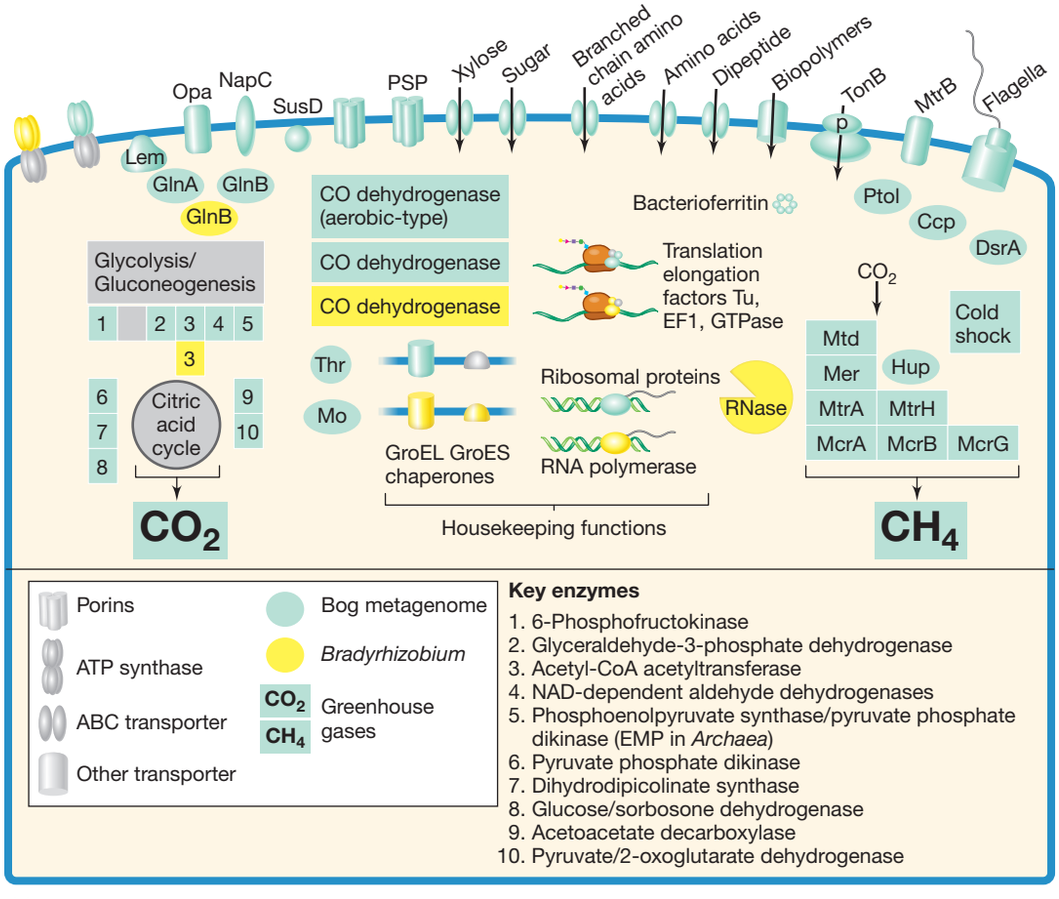
Utility of Metabolomics: An Environmental Case Study of Biocrusts

A study of the microbial ecology of biological soil crusts (biocrusts) offers an example of how metabolomics can provide new understanding of food webs that sustain natural microbial communities. Biocrusts are microbe-dominated communities that develop in arid environments of limited vegetation that in part function to reduce soil erosion (**Figure 19.27**; ▶ Section 20.6). They are relatively simple communities in which a dominant filamentous cyanobacterium, in this case *Microcoleus vaginatus* (a member of the *Oscillatoriales*, ◀ Section 15.3), is the primary producer and the ultimate source of dissolved carbon used by associated heterotrophic microbes. These communities persist in a desiccated, dormant state for extended periods but rapidly become active after a rainfall. Analysis of the biocrust metabolome using advanced methods of mass spectrometry identified and quantified hundreds of metabolites (e.g., adenine, aspartate, uracil, methylguanine, 2-isopropylmalate, methionine, guanidinobutanoate, glycine betaine, malate, succinate). Many of these metabolites are produced by *Microcoleus* and released to the environment for consumption by other members of the soil crust community. Since this cyanobacterium is unable to fix nitrogen, its nitrogen requirement is largely supplied by nitrogen-containing metabolites supplied by heterotrophic members of the community.

Characterization of the intracellular metabolites and exometabolites of a pure culture of *Microcoleus* showed that a significant fraction of its intracellular metabolites were released during growth and thus made available to heterotrophic community members as exometabolites (**Figure 19.28*a***). In turn, the heterotrophic community produced metabolites used selectively by the *Microcoleus* or by other heterotrophs (Figure 19.28*b*). Bacterial cultures isolated from the crust were found to have strong substrate preferences, each depleting a certain fraction of the metabolites while ignoring others. There was also little overlap in utilization; only two metabolites, glutamate and a second, unidentified compound, were used by all members of a group of bacterial isolates. Thus, each isolate from the biocrust likely occupies a specialized nutritional niche, using only a subset of the total pool of metabolites as nutrients. Analysis of the patterns of metabolite uptake and release suggested 80 potential cross-feeding patterns. In addition to providing an example of the power of metabolomics for untangling complex food



(a)



(b)

Figure 19.26 Meta-omics of permafrost. *(a)* Metagenomic and metaproteomic analysis of an environmental sample. Total DNA and protein are extracted from a sample and analyzed to identify microorganisms and their associated proteins. Following sequencing, the DNA is assembled into partial and complete individual genomes. The 16S rRNA gene can also be profiled to determine the phylogenetic affiliation of *Bacteria* and *Archaea* present in the sample. After identifying the digested proteins by mass spectrometry, their microbial source can be determined by searching for corresponding DNA sequences in the metagenomic data. *(b)* Visualization of a subset of the proteins identified from metagenomic and metaproteomic analysis of a bog sample. Ccp: cytochrome *c* peroxidase, DsrA: sulfite reductase subunit A, GlnA/GlnB: nitrogen storage, Hup: heterosulfide reductase, Lem: peptidoglycan-associated lipoprotein, Mtd/MtrA/MtrB/MtrH/McrA/McrB/McrG/Mer/Mo: methane metabolism, NapC: NapC/NirT cytochrome *c*, Opa: opacity protein, PSP: phosphate-selective porin, Ptol: periplasmic component of the Tol biopolymer transport system, Thr: thermosome, SusD: sulfate ABC transport system ATP-binding protein, TonB: periplasmic protein. Data adapted from Hultman, J., et al. 2015. *Nature 521*: 208.

Figure 19.27 Desert biocrust of the Colorado Plateau. *(a)* The thin desert biocrust is stabilized by the filamentous cyanobacterium *Microcoleus*, which binds together soil particles and reduces erosion. *(b, c)* Visible *Microcoleus* filaments (arrows) show the fine architecture of the crust. *Microcoleus* nourishes a rich community of primarily heterotrophic bacteria, releasing metabolites consumed by community members, which in turn produce metabolites consumed by the cyanobacterium. Environmental metabolomics (see Figure 19.28) has the power to unravel the complex food web that sustains these critical microbial communities in arid lands.

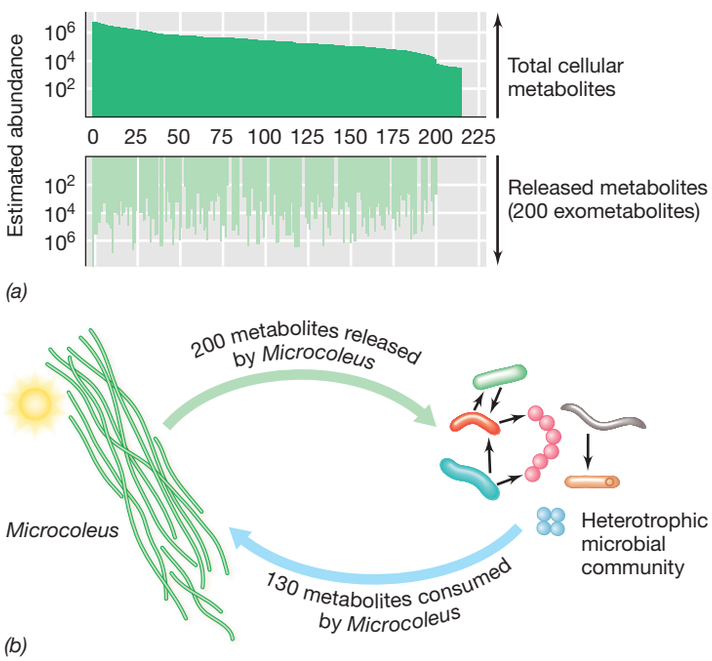


Figure 19.28 Metabolic network supporting the desert biocrust microbial community. *(a)* Abundance of each metabolite arranged in decreasing order (upper panel) The primary producer, the cyanobacterium *Microcoleus*, releases a large fraction of its intracellular metabolites (green) into the environment (blue). *(b).* These metabolites nourish a complex heterotrophic community that in turn produces metabolites nourishing *Microcoleus*. In addition, hundreds of additional metabolites consumed by the crust community were identified indicating additional metabolic interactions and cross-feeding among heterotrophs (arrows between bacterial cells). Data adapted from Baran, R., et al. 2015. *Nat. Commun. 6*: 8289, doi: 10.1038/ncomms9289.

webs, the use of this exometabolomic approach for identifying metabolites used by uncultured community members yields a second scientific benefit: It can guide researchers in formulating selective culture media for enrichment of heterotrophic bacteria that eluded initial cultivation efforts.

In the final part of this chapter, up next, we look at some other tools in the microbial ecologist's toolbox that can measure total microbial community activities.

Check Your Understanding

- What is a metaproteome, and how does it differ from a metagenome and from a metatranscriptome?
- How do environmental multi-omics approaches differ from environmental genomics or single-gene analyses, such as that based on 16S rRNA gene analysis for microbial community characterization?
- How can the most metabolically active cell populations in a community be identified using environmental omics methods?

IV • Measuring Microbial Activities in Nature

UNIT 5

Microbial communities manifest their presence through the concerted activities of individual microorganisms that together drive major biogeochemical processes. A powerful suite of isotopic and genetic methods combined with high-resolution microscopy and sensor technology have made it possible for the microbial ecologist to map these processes from the level of individual cells to entire environmental systems.

We wrap up this chapter by considering methods for measuring microbial activities in natural samples beyond those already discussed in Parts II and III. The techniques we consider here include the use of radioisotopes, microsensors, nanosensors, stable isotopes, and genomic methods. Activity measurements in a natural sample are *collective* estimates of the physiological reactions occurring in the entire microbial community, although several techniques to be discussed later (Sections 19.10–19.12) allow for a more targeted assessment of physiological activity. Activity measurements reveal both the types and rates of the major metabolic reactions occurring in a habitat, and the various techniques can be used alone or in combination in microbial community analyses. In conjunction with multi-omics analyses, these methods help define the structure and function of microbial ecosystems—the ultimate goal of microbial ecology—and often inform the design of enrichment cultures to bring microbial community members into the laboratory.

19.9 Chemical Assays, Radioisotopic Methods, Microsensors, and Nanosensors

In many studies, direct chemical measurements of microbial reactions are sufficient for assessing microbial activity in an environment. For example, the fate of lactate oxidation by sulfate-reducing bacteria in a sediment sample can be tracked easily. If sulfate-reducing bacteria are present and active in a sediment sample, then lactate added to the sediment will be consumed and SO_4^{2-} will be reduced to hydrogen sulfide (H_2S). Since lactate, SO_4^{2-}, and H_2S can all be measured specifically and with high sensitivity by chemical assay, the transformations of these substances relative to one another in a sample can easily be followed (**Figure 19.29*a***). However, when very high sensitivity is required, or when turnover rates need to be determined, *radioisotopes* are more useful than strictly chemical assays.

Another approach to the sensitive measurement of certain microbial process rates takes advantage of selective chemical inhibition of an enzyme specific to the process. For example, a widely employed chemical inhibitor uses acetylene to inhibit some processes, such as nitrification, or as an alternative substrate in another, such as nitrogen fixation. The final set of tools we will describe in this section are different types of microsensors and nanosensors used to measure an active microbial process by quantifying changes in the concentration of specific chemicals in time and space.

Radioisotopes

When very high sensitivity is required, or turnover rates need to be determined, or the fate of portions of a molecule needs to be followed, *radioisotopes* are more useful than strictly chemical assays. For instance, if measuring photoautotrophy is the goal, the light-dependent uptake of radioactive carbon dioxide ($^{14}CO_2$) into microbial cells can be measured (Figure 19.29*b*). If sulfate reduction is of interest, the rate of conversion of $^{35}SO_4^{2-}$ to $H_2{}^{35}S$ can be assessed (Figure 19.29*c*). Heterotrophic activities can be measured by tracking the release of $^{14}CO_2$ from ^{14}C-labeled organic compounds (Figure 19.29*d*), and so on.

Both isotopic and chemical methods are widely used in microbial ecology. To be valid, however, these must employ proper controls because some isotopic transformations might be due to abiotic processes. The *killed cell control* is an essential control in such experiments. That is, it is essential to show that the transformation being measured stops when chemical agents or heat treatments that kill microorganisms are applied to the sample. Formalin at a final concentration of 4% is commonly used as a chemical sterilant in microbial ecology studies. This kills all cells, and transformations that are insensitive to the presence of 4% formalin can be ascribed to abiotic processes (Figure 19.29*a*). Additional controls are also necessary. For example, if H_2-driven sulfate reduction is to be measured in a bottle, an essential control is to have a separate bottle identical in every way except that it lacks H_2 (Figure 19.29*c*). If photosynthesis is to be measured, a dark control is essential (Figure 19.29*b*), and so on. The art of doing good experimental science is in designing experiments with the correct controls, and this is especially true of bulk assays of microbial activity where particular subsets of the entire microbial community are targeted.

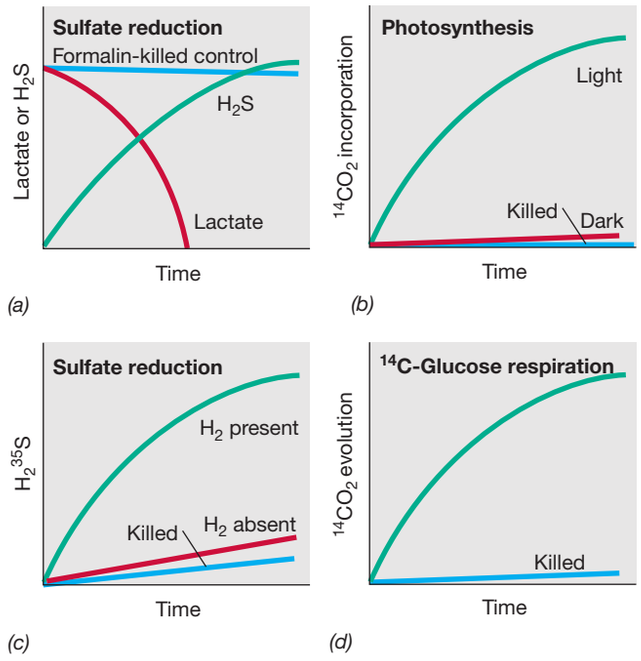


Figure 19.29 Microbial activity measurements. *(a)* Chemical measurements of lactate and H_2S transformations during SO_4^{2-} reduction. Radioisotopic measurements: *(b)* photosynthesis measured with $^{14}CO_2$; *(c)* SO_4^{2-} reduction measured with $^{35}SO_4^{2-}$ conversion to $H_2{}^{35}S$. *(d)* production of $^{14}CO_2$ from ^{14}C-glucose.

Acetylene as an Inhibitor and an Alternative Substrate

In some activity measurements, it is useful to inhibit the activities of certain organisms in order to focus on the activities of others. For example, the short-chain hydrocarbon acetylene ($HC{\equiv}CH$) is an inhibitor of several metabolisms, including nitrification (◀ Section 14.9), denitrification (◀ Section 14.11), and methanogenesis (◀ Section 14.15). In nitrification, acetylene inhibits the first step, ammonia oxidation to nitrite, while in denitrification, acetylene inhibits the terminal step, the reduction of nitrous oxide (N_2O) to N_2. Thus, acetylene can be used to focus on particular steps in these processes with the knowledge that the inhibited steps are not occurring. For example, the use of acetylene to block reduction of N_2O is a common assay for denitrification, using the production rate of N_2O (an easily assayed gas) in the presence of acetylene as a measure of the denitrification rate in an environmental sample. Acetylene and other metabolic inhibitors are useful in microbial ecology but must be used with the caveat that the inhibitor might also affect some unrecognized metabolic process that contributes to the activity being measured.

Acetylene is also commonly used for another purpose. The capacity for nitrogen fixation is widely distributed among *Bacteria* and *Archaea* (**Table 19.4**; ◀ Section 3.12). Nitrogenases are not specific for N_2 and also reduce other triply bonded compounds, such as acetylene, as alternative substrates. Unlike the reduction of N_2 to 2 NH_3 —a six-electron process—the reduction of acetylene by nitrogenase is only a two-electron process, *ethylene* ($H_2C{=}CH_2$) being the final product. Hence, the reduction of acetylene to ethylene provides a straightforward method for measuring nitrogenase activity

TABLE 19.4 Some nitrogen-fixing organisms[a]

Free-living aerobes		
Chemoorganotrophs	**Phototrophs**	**Chemolithotrophs**
Azotobacter *Azomonas* *Azospirillum* *Klebsiella*[b] *Methylomonas*	Cyanobacteria (e.g., *Anabaena, Nostoc, Gloeothece, Aphanizomenon*)	*Alcaligenes* *Acidithiobacillus*
Free-living anaerobes		
Chemoorganotrophs	**Phototrophs**	**Chemolithotrophs[c]**
Clostridium *Desulfotomaculum*	Purple bacteria (e.g., *Chromatium, Methanococcus, Rhodobacter*) Green sulfur bacteria (e.g., *Chlorobium*) Heliobacteria	*Methanosarcina* *Methanocaldococcus*
Symbiotic		
With leguminous plants	**With nonleguminous plants**	
Soybeans, peas, clover, etc. with *Rhizobium, Bradyrhizobium, Sinorhizobium*	Alder, bayberry, autumn olive, many other bushy plants, with the actinomycete *Frankia*	

[a]Only some common genera are listed in each category; many other genera are known. Except where indicated, all organisms are *Bacteria*.
[b]Nitrogen fixation occurs only under anoxic conditions.
[c]All are *Archaea*.

(**Figure 19.30**). This technique, known as the *acetylene reduction assay*, is widely used in microbiology to detect and quantify nitrogen fixation. Nitrogen fixation can also be assayed directly using the stable (nonradioactive) isotope $^{15}N_2$ and measuring its reduction to $^{15}NH_3$; however, acetylene reduction is a more rapid and sensitive method for measuring N_2 fixation and can easily be used in laboratory studies of pure cultures or ecological studies of nitrogen-fixing bacteria directly in their habitat. To do this, a sample, which may be soil, water, or a culture, is incubated in a vessel with $HC{\equiv}CH$ and the gas phase is later analyzed by gas chromatography for the production of $H_2C{=}CH_2$ (Figure 19.30). The unique enzymatic activity of nitrogenase—reduction of a triple bond—provides high confidence that acetylene reduction to ethylene measured in a natural sample is due to the activity of nitrogen-fixing bacteria.

Microsensors

Microsensors in the form of glass needles containing a sensing mechanism at the tip have been used to study the activity of microorganisms in nature. Microsensors have been constructed that measure many chemical species including pH, O_2, NO_2^-, NO_3^-, nitrous oxide (N_2O), CO_2, H_2, and H_2S. As the name *micro*sensor implies, these devices are very small, their tips ranging in diameter from 2 to 100 μm (**Figure 19.31**). The sensors are carefully inserted into the habitat in small increments to follow microbial activities over very short distances.

Microsensors have many applications. For example, O_2 concentrations in microbial mats (▶ Figure 20.8*c*), aquatic sediments, or soil particles (▶ Figure 20.3) can be accurately measured over extremely fine intervals using microsensors. A micromanipulator is used to insert the sensors gradually through the sample such that measurements can be taken every 50–100 μm (**Figure 19.32**). Using a bank of microsensors, each sensitive to a different chemical, simultaneous measurements of several transformations in a habitat can be made.

Microbial processes in the sea are extensively studied because they have a profound impact on nutrient cycles and the overall health of the planet. As it is difficult to reproduce in the laboratory the conditions found at great depths, it is useful to use microsensors on robotic devices to analyze microbial activities on the seafloor. **Figure 19.33** shows deployment of an instrument "lander" equipped with a suite of microsensors to detect various chemicals in the sediment. These data can then be analyzed and compared with that in overlying ocean water to explore the exchange of nutrients and other chemicals between water and sediment.

One of the biologically most important chemical species in the oceans is nitrate (NO_3^-), but electrochemical sensors cannot measure NO_3^- in seawater, as the high concentrations of salts interfere. To circumvent this problem, a "living" microsensor was designed

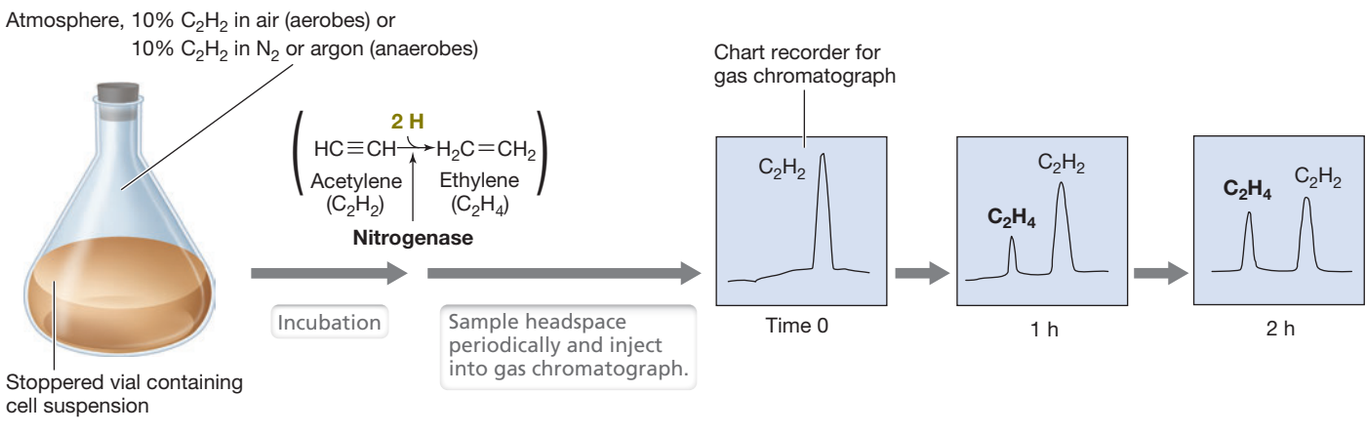


Figure 19.30 The acetylene reduction assay of nitrogenase activity in nitrogen-fixing bacteria. The results show no ethylene (C_2H_4) at time 0 but increasing production of C_2H_4 as the assay proceeds. As C_2H_4 is produced, a corresponding amount of C_2H_2 is consumed. Because the reduction of N_2 to 2 NH_3 requires 6 e^- and the reduction of C_2H_2 to C_2H_4 only 2 e^-, each three C_2H_4 produced is equivalent to the reduction of one N_2.

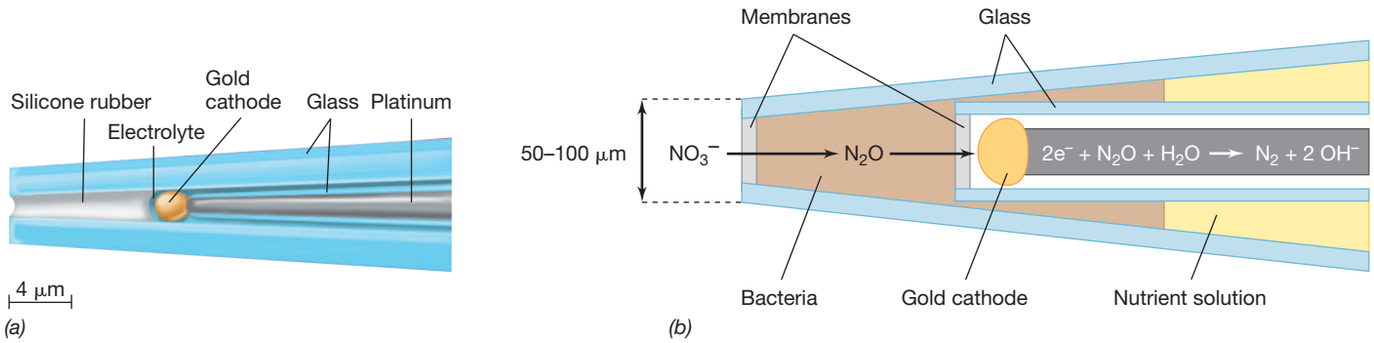


Figure 19.31 **Microsensors.** *(a)* Schematic drawing of an oxygen (O_2) microsensor. Oxygen diffuses through the silicone membrane in the microsensor tip and reacts with electrons on the gold surface of the cathode, forming hydroxide ions (OH^-); the latter generates a current proportional to the O_2 concentration in the sample. Note the scale of the electrode. *(b)* Biological microsensor for the detection of nitrate (NO_3^-). Bacteria immobilized at the sensor tip denitrify NO_3^- or NO_2^- to N_2O, which is detected by electrochemical reduction to N_2 at the cathode. Based on drawings by Niels Peter Revsbech.

that contains bacteria within its tip that reduce NO_3^- (or NO_2^-) to N_2O. The N_2O produced by the bacteria is then detected following its abiotic reduction to N_2 at the cathode of the microsensor (Figure 19.31*b*); this provides an electrical impulse signaling the presence of NO_3^-. In the oxic layer of marine sediments, NO_3^- is produced from the oxidation of NH_4^+ (nitrification, ◀ Section 14.9), so there is often a peak of NO_3^- in the sediment surface layer (Figure 19.32). In the deeper, anoxic layers of the sediment, NO_3^- is consumed by denitrification and dissimilative nitrate reduction to ammonia (DNRA) (◀ Section 14.11), and NO_3^- therefore disappears a few millimeters below the oxic–anoxic interface (Figure 19.32).

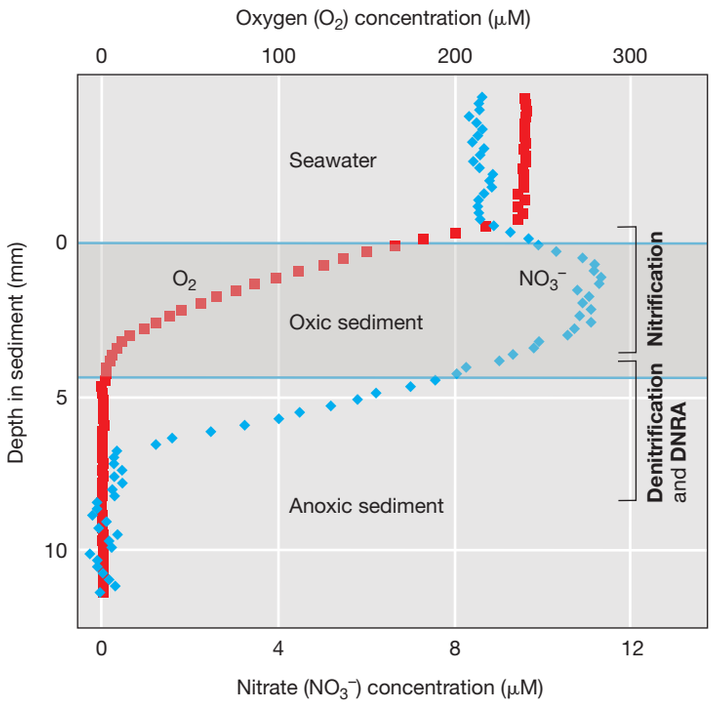


Figure 19.32 **Depth profiles of O_2 and NO_3^-.** Data obtained using the lander shown in Figure 19.33 equipped with microelectrode sensors for remote chemical characterization of deep-sea sediments. Note the zones of nitrification and denitrification and the fine scale resolution. DNRA, dissimilative nitrate (NO_3^-) reduction to ammonia (NH_4^+). Based on data and drawings by Niels Peter Revsbech.

Sensor Nanoparticles for Spatially Resolved Chemical Imaging

An entirely different sensor approach to measuring biologically significant processes in the environment uses nanotechnology. Traditional microsensors only generate a single point measurement, and because of this, multiple measurements must be made, for example,

Figure 19.33 **Deployment of a deep-sea lander.** The lander is equipped with a bank of microsensors (arrow) to measure distribution of chemicals in marine sediments.

when measuring depth-related changes in O_2 concentration in a marine sediment (Figure 19.32). Hence, this approach is essentially unusable for examining chemical gradients in more than one dimension, such as on an uneven biological surface. To explore such microbial habitats, *sensor nanoparticles* have been developed that can be sprayed in a thin layer over the uneven surface. These tiny particles (from a few to several hundred nanometers in diameter) made from silica or synthetic polymers incorporate fluorescent or phosphorescent compounds that have a concentration-dependent luminescent response to specific chemicals. Biological nanosensors have been developed for assaying temperature, pH, O_2, and a few ions of biological importance, such as Ca^{2+} and K^+.

A study to measure changing O_2 distribution over a coral surface demonstrates the power of sensor nanotechnology (**Figure 19.34**). As we will learn in Chapter 23, stony corals are marine animals whose cells contain the symbiotic dinoflagellate *Symbiodinium*, an alga that nourishes the coral cells by carrying out photosynthesis. However, *Symbiodinium* is extremely sensitive to changes in temperature and irradiation associated with climate change. When growth conditions become unfavorable, the symbiont can be expelled and the coral bleached, and this has resulted in the extensive loss of living coral reefs worldwide in recent years (▶ Figure 23.41). To document these changes experimentally, ecological studies of oxygen-producing coral tissues were undertaken to measure the response of the symbiotic alga to changes in light intensity. To do this, a coral surface was covered with a thin layer of nanoparticles containing a fluorescent indicator for oxygen. Following calibration using an inert fluorescent reference dye also incorporated in the particles, the spatial distribution of O_2 concentration was determined by taking fluorescent images of the coral under different levels of illumination, where oxygen levels can be seen to change quickly (Figure 19.34). When combined with nanosensors that respond to temperature differences, similar experiments should also be possible to measure coral responses to increasing ocean temperatures, an environmental variable thought to be a major trigger of the massive coral die-offs seen in recent years. By establishing links between coral structural organization, light, temperature, and chemical microenvironments, these analyses should yield important insights into factors that control the coral symbiosis and allow for more accurate predictions of how this important marine symbiosis will respond to climate change.

Other applications of nanosensor technology have been deployed or are on the horizon. For example, nanoparticles sensitive to both O_2 and pH have been used to examine the delivery of oxygen to the root systems of the seagrass *Zostera marina*. The observation of reduced pH and elevated oxygen close to the seagrass roots was attributed to oxygen delivered by the plant to support the growth of sulfur-oxidizing chemolithotrophs (◀ Sections 14.7 and 15.12), bacteria that oxidize the toxic hydrogen sulfide (H_2S); the resulting H_2SO_4 acidifies the roots, lowering the pH.

Although ecological applications of nanotechnology are still in their infancy, it is likely that in coming years more chemically diverse sensor particles will be developed and their use will become more widespread because of their unique ability to track gradients of microbial activity along uneven surfaces.

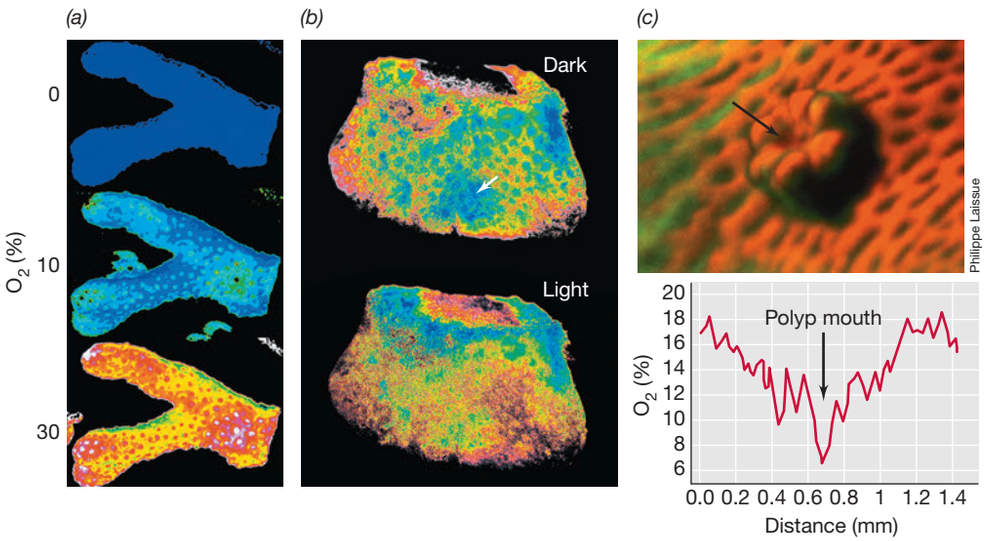


Figure 19.34 Oxygen nanosensor analysis of coral photosynthetic activity. *(a)* Calibration of nanosensor response to different dissolved oxygen concentrations using a fragment of a coral skeleton painted with the nanosensor octaethylporphine ketone platinum (II). *(b)* Living coral response to transition from darkness (top panel) to light (bottom panel). Note how the oxygen-depleted region due to coral respiration in the dark panel (arrow) quickly becomes oxygenated in the light. *(c)* Upper panel, photomicrograph of a coral polyp showing the polyp mouth (arrow). The red color is not from nanoparticles but is due to autofluorescence of chlorophyll in *Symbiodinium* cells present in the polyp and surrounding coral tissue. Lower panel, high-resolution profile from nanosensor data of an oxygen gradient across a polyp similar to that shown in the upper panel following transition to darkness. The scale on the abscissa indicates the small size of a polyp. The figure data and components in parts *a* and *b* were generated by Dr. Klaus Koren (Department of Bioscience, University of Aarhus) and Dr. Michael Kühl (Department of Biology, University of Copenhagen) with technical assistance from Sofie Lindegaard Jakobsen (Department of Biology, University of Copenhagen).

Check Your Understanding

- How can a microbial ecologist be sure that transformation of a radioisotope is actually caused by microbes?
- If a large pulse of organic matter entered the sediment, how would that change the profiles of NO_3^- and O_2 shown in Figure 19.32?
- Suggest additional applications of sensor nanoparticles in environmental microbiology.

19.10 Stable Isotopes and Stable Isotope Probing

Many of the chemical elements have more than one isotope, which differ in their number of neutrons. Certain isotopes are unstable and break down as a result of radioactive decay. Others, called *stable isotopes*, are not radioactive, but are metabolized differently by microorganisms and can be used to study microbial transformations in nature. There are two methods in which stable isotopes can yield information on microbial activities, *isotopic fractionation* and *stable isotope probing*.

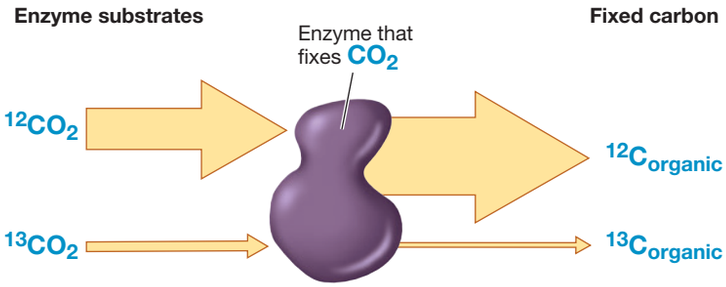


Figure 19.35 Mechanism of isotopic fractionation with C as an example. Enzymes that fix CO_2 preferentially fix the lighter isotope (^{12}C). This results in fixed carbon being enriched in ^{12}C and depleted in ^{13}C relative to substrate CO_2. The size of the arrows indicates the relative abundance of each isotope of carbon.

Isotopic Fractionation

The two elements most useful for stable isotope studies in microbial ecology are carbon (C) and sulfur (S), although the heavy isotope of nitrogen, ^{15}N, is also widely used. Carbon (C) exists in nature primarily as ^{12}C, but about 5% exists as ^{13}C. Likewise, S with its four stable isotopes exists primarily as ^{32}S. Some S is found as ^{34}S, and very small amounts as ^{33}S and ^{36}S. The relative abundance of these isotopes changes when certain C or S compounds are metabolized by microorganisms because the enzymes that act on these compounds typically favor the *lighter* isotope of C or S. That is, relative to the lighter isotope, the heavier isotope is discriminated against when both are metabolized by an enzyme (**Figure 19.35**).

For example, when CO_2 is fixed into cell material by an autotrophic organism, the cellular C becomes *enriched* in ^{12}C and *depleted* in ^{13}C, relative to an inorganic carbon standard of known isotopic composition. Likewise, the S atom in H_2S produced from the bacterial reduction of SO_4^{2-} is isotopically lighter than H_2S that has formed geochemically. These discriminations are called **isotopic fractionations** (Figure 19.35) and are typically the result of biological activities. Thus this technique can be used as a measure of whether a particular transformation has been catalyzed by microorganisms.

The isotopic fractionation of C in a sample is calculated as the extent of ^{13}C depletion relative to a standard having an isotopic composition of geological origin. The standard for C isotope analysis is rocks from a Cretaceous (65- to 150-million-year-old) limestone formation (the Pee Dee belemnite). Because the magnitude of fractionation is usually very small, depletion is calculated as "per mil" (‰, or parts per thousand) and reported as the $\delta^{13}C$ (pronounced "delta C 13") of a sample using the following formula:

$$\delta^{13}C = \frac{(^{13}C/^{12}C \text{ sample}) - (^{13}C/^{12}C \text{ standard})}{(^{13}C/^{12}C \text{ standard})} \times 1000‰$$

The same formula is used to calculate the fractionation of S isotopes, in this case using iron sulfide (FeS) mineral from the Canyon Diablo meteorite as the standard:

$$\delta^{34}S = \frac{(^{34}S/^{32}S \text{ sample}) - (^{34}S/^{32}S \text{ standard})}{(^{34}S/^{32}S \text{ standard})} \times 1000‰$$

Use of Isotopic Fractionation in Microbial Ecology

The isotopic composition of a material can reveal its biological or geological past. For example, plant material and petroleum (which is derived from plant material) have similar isotopic compositions (**Figure 19.36**). Carbon from both plants and petroleum is isotopically lighter than the CO_2 from which it was formed because the biochemical pathway used to fix CO_2 discriminated against $^{13}CO_2$ (Figures 19.35 and 19.36). Moreover, methane (CH_4) produced by methanogenic *Archaea* (◀ Section 17.2) is isotopically extremely light, indicating that methanogens discriminate strongly against $^{13}CO_2$ when they reduce CO_2 to CH_4 (◀ Section 14.15). By contrast, carbon in isotopically heavier marine carbonates is clearly of geological origin (Figure 19.36).

Because of the differences in the proportion of ^{12}C and ^{13}C in carbon of biological versus geological origin, the $^{13}C/^{12}C$ ratio of rocks of different ages has been used as evidence for or against past biological activity in Earth's ancient environments. Organic C in rocks as old as 3.5 billion years shows evidence of isotopic fractionation (Figure 19.36), supporting the idea that autotrophic life existed at this time. Indeed, we now believe that the first life on Earth appeared somewhat before this, about 3.8–3.9 billion years ago (◀ Sections 1.5 and 13.1).

The activity of sulfate-reducing bacteria is easy to recognize from their fractionation of stable S isotopes in sulfides (**Figure 19.37**). As compared with an H_2S standard, sedimentary H_2S is highly enriched in ^{32}S (depleted in ^{34}S, Figure 19.37). Fractionation during sulfate reduction allows one to identify biologically produced S and has been widely used to trace the activities of sulfur-cycling *Bacteria* and *Archaea* through geological time. Sulfur isotopic analyses have also been used as evidence for the lack of life on the Moon. For example, the data in Figure 19.37 show that the isotopic composition of

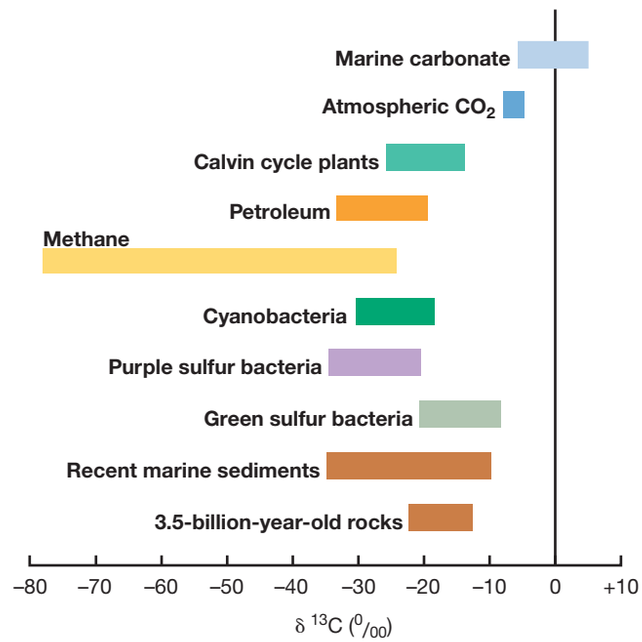


Figure 19.36 Isotopic geochemistry of ^{13}C and ^{12}C. Note that C fixed by autotrophic organisms is enriched in ^{12}C and depleted in ^{13}C. Methane formed from the reduction of CO_2 with H_2 by methanogenic *Archaea* shows extreme isotopic fractionation.

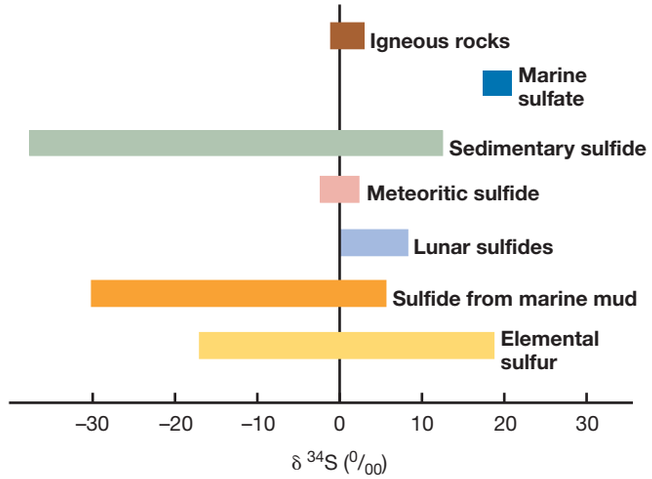


Figure 19.37 Isotopic geochemistry of ^{34}S and ^{32}S. Note that H_2S and S^0 of biogenic origin are enriched in ^{32}S and depleted in ^{34}S.

sulfides in lunar rocks closely approximates that of the H_2S standard, which represents primordial Earth, and differs from that of microbially produced H_2S.

Stable Isotope Probing

Beyond stable isotope fractionation, an alternative stable isotope method called **stable isotope probing (SIP)** can be used to identify an organism or organisms carrying out the transformation of a nutrient labeled with a specific stable heavy isotope, such as ^{13}C or ^{15}N or even ^{18}O (the lighter, more common isotopes of these elements are ^{12}C, ^{14}N, and ^{16}O, respectively). The idea behind SIP is that the label will be selectively incorporated only into the cellular material of organisms actively metabolizing the nutrient. Then, following the isolation and sequencing of isotopically labeled DNA, the organisms carrying out the transformation can be identified.

Stable isotope probing can be used to ask general to more specific questions. For example, if ^{13}C-labeled benzoate were added to a sediment sample and the sample incubated for an appropriate period, the ^{13}C label would end up in the DNA of the organism (or organisms) that metabolized the benzoate (**Figure 19.38**). Thus, although all DNA from the sample would be isolated, the ^{13}C-DNA is heavier, albeit only slightly heavier, than ^{12}C-DNA, and this difference is sufficient to separate the DNAs by a special type of centrifugation technique (Figure 19.38). Once the ^{13}C-DNA is isolated, it can be analyzed for phylogenetic genes or metabolic genes to yield genomic information about the benzoate degrader(s) in the sample. If instead of an organic compound, the experimental study focused on nitrogen fixation (conversion of N_2 to cell nitrogen, ◀ Section 3.12), $^{15}N_2$ could be supplied to a sample. When nitrogen fixers in the sample incorporate this, they will produce slightly heavier DNA than organisms that cannot fix nitrogen, and the heavier DNA can be isolated and analyzed for genes of interest.

SIP can also be used in combination with metagenomics to pinpoint organisms carrying out a specific metabolism (from SIP results) in the context of all the other species and metabolisms present in the sample (as revealed by metagenomic results). Besides a phylogenetic "hit" from the SIP results, additional genomic analyses of the labeled DNA could reveal functional genes required for the specific metabolism. Moreover, the phylogenetic and functional results from the SIP experiment could be further confirmed from the metagenomic profile. Clearly many opportunities exist for blending SIP and metagenomics to study important ecological problems.

Check Your Understanding

- What is the simplest explanation for why lunar sulfides are isotopically similar to those of the primordial Earth?
- What is the expected isotopic composition of carbon in methanotrophs (bacteria that consume CH_4)?
- How might exchange of metabolites among members of a microbial community complicate interpretation of a stable isotope probe experiment?

UNIT 5

19.11 Linking Functions to Specific Organisms

The isotopic methods described thus far used samples containing large numbers of cells to infer that specific metabolisms were occurring within a community or in particular species within the

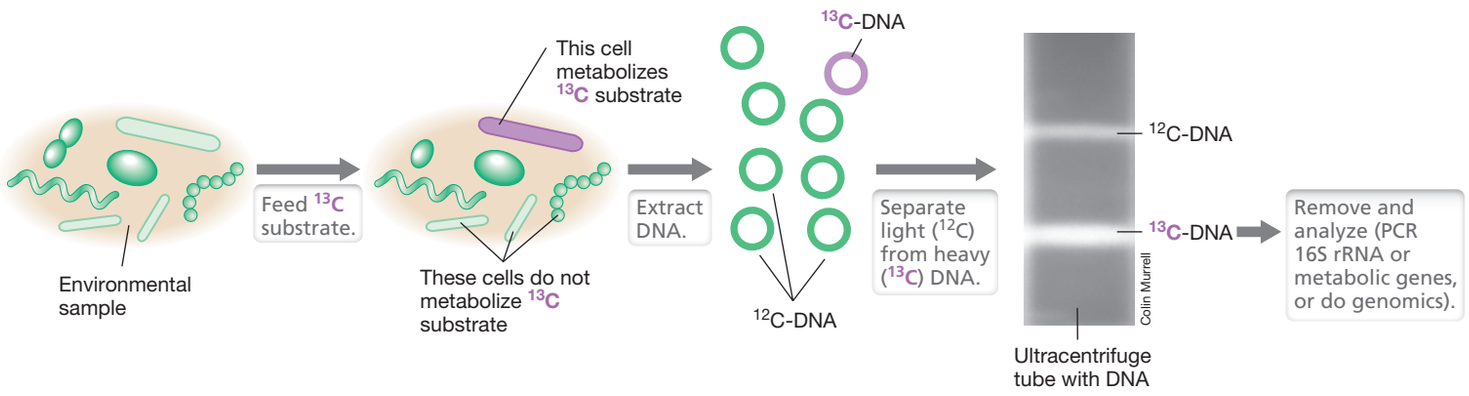


Figure 19.38 Stable isotope probing. The microbial community in an environmental sample is fed a ^{13}C substrate. Organisms that can metabolize the substrate produce ^{13}C-DNA as they grow and divide; ^{13}C-DNA can be separated from lighter ^{12}C-DNA by density gradient centrifugation (photo). The isolated DNA is then subjected to specific gene analysis or entire genomic analysis.

community. These methods give an overview of community activities but do not reveal the contribution of individual cells. To do this, new methods have been developed that can measure the activity and the elemental and isotopic composition of single cells. These are powerful methods for connecting cells of a specific microbial population with a specific activity or ecological niche, but in most cases, the phylogeny of the organisms of interest must be known in order to develop the necessary FISH probes (Section 19.5). Single-cell methods have been of particular importance to the study of metabolite exchange among microorganisms, such as exchanges involved in syntrophic relationships (◀ Section 14.22), where close physical association is essential for efficient exchange.

Single-Cell Metabolisms Imaged by Secondary Ion Mass Spectrometry (SIMS)

Secondary ion mass spectrometry (*SIMS*) is based on the detection of ions released from a sample placed under a focused high-energy primary ion beam, for example, of cesium (Cs^+); from the data generated, the elemental and isotopic composition of released materials can be obtained. When the primary ion beam impacts the sample, most chemical bonds are broken and atoms or polyatomic fragments are ejected from a very thin layer (1–2 nm) of the surface as either neutral or charged particles (secondary ions), a process called *sputtering*. These secondary ions are directed to a mass spectrometer, an instrument that can determine their mass-to-charge ratio.

NanoSIMS instruments are SIMS devices designed to yield information on single cells. The instrument is equipped with Cs^+ and O_2 primary beam sources with a resolution of 50 nm for the Cs^+ ion beam and 200 nm for the O_2 beam. The O_2 beam generates positive secondary ions and is used to analyze metals (e.g., Fe, Na, Mg) while the Cs^+ beam generates negative secondary ions for the analysis of major cellular elements (C, N, P, S, O, H) and halogens. The NanoSIMS instrument also records where on the specimen the ion beam is directed such that a two-dimensional image of the distribution of specific ions on the sample surface is obtained. In addition, by focusing the ion beam on the same spot during repeated cycles of sputtering, material can be slowly burned away to expose deeper regions of the sample. This high-resolution SIMS analysis is where the term *NanoSIMS* got its name. NanoSIMS instruments have multiple detectors that provide for the simultaneous analysis of ions of different mass-to-charge ratios originating from the same sample location (**Figure 19.39*a***).

When NanoSIMS is combined with FISH (Section 19.5) in a technology called *FISH-SIMS*, the incorporation of different elements, natural isotopes, or isotope-labeled substrates can be tracked into individual cells of specific cell populations. A variation on the FISH-SIMS method that simplifies the identification of cells scanned by NanoSIMS uses probe-conferred deposition of a halide (Br, F, I), either through direct incorporation of the halide into an oligonucleotide probe or by using a halide-containing tyramide substrate (see CARD-FISH, Section 19.5). Halogens possess a high ionization yield compared with other elements and are thus easy to detect and are typically of low natural abundance. Using this technology, one of the NanoSIMS detectors is dedicated to identifying cells to which the probe has hybridized (Figure 19.39*d*) by halogen ionization while the remaining detectors are used for assessing elemental composition (Figure 19.39*c*).

Because of its excellent spatial resolution, NanoSIMS technology is being increasingly used to examine metabolite transfer among single cells of interacting microorganisms. For example, labeling with $^{15}N_2$ followed by NanoSIMS was used to demonstrate the transfer of N_2 fixed by filamentous cyanobacteria to attached heterotrophic bacteria (Figure 19.39*c*). Labeling with $^{15}NH_4$ and ^{13}C-labeled CO_2 or organic substrates is also being used to explore the assimilation of key nutrients and the transfer of metabolites among microbial species in both aquatic and soil microbial communities.

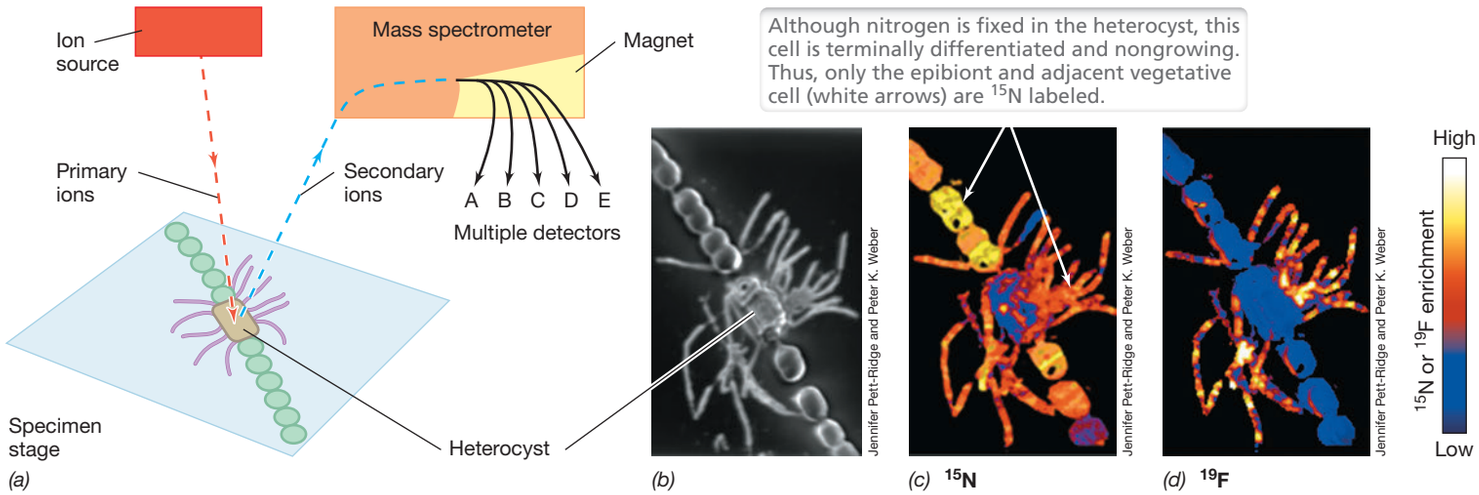


Figure 19.39 NanoSIMS technology. *(a)* Schematic of NanoSIMS operation showing the beams of primary (red) and secondary (blue) ions and five different detectors, each of which identifies ions of a different mass-to-charge ratio. *(b–d)* Demonstration of interspecies nutrient transfer from a filamentous cyanobacterium (*Anabaena*) to a *Rhizobium* species attached to the cyanobacterial heterocyst. The coculture was incubated with $^{15}N_2$, and the transfer of ^{15}N-labeled compounds from *Anabaena* to *Rhizobium* was imaged using a combination of enhanced elemental labeling–FISH (EL-FISH, a technique that allows for simultaneous fluorescence and halogen labeling) and NanoSIMS. *(b)* Total ^{12}C abundance shown by gray tones. *(c)* ^{15}N enrichment. *(d)* ^{19}F abundance conferred by a probe that hybridizes only to the attached rhizobial cells (EL-FISH).

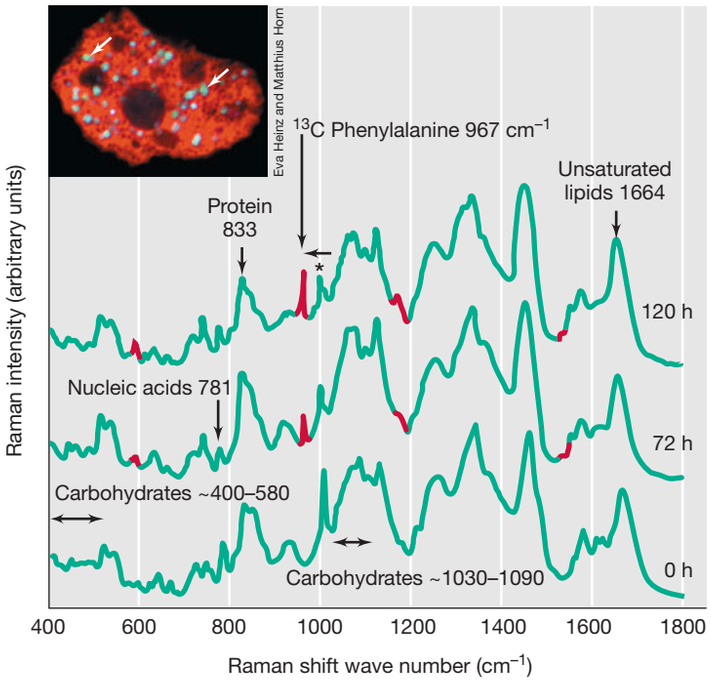


Figure 19.40 Raman microspectroscopic analysis of single cells. A cell of *Acanthamoeba* containing FISH-stained chlamydial symbionts (inset photo, arrows point to blue chlamydial cells) and Raman spectroscopy of isolated chlamydial cells. After incubation for 72 and 120 h in medium containing ^{13}C phenylalanine, symbionts were released by lysis of the amoeba and their Raman spectra recorded. Peaks diagnostic for labeled phenylalanine are shown in red. Since the Raman wave number of ^{13}C phenylalanine at 967 cm^{-1} is well resolved from other spectral features, the ratio of 1003 cm^{-1} (unlabeled phenylalanine, at *) to 967 cm^{-1} (labeled phenylalanine) peak areas corresponds to the relative amount of labeled phenylalanine incorporated by single cells (note how this increases with time). Other red peaks correspond to spectral peaks specific for different chemical bonds in ^{13}C phenylalanine. Also shown are selected peaks and their wave numbers for other cellular components of the symbiont.

Raman Microspectroscopy

Raman microspectroscopy can be used to characterize the molecular and isotopic composition of single cells by nondestructive illumination with monochromatic light generated by a laser. Raman is a form of spectroscopy that measures light scattering and can yield both qualitative and quantitative results. Compositional analysis is based on photon scattering after interaction with different cellular components. Although most of the scattered photons have the same energy as the incident photons, a small fraction of scattered photons are shifted in wavelength (relative to the incident wavelength) to either a longer wavelength (a phenomenon known as *Stokes Raman scattering*) or shorter wavelength (*anti-Stokes scattering*).

Raman spectrometers separate the more abundant Stokes scattered photons for analysis and, when combined with confocal microscopy (◀ Section 1.9), can generate a compositional spectrum of a single microbial cell (**Figure 19.40**). Although the spectrum is complex, several compounds and molecules have characteristic peaks that can identify specific cell types, physiological states, or metabolic activities following incorporation of compounds labeled with stable isotopes.

Major advantages of Raman microspectroscopy include the following: It is nondestructive and can be used on living cells; water does not cause interference; it can be combined with FISH; and cells of interest can be further manipulated, for example, through capture by laser tweezers (Section 19.3). In the example of Raman shown in Figure 19.40, the incorporation of phenylalanine (an amino acid) by an obligate chlamydial symbiont of an amoeba has been tracked following addition of phenylalanine labeled with ^{15}N and ^{13}C to the amoeba growth medium. A less specific but still useful application of Raman is based on the incorporation of heavy protons from deuterated water ($^{2}H_2O$). Since water is a reactant in many biochemical reactions, metabolically active microorganisms that incorporate the heavy hydrogen atom can be identified by Raman microspectroscopy for further processing, including characterization by single-cell genomics (◀ Section 10.11).

Radioisotopes in Combination with FISH: Microautoradiography-FISH

Radioisotopes are used as measures of microbial activity in a microscopic technique called **microautoradiography (MAR)**. In this method, cells from a microbial community are exposed to a substrate containing a radioisotope, such as an organic compound or CO_2. Heterotrophs take up the radioactive organic compounds and

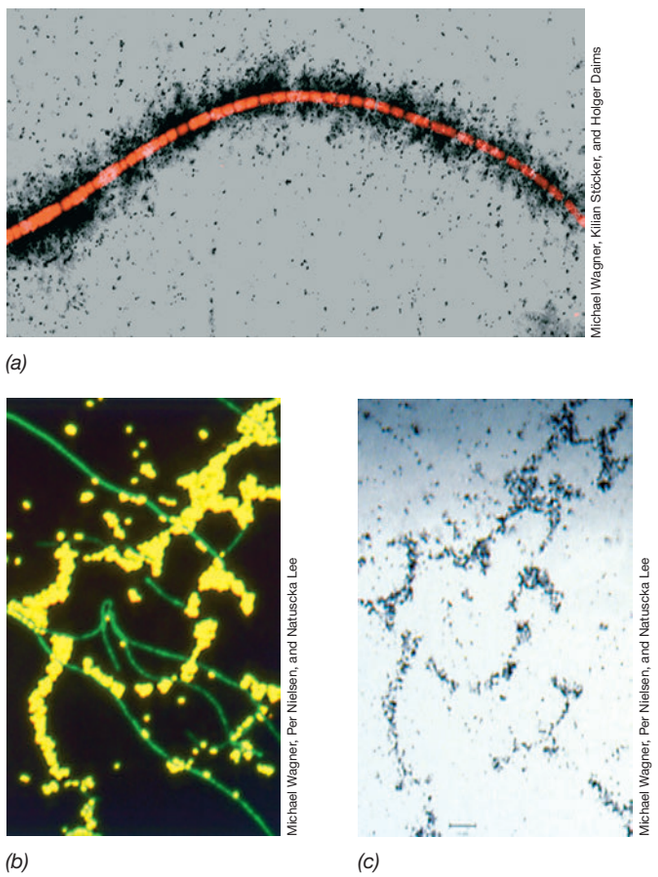


Figure 19.41 MAR-FISH. Fluorescence in situ hybridization (FISH) combined with microautoradiography (MAR). *(a)* An uncultured filamentous cell belonging to the *Gammaproteobacteria* (as revealed by FISH) is shown to be an autotroph (as revealed by MAR-measured uptake of $^{14}CO_2$). *(b)* Uptake of ^{14}C-glucose by a mixed culture of *Escherichia coli* (yellow cells) and *Herpetosiphon aurantiacus* (filamentous green cells). *(c)* MAR of the same field of cells shown in part *b*. The radioactivity of incorporated glucose exposes the film and shows that glucose was assimilated mainly by cells of *E. coli*.

autotrophs take up the radioactive CO_2. Following incubation in the substrate, cells are affixed to a slide and the slide is dipped in photographic emulsion. While the slide is left in darkness for a period, radioactive decay from the incorporated substrate induces formation of silver grains in the emulsion; these appear as black dots above and around the cells. **Figure 19.41*a*** shows a MAR experiment in which an autotrophic cell has taken up $^{14}CO_2$.

Microautoradiography can be done simultaneously with FISH (Section 19.5) in **MAR-FISH**, a powerful technique that combines identification with activity measurements. MAR-FISH allows a microbial ecologist to determine (by MAR) which organisms in a natural sample are metabolizing a particular radiolabeled substance while at the same time identifying these organisms (by FISH) (Figure 19.41*b, c*). MAR-FISH thus goes a step beyond phylogenetic identification by revealing physiological information about the organisms, as is also true of NanoSIMS. Such data are useful not only for understanding the activity of the microbial ecosystem but also for guiding enrichment cultures. For example, knowledge of the phylogeny and morphology of an organism metabolizing a particular substrate in a natural sample can be used to design an enrichment protocol to isolate the organism. In addition, MAR-FISH results can be quantified by counting the silver grains as a measure of the amount of substrate consumed by single cells, allowing the activity distribution in a community to be described. The technique is limited only by the availability of suitable radioactive isotopes. For example, although C-labeled substrates work well, it is not feasible to track N incorporation using MAR-FISH because the radioactive isotope ^{13}N has a very short half-life. However, it is feasible to track N incorporation using the nonradioactive stable isotope ^{15}N with NanoSIMS, as we saw earlier (Figure 19.39).

Check Your Understanding

- How could NanoSIMS be used to identify a nitrogen-fixing bacterium?
- Why is Raman microspectroscopy suited for the selective isolation of microorganisms and NanoSIMS is not?
- How does MAR-FISH link microbial diversity and activity?

19.12 Linking Genes and Cellular Properties to Individual Cells

We have seen in the previous section how the combination of FISH with MAR or FISH with NanoSIMS allows for analyses of both microbial diversity and activity. Coupled with advanced DNA sequencing methods that can determine a genome sequence from the DNA contained in a single cell (◀ Section 10.11), these techniques are at the cutting edge of microbial ecology today. Improvements in single-cell DNA sequencing technology, combined with high-throughput analysis and isolation of single cells by flow cytometry, now allow for gene identification and selected physiological analyses (e.g., size and intrinsic fluorescence) to be performed on selected populations and single cells in the environment.

Mastering Microbiology
Art Activity: Figure 19.36 Flow cytometric cell sorting

Flow Cytometry and Multiparametric Analyses

Because of the large population sizes of natural microbial communities, methods that rely on microscopy can examine only a very small part of a whole community. It is difficult to assess cell numbers by counting cells microscopically, and this problem is compounded if populations are present in low numbers. However, flow cytometry (Section 19.3) offers an alternative to more labor-intensive microscopic methods.

Flow cytometers can examine specific cell parameters such as size, shape, or fluorescent properties as the cells pass through a detector at rates of many thousands of cells per second (**Figure 19.42**). Fluorescence may be intrinsic (for example, chlorophyll fluorescence of phototrophic microorganisms); or it may be conferred by DNA staining, by differential staining of live versus dead cells (vital stains), or by fluorescent DNA probes (FISH or BONCAT-FISH), all methods discussed earlier in this chapter.

A major advantage of flow cytometry is the ability to carry out *multiparametric analyses*, that is, the capacity to combine multiple parameters in the analysis of a microbiological sample or to sort

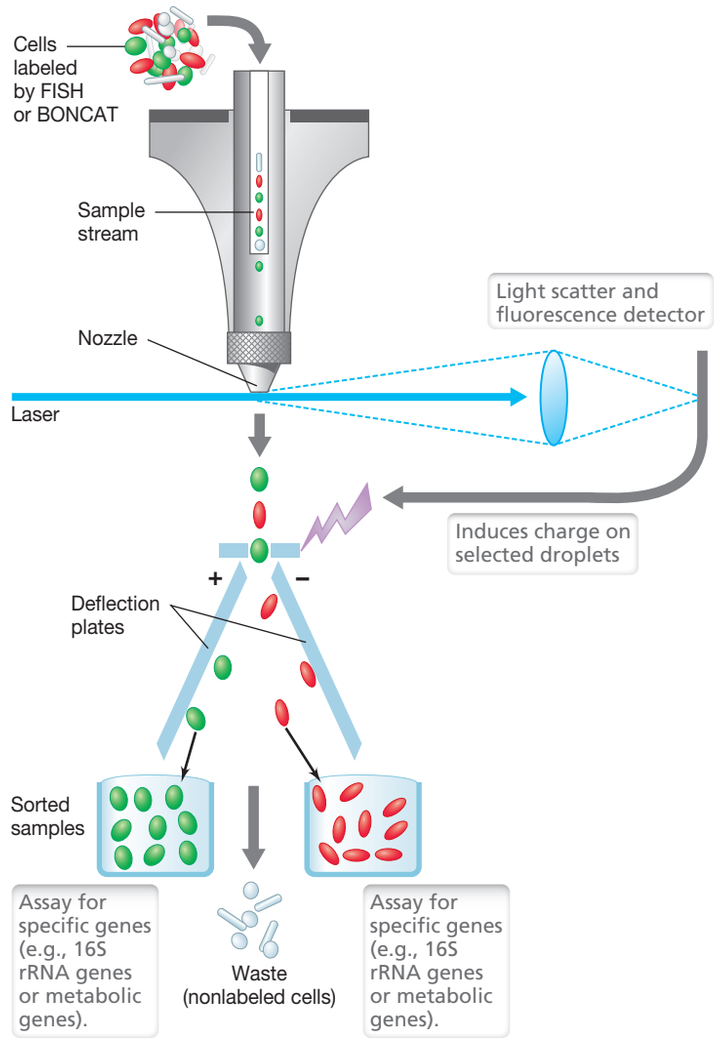


Figure 19.42 Flow cytometric cell sorting. As the fluid stream exits the nozzle, it is broken into droplets containing no more than a single cell. Droplets containing desired cell types (detected by fluorescence or light scatter) are charged and collected by redirection into collection tubes or microtiter plates by positively or negatively charged deflection plates. DNA recovered from a specific population of cells or amplified from a single cell (◀ Figure 10.30) is characterized by PCR amplification and sequencing of specific genes or by full genome sequence analysis.

cells in order to find a specific population. A good example of this was the discovery in the late 1980s of a novel and abundant community of marine cyanobacteria, all species of the genus *Prochlorococcus*. *Prochlorococcus* cells are smaller and have different fluorescent properties than another common marine cyanobacterium, *Synechococcus*. Based on differences in size and fluorescence, flow cytometry resolved these two populations and *Prochlorococcus* was subsequently shown to be the predominant oxygenic phototroph in ocean waters between 40°S and 40°N latitude, reaching concentrations greater than 10^5 cells/ml. Metagenomics has also been used to identify the unique genomic features of different natural populations of *Prochlorococcus* (Figure 19.24). These findings have led to the conclusion that *Prochlorococcus* is the most abundant phototrophic organism on Earth. We discuss the biology of *Prochlorococcus* in more detail in Section 20.11.

Single-Cell Genomics

A major stumbling block in a PCR-based gene recovery method is the requirement that a specific gene that will react with the primers used in the amplification be identified prior to analysis. Newer methods of DNA amplification now provide an alternative method for associating specific genes with a specific organism without the problems and biases associated with PCR. These methods employ *single-cell genomics* (◀ Section 10.11), one of the more recent tools to enter the microbial ecologist's toolbox. As discussed in Chapter 10, when combined with methods for recovery of single cells and high-throughput DNA sequencing methods, single-cell genome sequencing provides a powerful tool for linking specific metabolic functions to individual cells that have never been grown in laboratory culture.

Multiple displacement amplification (MDA) (Figure 19.43) is key to single-cell genomics because it can amplify chromosomal DNA from a single cell isolated from a natural environment using a cell sorting technique, such as flow cytometry (Figure 19.42). MDA uses a specific bacteriophage DNA polymerase to initiate replication of cell DNA at random points in the chromosome, displacing the complementary strand as each polymerase molecule synthesizes new DNA. The phage polymerase has strong strand displacement activity, resulting in the synthesis of numerous high-molecular-weight DNA products at very high fidelity. The number of genome copies produced by MDA is usually sufficient to assemble the complete, or nearly complete, genome of the cell that yielded the DNA using next-generation sequencing platforms and powerful sequence analysis software. In this way, both phylogenetic and metabolic functions can be inferred from the genome sequence and PCR is not required. PCR can be a problem if there exists a bias in the genome against the primers used. MDA avoids this by annealing random rather than specific primers to the genomic DNA such that amplification of the entire genome is highly likely.

Not surprisingly, MDA requires stringent control over purity to eliminate contaminating DNA, but when combined with high-throughput DNA sequencing methods, MDA provides a powerful tool for linking specific metabolic functions to individual cells that have thus far eluded laboratory culture. Information about the metabolic capacities of these uncultured organisms can then be used to develop strategies to recover them by either classical enrichment and isolation methods (Sections 19.1 and 19.2) or by any of the several single-cell isolation culturing techniques now available to tease out individual cells and get them growing in the laboratory (Section 19.3 and Figure 19.42).

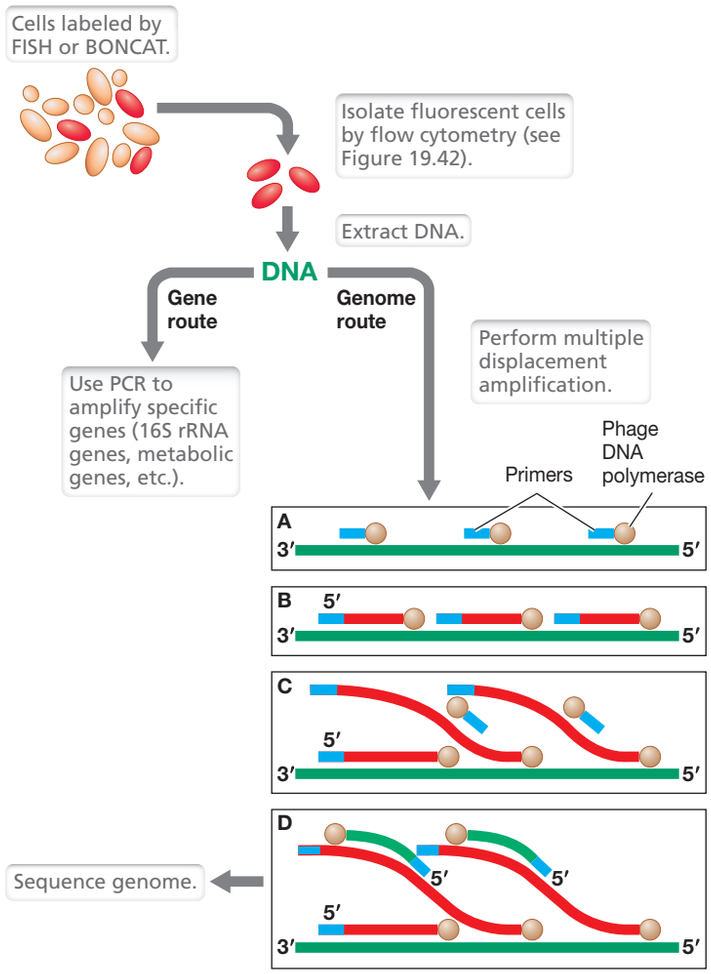


Figure 19.43 Genetic analyses of sorted cells. DNA is recovered from a specific population of cells following FISH labeling and flow cytometric sorting (Figure 19.42). DNA is characterized by PCR amplification and sequencing of specific genes, or by amplification of the entire genome by multiple displacement amplification (MDA) followed by sequencing. For MDA, an amount of DNA sufficient for full genome sequence determination is produced using short DNAs of random sequence as primers (A) to initiate genome replication by a bacteriophage DNA polymerase. The bacteriophage polymerase copies DNA from multiple points in the genome and also displaces newly synthesized DNA (B, C), thereby freeing additional DNA for primer annealing and (D) initiation of polymerization.

A Preview of What's to Come

Now that we have some background on how we can track and identify microbes in nature and assess their activities, we will use these tools as we move on to the next chapter to consider where microbes actually live in nature. In Chapter 20 we will link microbial diversity with major habitats to answer the questions "Who is where?" and

Mastering Microbiology
Art Activity: Figure 19.37 Genetic analysis of sorted cells

"Why are they there?" In Chapter 21 we will connect diversity with metabolism to explore the major nutrient cycles in nature that are driven by microbial activities. In Chapter 22 we will consider microbes and their habitats once again but in the context of microbes that reside in our buildings, subways, and other major structures constructed by humans. Chapters 23 and 24 also link microbes to habitats but in the context of the many symbiotic associations that microbes have developed with plants and animals, including ourselves. Background on where microbes live in nature and why is the natural introduction to the final two units in this text where we focus on microbe–human relationships, both the highly beneficial and the life-threatening.

Check Your Understanding

- Compared with microscopy, what are the advantages and disadvantages of flow cytometry for characterizing a microbial community?
- What key method is required to do genomics on a single cell?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Culture-Dependent Analyses of Microbial Communities

19.1 The enrichment culture technique is a means of obtaining microorganisms from natural samples. Successful enrichment and isolation prove that an organism of a specific metabolic type was present in the sample, but do not indicate its ecological importance or abundance. Enrichments following dilution of the sample often yield different organisms than enrichments with undiluted samples.

Q What exactly constitutes an enrichment culture? What should an effective enrichment culture duplicate?

19.2 Once a successful enrichment culture has been established, pure cultures can often be obtained by conventional microbiological procedures, including streak plates, agar dilution, and liquid dilution methods.

Q Describe how an appropriate inoculum can be used to successfully start an enrichment culture.

19.3 Several methods are available to isolate and culture single cells. Laser tweezers allow one to isolate a cell from a microscope field and move it away from contaminants. Flow cytometric sorting combined with high-throughput culturing technology allow for isolated cells to be cultured in a large variety of culture media simultaneously to identify the resources and conditions best suited to the growth of the isolated cell.

Q It is thought that every microbe has a fundamental niche and a realized niche. How does a fundamental niche differ from a realized niche?

II • Culture-Independent Microscopic Analyses of Microbial Communities

19.4 DAPI, acridine orange, and SYBR Green are general stains for quantifying microorganisms in natural samples. Some stains can differentiate live versus dead cells. The GFP makes cells autofluorescent and is a means for tracking cells introduced into the environment and reporting gene expression. In natural samples, morphologically identical cells may actually be genetically distinct.

Q What are dyes that stain DNA used for? Is background staining a problem when using such dyes?

19.5 FISH methods have combined the power of nucleic acid probes with fluorescent dyes and are thus highly specific in their staining properties. FISH methods include phylogenetic stains, CARD-FISH, and BONCAT-FISH. Using fluorescence microscopy, CARD-FISH can identify transcriptionally active cells while BONCAT-FISH can identify translationally active cells.

Q Compare and contrast CARD-FISH and BONCAT-FISH. Why are these methods more suitable than FISH for characterizing very slowly growing microorganisms in the environment?

III • Culture-Independent Molecular Analyses of Microbial Communities

19.6 PCR can be used to amplify specific target genes such as rRNA genes or key metabolic genes for subsequent analysis of community structure and potential functions. DGGE and T-RFLP can identify the different variants of these genes among the species in a community. Application of ARISA is limited to amplification of the internal transcribed spacer region separating the 16S and 23S rRNA genes.

Q Which method, ARISA or T-RFLP, would provide more detail about microbial community complexity? Why?

19.7 In current application, microarrays used for environmental studies primarily consist of thousands of DNA probes that hybridize to specific genes encoding key biochemical processes.

Q Why might a microarray be superior to using high-throughput sequencing to quantify both abundant and rare gene sequences in a complex microbial community? What are the limitations of functional microarrays, such as the GeoChip, for analysis of microbial communities?

19.8 Environmental multi-omics uses different combinations of metagenomics, metatranscriptomics, metaproteomics, and metabolomics to derive deeper understanding of how microbial diversity relates to the complex metabolic food webs sustaining natural microbial communities and associated biogeochemical processes.

Q Give an example of how multi-omics has contributed to a better understanding of microbial food web structure. Why is the use of environmental genomic information alone often insufficient to discover new biochemical properties?

IV • Measuring Microbial Activities in Nature

19.9 The activity of microorganisms in natural samples can be assessed very sensitively using radioisotopes or microsensors, or both. The measurements obtained give the net activity of the microbial community.

Q What are the major advantages of radioisotopic methods in the study of microbial ecology? What type of controls (discuss at least two) would you include in a radioisotopic experiment to show $^{14}CO_2$ incorporation by cyanobacteria or to show ^{14}C-glucose incorporation by anaerobic bacteria?

19.10 Natural isotopic composition, the result of isotopic fractionation by enzymes that discriminate against the heavier form of an element, can reveal the biological origin and/or biochemical mechanisms involved in the formation of various substances. Stable isotope probing (SIP) uses compounds labeled with isotopes not naturally abundant to identify microorganisms metabolizing and assimilating the compound added to a community.

Q Will autotrophic organisms contain more or less ^{12}C in their organic compounds than was present in the CO_2 that fed them? Why would SIP using $^{15}NO_3^-$ not be useful for identifying bacteria carrying out nitrate respiration?

19.11 A variety of advanced technologies such as NanoSIMS, MAR-FISH, BONCAT-FISH, and Raman microspectroscopy make it possible to examine metabolic activity, gene content, and gene expression of single cells in natural microbial communities. NanoSIMS employs secondary ion mass spectrometry technology. MAR-FISH combines the uptake of radiolabeled substrates (MAR) along with phylogenetic identification (FISH). Raman microspectroscopy is a nondestructive method (retaining cell viability).

Q What can MAR-FISH tell you that FISH alone cannot? How might you combine SIP and NanoSIMS to identify novel methane-consuming cells in a natural community?

19.12 Flow cytometry combined with cell sorting can rapidly evaluate many thousands of single cells in a natural environment for basic cellular properties (size, shape) or gene content (using specific fluorescent probes). Single-cell genomics incorporates methods for use on individual cells from a natural microbial community, for example, by flow cytometric isolation of cells and multiple displacement amplification to obtain genome sequences of single cells.

Q How would you use cytometric cell sorting to evaluate genome sequence variation among a population of marine bacteria present in low abundance?

APPLICATION QUESTIONS

1. Design an experiment for measuring the activity of sulfur-oxidizing bacteria in soil. If only certain species of the sulfur oxidizers present were metabolically active, how could you tell this? How would you prove that your activity measurement was due to biological activity?
2. You wish to know whether *Archaea* exist in a lake water sample but are unsuccessful in culturing any. Using techniques described in this chapter, how could you determine whether *Archaea* exist in the sample, and if they do, what proportion of the cells in the lake sample are *Archaea*?
3. Design an experiment to solve the following problem: Determine the rate of methanogenesis ($CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$) in anoxic lake sediments and whether or not it is H_2-limited. Also, determine the morphology of the dominant methanogen (recall that these are *Archaea*, ◀ Section 17.2). Finally, calculate what percentage the dominant methanogen is of the total archaeal and total prokaryotic populations in the sediments. Remember to specify necessary controls.
4. Design a SIP experiment that would allow you to determine which organisms in a lake water sample were capable of oxidizing the hydrocarbon hexane (C_6H_{14}). Assume that four different species could do this. How would you combine SIP with other molecular analyses to identify these four species?

CHAPTER GLOSSARY

Acridine orange a nonspecific fluorescent dye used to stain DNA in microbial cells in a natural sample

BONCAT-FISH a method to identify translationally active cells labeled by FISH by incorporating a noncanonical amino acid into their proteins followed by coupling it to a fluorescent reporter molecule (BONCAT stands for bioorthogonal noncanonical amino acid tagging)

DAPI a nonspecific fluorescent dye that stains DNA in microbial cells; used to obtain total cell numbers in natural samples

Denaturing gradient gel electrophoresis (DGGE) an electrophoretic technique

capable of separating nucleic acid fragments of the same size that differ in base sequence

Enrichment bias a problem with enrichment cultures in which "weed" species tend to dominate in the enrichment, often to the exclusion of the most abundant or ecologically significant organisms in the inoculum

Enrichment culture a culture that employs highly selective laboratory methods for obtaining microorganisms from natural samples

Environmental genomics (metagenomics) the use of genomic methods (sequencing and analyzing genomes) to characterize natural microbial communities

Exometabolite a product of metabolism released from a cell that may be metabolized by other organisms

Flow cytometry a technique for counting and examining microscopic particles by suspending them in a stream of fluid and passing them by an electronic detection device

Fluorescence in situ hybridization (FISH) a method employing a fluorescent dye covalently bonded to a specific nucleic acid probe for identifying or tracking organisms in the environment

Fluorescent protein any of a large group of proteins that fluoresce different colors, including the green fluorescent protein, for tracking genetically modified organisms and determining conditions that induce the expression of specific genes

Fundamental niche the range of environments in which a species will be sustained when it is not resource-limited, such as may result from competition with other species

Green fluorescent protein (GFP) a protein that fluoresces green and is widely used in genetic analysis

High-throughput culturing methods the use of microtiter plates whose wells contain various culture media that can be inoculated with single cells whose growth or target gene content is measured robotically

Isotopic fractionation the discrimination by enzymes against the heavier isotope of the various isotopes of C or S, leading to enrichment of the lighter isotopes

Laser tweezers a device for obtaining pure cultures by optically trapping a single cell with a laser beam and moving it away from surrounding cells into sterile growth medium

MAR-FISH a technique that combines identification of microorganisms by FISH with measurement of metabolic activities by microautoradiography (MAR)

Metabolomics the comprehensive analysis of cellular and extracellular metabolites of a cell, organism, or microbial community

Metaproteomics the measurement of whole-community protein expression using mass spectrometry to assign peptides to the amino acid sequences encoded by unique genes

Metatranscriptomics the measurement of whole-community gene expression using RNA sequencing

Microautoradiography (MAR) the measurement of the uptake of radioactive substrates by visually observing the cells in an exposed photographic emulsion

Microbial ecology the study of the interaction of microorganisms with each other and their environment

Microfluidic devices miniaturized systems for fluid handling that are increasingly used for high-throughput culturing of microorganisms

Microsensor a small glass sensor or electrode for measuring pH or specific compounds such as O_2, H_2S, or NO_3^- that can be immersed into a microbial habitat at microscale intervals

Most-probable-number (MPN) technique the serial dilution of a natural sample to determine the highest dilution yielding growth

Multi-omics the integration of data from multiple omics platforms to more fully characterize microbial community structure and function

Multiple displacement amplification (MDA) a method to generate multiple copies of chromosomal DNA from a single organism

Nucleic acid probe a strand of nucleic acid that can be labeled and used to hybridize to a complementary molecule from a mixture of other nucleic acids

Phylotype one or more organisms with the same or related sequences of a phylogenetic marker gene

Realized niche the range of natural environments supporting a species when that organism is confronted with factors such as resource limitation, predation, and competition from other species

Stable isotope probing (SIP) a method for characterizing an organism that incorporates a particular substrate by supplying the substrate in ^{13}C or ^{15}N form and then isolating heavy isotope–enriched DNA and analyzing the genes

Winogradsky column a glass column packed with mud and overlaid with water to mimic an aquatic environment, in which various bacteria develop over a period of months

Microbial Ecosystems 20

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II The Microbial Environment 690

III Terrestrial Environments 697

IV Aquatic Environments 705

MICROBIOLOGYNOW

Living on Fumes

Antarctica is one of the harshest environments on Earth, experiencing extremely cold temperatures, high levels of ultraviolet radiation, and limited availability of carbon, nitrogen, and water. Even so, rocky desert Antarctic soils (photo) support microbial communities whose diversity rivals that of temperate soils. Cyanobacteria fix enough carbon dioxide (CO_2) to support heterotrophic communities in many soils of Antarctica. However, the virtual absence of cyanobacteria in some other Antarctic soils raises the question of what energy source (other than light) sustains these microbial communities.

Researchers combined metagenomics with biochemical measurements to probe this mystery. The first hint was the discovery of Calvin cycle genes for CO_2 fixation in *Actinobacteria* that composed about half of the total soil microbiota (the Calvin cycle is a major autotrophic pathway). Since these *Actinobacteria* are not phototrophic, CO_2 fixation—which requires ATP—had to be supported by energy sources other than sunlight. The answer again emerged from metagenomics by identifying genes in the *Actinobacteria* encoding aerobic respiration of molecular hydrogen (H_2) and carbon monoxide (CO) and then showing that these genes were actively transcribed at below-freezing temperatures. Additionally, comparison of these gene sequences to those of cultured close relatives suggested these enzymes could scavenge H_2 and CO at the parts per billion concentrations typically found in Earth's lower atmosphere. This was subsequently confirmed by direct measurements of activity where it was shown that atmospheric concentrations of H_2 stimulated rates of CO_2 fixation sufficient to support about 10^7 bacteria per gram of Antarctic soil.

Because low concentrations of H_2 and CO are constantly available, they are dependable energy sources for the slow-growing soil microbial communities in Antarctica. Whereas most ecosystems are sustained by solar or geologically derived energy, it appears that bacteria in some Antarctic surface soils are supported by atmospheric trace gases. In other words, these microbes are "living on fumes"!

Source: Mukan Ji, Chris Greening, Inka Vanwonterghem, et al. 2017. Atmospheric trace gases support primary production in Antarctic desert surface soil. *Nature* 552: 400.

Microorganisms do not live alone in nature but instead interact with other organisms and with their environment. In so doing, microorganisms carry out many essential activities that support all life on Earth. In this chapter we explore some of the major habitats of microorganisms; these include soil, freshwater, and the oceans. In addition to these, microbes have also established more specific, and often very intimate, associations with plants and animals. We examine a few examples of such microbial partnerships and symbioses in Chapters 23 and 24.

I • Microbial Ecology

Microbial ecology borrows many concepts from classical ecology but differs in the enormous diversity of microbial habitats and metabolisms it must consider—habitats and metabolisms that collectively drive the major nutrient cycles in nature and provide life support to all macroorganisms.

We begin with a broad overview of the science of microbial ecology, including ways that organisms interact with each other and their environments and the difference between species *diversity* and species *abundance*. These basic ecological concepts pervade this and the next three chapters.

20.1 General Ecological Concepts

The distribution of microorganisms in nature resembles that of macroorganisms in the sense that a given species resides in certain places but not others; that is, everything is not everywhere. Also, environments differ in their abilities to support diverse microbial populations, from the highly diverse microbial world of undisturbed fertile soil to the rather restricted world of some highly extreme environments.

Ecosystems and Habitats

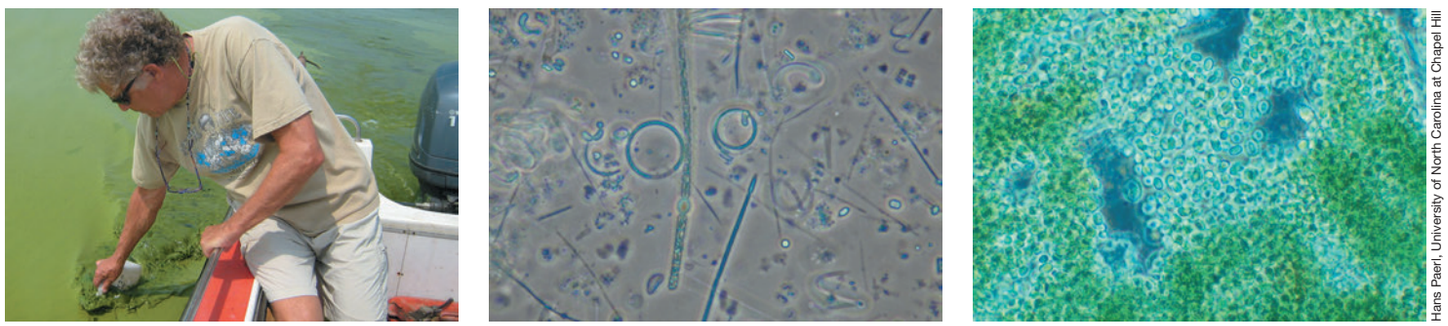
An **ecosystem** is a dynamic complex of plant, animal, and microbial communities and their abiotic surroundings, all of which interact as a functional unit. An ecosystem contains many different **habitats**, parts of the ecosystem best suited to one or a few populations. Although microorganisms are present in any habitat containing plants and animals, many microbial habitats are unsuitable for plants and animals. For example, microorganisms are ubiquitous on Earth's surface and even deep within it; they inhabit boiling hot springs and solid ice, acidic environments near pH 0, saturated brines, environments contaminated with radionuclides and heavy metals, and the interior of porous rocks that contain only traces of water. Therefore, some ecosystems are mostly or even exclusively microbial.

Collectively, microorganisms show great metabolic diversity and are the primary catalysts of nutrient cycles in nature (Chapter 21). The *types* of microbial activities possible in an ecosystem are a function of the species present, their population sizes, and the physiological state of the microorganisms in each habitat. By contrast, the *rates* of microbial activities in an ecosystem are controlled by the nutrients and growth conditions that prevail. Depending on several factors, microbial activities in an ecosystem can have minimal or profound impacts and can diminish or enhance the activities of both the microorganisms themselves and the macroorganisms that may coexist with them.

Species Diversity in Microbial Habitats

A group of microorganisms of the same species that reside in the same place at the same time constitutes a microbial **population** and may be descendants of a single cell. A microbial population differs from a microbial **community**. A community consists of populations living in association with other populations. The microbial species that reside in a certain habitat are those best able to grow with the nutrients and conditions that prevail there, and the number of different populations present is a measure of the community's complexity.

The diversity of microbial species in a community can be expressed in two ways: richness and abundance (Figure 20.1). **Species richness** is an expression of the total *number* of different species present. Identifying cells is, of course, basic to determining microbial species richness, but this need not require their isolation and culture. Species richness may also be expressed in molecular terms by the diversity of phylotypes (for example, the diversity of ribosomal RNA genes, ◀ Section 19.6) observed in a given community. **Species abundance**, by contrast, is the *proportion* of each species in the community (compare Figure 20.1*b* and *c*). Species richness and



Hans Paerl, University of North Carolina at Chapel Hill

(a) (b) (c)

Figure 20.1 Microbial species diversity: Richness versus abundance. *(a)* Collecting samples from Lake Taihu, China, following a bloom of the cyanobacterium *Microcystis*. *(b)* High species richness in St. John's River, Florida, shown by microscopy of planktonic microorganisms including cyanobacteria, diatoms, green algae, flagellates, and bacteria. *(c)* Shift of St. John's River community to low richness but high abundance following a bloom of the cyanobacterium *Microcystis*.

TABLE 20.1 Resources and conditions that govern microbial growth in nature

Resources
Carbon (organic, CO_2)
Nitrogen (organic, inorganic)
Other macronutrients (S, P, K, Mg)
Micronutrients (Fe, Mn, Co, Cu, Zn, Mn, Ni)
O_2 and other electron acceptors (NO_3^-, SO_4^{2-}, Fe^{3+})
Inorganic electron donors (H_2, H_2S, Fe^{2+}, NH_4^+, NO_2^-)
Conditions
Temperature: cold → warm → hot
Water potential: dry → moist → wet
pH: 0 → 7 → 14
O_2: oxic → microoxic → anoxic
Light: bright light → dim light → dark
Osmotic conditions: freshwater → marine → hypersaline

abundance can change quickly over a short time as shown by the change in abundance of cyanobacteria in a lake receiving nutrient-rich agricultural runoff (Figure 20.1*a*). One goal of microbial ecology is to understand what factors control species richness and abundance in microbial communities along with the community's associated activities and the abiotic environment. Once all of these factors are known, microbial ecologists can model the ecosystem by perturbing it in some way and observing whether predicted changes match experimental results.

The microbial species richness and abundance of a community are functions of the conditions that prevail and the kinds and amounts of nutrients available in the habitat. Table 20.1 lists common nutrients and conditions relevant to microbial growth. In some microbial habitats, such as undisturbed organic-rich soils, high species richness is common (see Figure 20.14), with most species present at only moderate abundance. Nutrients in such a habitat are of many different types, and this helps select for high species richness. In other habitats, such as some extreme environments, species richness is often very low and abundance of one or a few species very high. This is because the physical and chemical conditions in the environment exclude all but a handful of species, and key nutrients are present at such high levels (Figure 20.1*a*) that the highly adapted species can grow to high cell densities. Bacteria that catalyze acid mine runoff from the oxidation of iron are an example of this. These organisms thrive in highly acidic, iron-rich but organic-poor waters, where the acidic conditions and the dearth of organic carbon limit species richness. However, the elevated levels of ferrous iron (Fe^{2+}) present, which is oxidized to Fe^{3+} in energy-yielding reactions (◀ Section 14.8), fuel high species abundance. We examine the activities of acidophilic iron-oxidizing microorganisms in Sections 21.6, 21.1, and 22.2. Although the microbial diversity of extreme environments may at first seem mundane, extreme environments have been well-studied by microbial ecologists because the limited community complexity makes it easier to dissect the ecological interactions of the microbes that inhabit them.

Check Your Understanding

- What is the difference between species richness and species abundance?
- How does an ecosystem differ from a habitat?
- What are the characteristics of a microbial population?
- How does a microbial population differ from a microbial community?

20.2 Ecosystem Service: Biogeochemistry and Nutrient Cycles

In any ecosystem whose resources and growth conditions are suitable, microorganisms will grow to form populations. Metabolically similar microbial populations that exploit the same resources in a similar way are called **guilds**. A habitat that is shared by a guild and supplies the resources and conditions the cells require for growth is called a **niche**. Sets of guilds form microbial communities (Figure 20.2). Microbial communities interact with macroorganisms and abiotic factors in the ecosystem in a way that defines the workings of that ecosystem.

Energy Inputs to the Ecosystem

Energy enters ecosystems as sunlight, organic carbon, and reduced inorganic substances. Light is used by phototrophs to make ATP and synthesize new organic matter (Figure 20.2). In addition to carbon

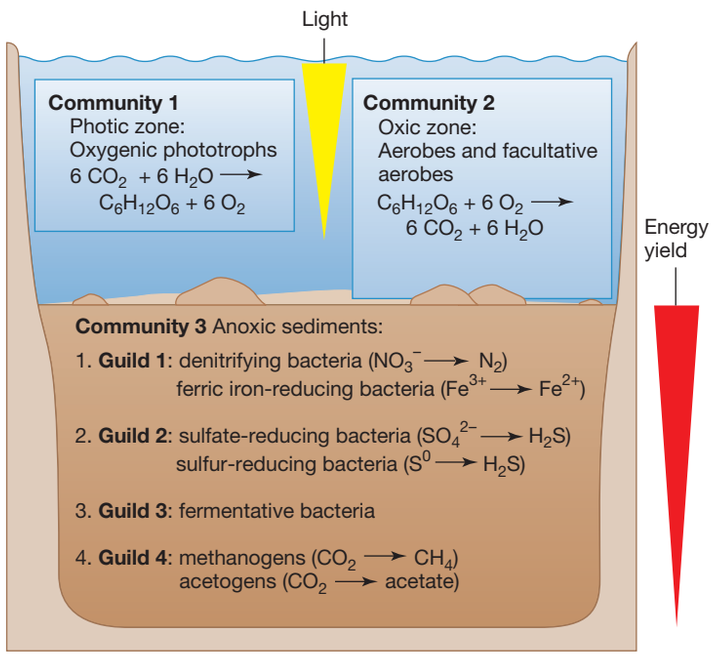


Figure 20.2 Populations, guilds, and communities. Microbial communities consist of populations of cells of different species. A freshwater lake ecosystem would likely have the communities shown here. The reduction of NO_3^-, Fe^{3+}, SO_4^{2-}, S^0, and CO_2 are examples of anaerobic respirations. The region of greatest activity for each of the different respiratory processes would differ with depth in the sediment. As more energetically favorable electron acceptors are depleted by microbial activity near the surface, less favorable reactions occur deeper in the sediment. All guild metabolisms shown are described in Chapter 14.

(C), new organic matter contains nitrogen (N), sulfur (S), phosphorus (P), iron (Fe), and the other elements of life (◀ Section 3.1). This newly synthesized organic material along with organic matter that enters the ecosystem from the outside (called *allochthonous* organic matter) fuels the catabolic activities of chemoorganotrophic organisms. These activities oxidize the organic matter to CO_2 by respiration or ferment it to various reduced substances. If chemolithotrophs are present and metabolically active in the ecosystem, they can conserve energy from the oxidation of inorganic electron donors, such as H_2, Fe^{2+}, S^0, or NH_3 (Chapters 14 and 15), and contribute new organic matter through their autotrophic activities.

Biogeochemical Cycling

Microorganisms play an essential role in cycling elements, in particular C, N, S, and Fe, between their different chemical forms. The study of these transformations is part of **biogeochemistry**, an interdisciplinary science that includes biology, geology, and chemistry. Figure 20.2 shows how the activities of different guilds of microorganisms influence the chemistry of one environment, a lake ecosystem. The sequence of changing chemistry with increasing depth in the sediments corresponds to the layers of different microbial guilds. The location of each guild in the ecosystem is primarily determined by the availability of electron donors and acceptors, both of which tend to decrease with increasing depth in the sediments.

A *biogeochemical cycle* defines the transformations of an element that are catalyzed by either biological or chemical means (or both). Many different microorganisms participate in biogeochemical cycling reactions, and in many cases, microorganisms are the *only* biological agents capable of regenerating forms of the elements needed by other organisms, particularly plants. Thus, biogeochemical cycles are often also *nutrient cycles*, reactions that generate important nutrients for other organisms.

Most biogeochemical cycles proceed by oxidation–reduction reactions as the element moves through the ecosystem and are often tightly *coupled*, with transformations in one cycle affecting one or more other cycles. For example, hydrogen sulfide (H_2S) is oxidized by phototrophic and chemolithotrophic microbes to sulfur (S^0) and sulfate (SO_4^{2-}), the latter being a key nutrient for plants. Phototrophs and chemolithotrophs are also autotrophs, and thus affect the carbon cycle by producing new organic carbon from CO_2. However, SO_4^{2-} can be reduced to H_2S by the sulfate-reducing bacteria, organisms that consume organic carbon, and this reduction closes the biogeochemical sulfur cycle while regenerating CO_2. The cycling of nitrogen is also a microbial process and is key to the regeneration of forms of nitrogen usable by plants and other organisms. The nitrogen cycle is driven by both chemolithotrophic and chemoorganotrophic bacteria, organisms that produce and consume organic carbon, respectively. We considered the microbiology of biogeochemical cycles and their coupled nature in Chapters 14 and 15 and will revisit this theme in more detail in Chapter 21.

Check Your Understanding

- How does a microbial guild differ from a microbial community?
- What is a biogeochemical cycle? Give an example based on sulfur. Why are biogeochemical cycles also called nutrient cycles?

II • The Microbial Environment

Being so small, microbes directly experience only a tiny portion of their environment. Microbial interactions, often as part of attached or layered communities, are based on consumption, generation, and diffusion of nutrients and the products of metabolism.

Microorganisms define the limits of life throughout aquatic and terrestrial environments on our planet. Specific conditions required by a particular organism or group of organisms may be subject to rapid change as a result of inputs to and outputs from their habitat or as a result of microbial activities or physical disturbances. Thus, within one environment there can be multiple habitats, some relatively stable and others changing rapidly over time and space.

20.3 Environments and Microenvironments

Besides living in the common habitats of soil and water, microorganisms thrive in extreme environments and also reside on and within the cells of other organisms. The intimate associations developed between microorganisms and other organisms will be explored in Chapters 23 and 24. Here we focus on terrestrial and aquatic microbial habitats.

The Microorganism, Niches, and the Microenvironment

The habitat in which a microbial community resides is governed by physicochemical conditions that are determined in part by the metabolic activities of the community. For example, the organic material used by one species may have been a metabolic by-product of a second species. Another example is oxygen (O_2), which can become limiting if biological consumption exceeds the rate at which it is supplied.

Because microbes are very small, they directly experience only a tiny local environment; this small space is called their **microenvironment**. For example, for a typical 3-μm rod-shaped bacterium, a distance of 3 mm is equivalent to that which a human would experience over a distance of 2 km! As a consequence of the smallness of microorganisms, the variable metabolic activities of nearby microbes, and the changes in physicochemical conditions over short intervals of time and distance, several microenvironments can exist within a given microbial habitat. The conditions supporting growth within a microenvironment correspond to the general requirements for growth we considered in Chapters 3, 4, and 8.

Ecological theory states that for every organism there exists at least one niche, the *realized niche* (also called the *prime niche*), where it will be most successful. The organism dominates the realized niche but may also inhabit other niches; in other niches it is less ecologically successful than in its realized niche, but it may still be able to compete. The full range of environmental conditions under which an organism can exist is called its *fundamental niche* (we considered the realized and fundamental niche in the context of enrichment culture and isolation in Sections 19.1–19.3). The word "niche" should not be confused with the word "microenvironment" because the microenvironment describes conditions at a specific location and can change rapidly. In other words, the general conditions that describe a specific niche may be transient at many places in a microenvironment.

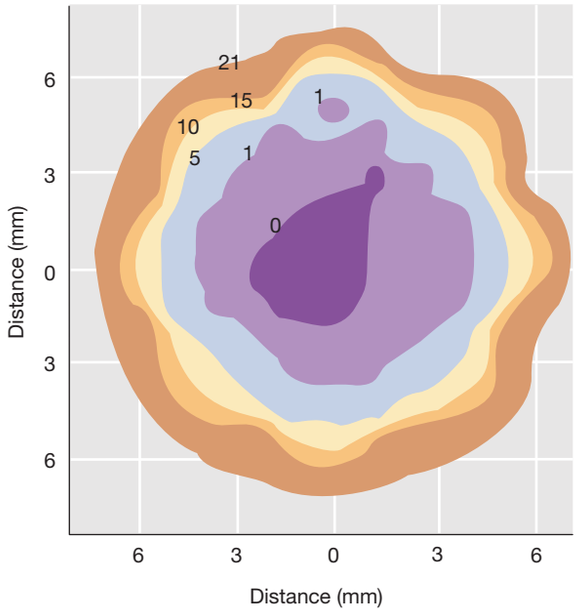


Figure 20.3 Oxygen microenvironments. Contour map of O_2 concentrations in a small soil particle as determined by a microsensor (◀ Section 19.9). The axes show the dimensions of the particle. The numbers on the contours are percentages of O_2 concentration (air is 21% O_2). Each zone can be considered a different microenvironment.

Another important consequence of microbes being so small is that diffusion often determines the availability of resources. Consider, for example, the distribution of an important microbial nutrient such as O_2 in a soil particle. Microsensors (◀ Section 19.9) can be used to measure oxygen concentrations throughout small soil particles. As shown in the data from an actual microsensor experiment (**Figure 20.3**), soil particles are not homogeneous in terms of their O_2 content but instead contain many adjacent microenvironments. The outer layer of the soil particle may be fully oxic (21% O_2) while the center, only a very short distance away (in human terms, but of course a great distance from a microbial standpoint), may be anoxic (O_2-free). The microorganisms near the outer edges consume all of the O_2 before it can diffuse to the center of the particle. Thus, anaerobic organisms could thrive near the center of the particle, microaerophiles (aerobes that require very low oxygen levels) farther out, and obligately aerobic organisms in the outermost region of the particle. Facultatively aerobic bacteria (organisms that can grow either aerobically or anaerobically, ◀ Section 4.16) could be distributed throughout the particle. Nutrient transfer is particularly important in thick assemblages of cells, such as biofilms and microbial mats, and we explore this in Section 20.4.

Physicochemical conditions in a microenvironment are subject to rapid change in both time and space. For example, the O_2 concentrations shown in the soil particle in Figure 20.3 represent "instantaneous" values. Measurements taken in the same particle following a period of intense microbial respiration or disturbance due to wind, rain, or disruption by soil animals could differ dramatically from those shown. During such events certain populations may temporarily dominate the activities in the soil particle and grow to high numbers, while others remain dormant or nearly so. However, if the microenvironments shown in Figure 20.3 are eventually reestablished, the various microbial activities characteristic of different regions of the soil particle will eventually return as well.

Nutrient Levels and Growth Rates

Resources (Table 20.1) typically enter an ecosystem intermittently. A large pulse of nutrients—for example, an input of leaf litter or the carcass of a dead animal—may be followed by a period of nutrient deprivation. Because of this, microorganisms in nature often face a "feast-or-famine" existence. It is thus common for them to produce storage polymers as reserve materials when resources are abundant and draw upon these reserves in periods of starvation. Examples of storage materials are poly-β-hydroxyalkanoates, polysaccharides, and polyphosphate (◀ Section 2.7).

Extended periods of exponential microbial growth in nature are typically rare, except in certain extreme environments—for example, a hot spring—where conditions and resources may stay optimal indefinitely. Instead, microorganisms typically grow in spurts, linked closely to the availability and types of resources and ideal growth conditions. Because all relevant physicochemical conditions in nature are rarely optimal for microbial growth at the same time, growth rates of microorganisms in nature are usually well below the maximum growth rates recorded in the laboratory. For instance, the generation time of *Escherichia coli* in the intestinal tract of a healthy adult eating at regular intervals is about 12 h (two doublings per day), whereas in pure culture it can grow much faster, with a minimum generation time of about 20 min under optimal conditions. In addition, research-based estimates indicate that most cultured soil bacteria typically grow in nature at less than 1% of the maximal growth rate measured in the laboratory.

These slower growth rates in nature than in laboratory culture reflect the facts that (1) resources and growth conditions (Table 20.1) are frequently suboptimal; (2) the distribution of nutrients throughout the microbial habitat is not uniform; and (3) except in rare instances, microorganisms in nature grow in mixed populations rather than pure culture. An organism that grows rapidly in pure culture may grow much slower in a natural environment where it must compete with other organisms that may be better suited to the resources and growth conditions available.

Microbial Competition and Cooperation

Competition among microorganisms for resources in a habitat may be intense, with the outcome dependent on several factors, including rates of nutrient uptake, inherent metabolic rates, and ultimately, growth rates. A typical habitat contains a mixture of different species (Figures 20.1 and 20.2), with the density of each population dependent on how closely its local environment resembles its realized niche.

Some microbes work together to carry out transformations that neither can accomplish alone—a process called *syntrophy*—and these microbial partnerships are particularly important for anoxic carbon cycling (◀ Section 14.22 and ▶ Section 21.2). Metabolic cooperation can also be seen in the activities of organisms that carry out *complementary* metabolisms. For example, we have previously considered metabolic transformations that are carried out by two distinct groups of organisms, such as those of the nitrifying *Bacteria* and *Archaea* (◀ Sections 14.9, 15.10, and 17.5). Together, these

nitrifiers oxidize ammonia (NH_3) to nitrate (NO_3^-). Because nitrite (NO_2^-), the final product of most ammonia-oxidizing nitrifiers (◀ Section 15.10 and ▶ Section 21.3), is the substrate for the nitrite-oxidizing bacteria, the two groups of organisms often live in nature in tight association within their habitats (◀ Figure 19.13*a*).

Winogradsky discovered nitrification in 1890 (◀ Section 1.13), but for over one hundred years no organism capable of complete ammonia oxidation was ever discovered. However, some *Nitrospira* species (*Bacteria*) can oxidize both ammonia and nitrite (◀ Section 15.10), a process that has been coined *comammox*. With the use of molecular tools (Chapter 19) to survey various habitats for comammox bacteria, related organisms have been identified in wetlands, riverbeds, aquifers, lake sediments, and wastewater treatment systems but not in the oceans. In some of these environments, comammox bacteria seem to outnumber classical nitrifying bacteria (species that catalyze only one step in nitrification), whereas in other environments, comammox organisms may be part of the rare biosphere (◀ Section 19.6), low in abundance yet nevertheless ecologically important microbes.

Check Your Understanding

- What characteristics define the realized niche of a particular microorganism?
- Why can many different physiological groups of organisms live in a single habitat?

20.4 Surfaces and Biofilms

Surfaces are important microbial habitats, typically offering microbes greater access to nutrients and protection from predation and physicochemical disturbances. Attachment to a surface also offers cells a means to remain in a favorable habitat, modify the habitat by their own activities, and not be washed away. In addition, flow across a colonized surface increases the flux of nutrients to the surface, providing more resources than are available to planktonic cells (cells that live a floating existence) in the same environment. A surface may be abiotic, such as a particle of organic matter, or provided by another organism. For example, plant root surfaces become heavily colonized by soil bacteria thriving on organic compounds excreted by the plant, as revealed when fluorescent stains such as acridine orange (◀ Section 19.4) are used on natural samples (**Figure 20.4*a***).

Virtually any natural or artificial surface exposed to microorganisms will be colonized. For example, microscope slides have been used as experimental surfaces to which organisms can attach and grow. A slide can be immersed in a microbial habitat, left for a period of time, and then retrieved and examined microscopically (Figure 20.4*b*). Clusters of a few cells that develop from a single colonizing cell—called *microcolonies*—form readily on such surfaces, much as they do on natural surfaces (Figure 20.4*a*). Periodic microscopic examination of immersed slides has been used to measure growth rates of attached organisms in nature.

Surface colonization may be sparse, consisting only of microcolonies not visible to the naked eye, or may consist of so many cells that microbial accumulation becomes visible as, for example, in a stagnant toilet bowl. Surface growth can be particularly problematic in a hospital setting where microbial colonization of indwelling devices such as catheters and intravenous lines can cause serious infection (◀ Figure 4.17*b*). In a few extreme environments that lack

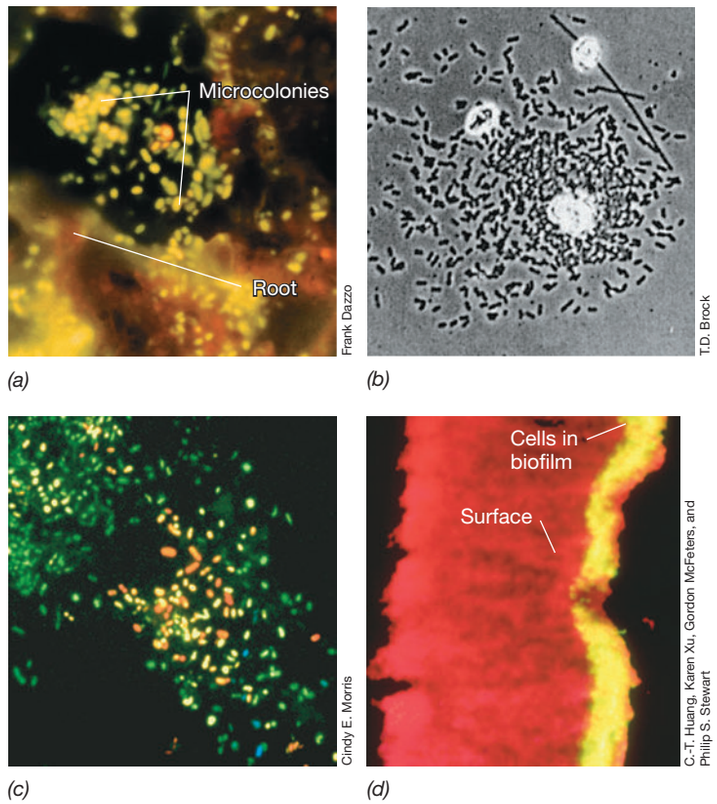


Figure 20.4 Microbial biofilms on surfaces. *(a)* Fluorescence photomicrograph of a natural microbial community living on plant roots in soil and stained with acridine orange. Note microcolony development. *(b)* Bacterial microcolonies developing on a microscope slide that was immersed in a river. The bright particles are mineral matter. The short, rod-shaped cells are about 3 μm long. *(c)* Confocal scanning laser microscopy through a natural biofilm (top view) on a leaf surface. The color of the cells indicates their depth in the biofilm: red, surface; green, 9-μm depth; blue, 18-μm depth. *(d)* A cross-sectional view of an experimental biofilm composed of cells of *Pseudomonas aeruginosa*. The yellow layer (about 15 μm in depth) contains cells and is stained by a reaction showing activity of the enzyme alkaline phosphatase.

small animal grazers (for example, hot springs), microbial accumulation on a surface can be layered and several centimeters thick. Called *microbial mats* (Section 20.5), such accumulations often contain highly complex yet very stable assemblages of phototrophic, chemolithotrophic, autotrophic, and heterotrophic microbes.

Layered communities can even form where light penetrates the near-surface interior of minerals, such as calcium sulfate (gypsum) crusts in solar salterns. Despite the extremely high salt content of these crystals, they are readily colonized by a community of halophilic phototrophs and heterotrophs (**Figure 20.5**). The phototrophic components in the gypsum crusts include cyanobacteria (◀ Section 15.3; orange- and green-colored layers in Figure 20.5) and purple sulfur bacteria (◀ Section 15.4, red layer in Figure 20.5). Purple bacteria grow in the deepest layer of the crust because of their need for H_2S (produced by sulfate-reducing bacteria deeper in the crust) and ability to grow at low light intensities.

Biofilms

As bacterial cells grow on surfaces, they commonly form **biofilms**—assemblages of bacterial cells attached to a surface and enclosed in

Marilyn B. Vogel and David J. Des Marais

Figure 20.5 Endolithic microbial biofilm. Section through a submerged calcium sulfate (gypsum) crust that formed in a Guerrero Negro (Baja California, Mexico) crystallizer pond showing layered phototrophic microbial communities. The orange layer just under the crust surface contains the unicellular cyanobacterium *Halothece*, the green layer a mixture of unicellular and filamentous cyanobacteria, and the red layer purple sulfur bacteria of the genera *Ectothiorhodospira* and *Halochromatium*. Various chemotrophic bacteria are present in each of the layers as well, including sulfate-reducing bacteria, the source of the H_2S that feeds the purple sulfur bacteria.

an adhesive matrix that is the product of excretion by cells and cell death (Figure 20.4*c*, *d*). The matrix is typically a mixture of polysaccharides, proteins, and nucleic acids that bind the cells together. Biofilms trap nutrients and help prevent the detachment of cells on dynamic surfaces, such as in flowing systems (**Figure 20.6**). We examined the basic properties of biofilms in Section 4.9 and genetic and regulatory features of biofilm formation in Section 8.10, and so here we focus on their ecological and medical consequences.

Biofilms typically contain multiple layers of cells embedded in the porous matrix material, and the cells in each layer can be examined by confocal scanning laser microscopy (◀ Section 1.9; Figure 20.4*c*). Biofilms may contain one or two species or, more commonly, many species of bacteria. The biofilms that form on tooth and soft surfaces of the mouth, for example, contain between 100 and 200 different phylotypes (◀ Section 19.6), including species of both *Bacteria* and *Archaea*; in total, the human mouth is a habitat for at least 700 phylotypes (▶ Sections 24.3 and 25.2). Biofilms are thus functional and growing microbial communities and not just cells trapped in a sticky matrix.

Wherever submerged surfaces are present in natural environments, biofilm growth is almost always more extensive and diverse than the planktonic growth in the liquid above the surface. Biofilms differ from planktonic communities in supporting critical transport and transfer processes, which generally control growth in biofilm environments. For example, if consumption of O_2 by populations near the surface exceeds diffusion of O_2 into deeper regions of the biofilm, the deeper regions will become anoxic, opening up new niches for colonization by obligate anaerobes or facultative aerobes. This is analogous to the depletion of O_2 in the interior of a soil particle that was depicted in Figure 20.3. Hence, different metabolisms can be active in different regions of a biofilm and this broadens the diversity of microbes that can exist there.

Why Bacteria Form Biofilms

At least three reasons have been proposed for the formation of biofilms. First, biofilms are a means of microbial self-defense that increase survival. Biofilms resist physical forces that could otherwise remove cells only weakly attached to a surface. Biofilms also resist phagocytosis by protozoa and cells of the immune system and retard the penetration of toxic molecules such as antibiotics. These advantages improve the chances for survival of cells in the biofilm. Second, biofilm formation allows cells to remain in a favorable niche. Biofilms attached to nutrient-rich surfaces, such as animal tissues, or to surfaces in flowing systems (Figure 20.6) fix bacterial cells in locations where nutrients may be more abundant or are constantly replenished. Third, biofilms form because they allow bacterial cells to live in close association with each other. This facilitates cell-to-cell communication, offers more opportunities for nutrient and genetic exchange, and in general increases chances for survival.

The fact that biofilms form on virtually any surface capable of supporting bacterial growth suggests that biofilms are the "default" growth mode for bacteria in natural habitats. Planktonic growth, so common in shaken or otherwise mixed laboratory cultures, may thus be an atypical growth mode and the norm only for those bacteria adapted to life at extremely low nutrient concentrations (oligotrophs, Sections 20.9, 20.10, and 20.12). For example, the rocky and pebbly substratum of rivers and streams is an important biofilm habitat, as any hiker attempting to cross over the slippery rocks of a mountain stream is aware. This type of biofilm is in part nourished

Anna Depetris and Tom Ian Battin

(a)

(b)

Figure 20.6 Phototrophic biofilms in rivers and streams. *(a)* Phototrophic biofilms colonizing the rocky bottom of a stream flowing from the Rhone Glaciers, Switzerland (water is flowing toward the viewer). The striped patterns emerge from microbial growth along different flow paths of the glacial outflow. *(b)* Cyanobacteria attached to the river rocky substratum form "tower-like" clusters with apical oxygen bubbles forming from high photosynthesis rates within the clusters. The clusters are about 0.5–1 cm high.

by phototrophic algae and cyanobacteria that rapidly colonize newly exposed surfaces (Figure 20.6*a*).

Although biofilms are known to provide some resistance to grazing, they are not immune to grazing and typically attract high densities of micrograzers, including heterotrophic flagellates, amoebae, and ciliates. These specialized biofilm micrograzers, together with small metazoans called *meiofauna* (rotifers, nematodes, microcrustaceans, oligochaetes, and flies), feed on components of the biofilm. In doing so, they incorporate a significant amount of the carbon fixed by biofilm autotrophs and also remove planktonic cells and organic carbon from the water column. Thus, by existing at the interface between an inert substratum and surface water, river and stream biofilms provide significant ecosystem services by coupling benthic and water column microbial communities and nourishing higher trophic levels in aquatic systems.

Biofilms on Microplastics

In addition to natural surfaces, plastics provide an artificial surface for microbial colonization, and biofilms that develop on plastics can have both beneficial and harmful aspects. Each year humans add millions of metric tons of plastic to the oceans alone. Biodegradation and weathering processes degrade this plastic into small particles and fibers less than 5 mm in diameter called *microplastics* (MPs, **Figure 20.7**), and the surfaces of MPs get colonized by microbes. On the one hand, the formation of biofilms on these surfaces signals the initial stages of biodegradation and can be considered a good thing. But on the other hand, more than 200 marine organisms are known to ingest microplastics, including zooplankton, fish, seabirds, marine mammals, and benthic invertebrates. Depending on the animal, MPs can cause a variety of health issues including neurotoxicity, DNA damage, reproductive defects, and decreased filtration rates in filter feeders such as mussels. Unfortunately, biofilm-coated (as opposed to clean) MP may actually escalate this environmental problem.

Surface colonization and biodegradation of plastic by microorganisms is suspected to increase harmful environmental effects by altering the surface chemistry, density, and sinking rates of MPs. In addition, since microbially colonized MPs (Figure 20.7) can be greatly enriched in hydrophobic organic contaminants relative to surrounding seawater, they are also a conduit for the delivery of these adsorbed contaminants to filter-feeding biota. Thus, apart from the possibility that filter feeders may preferentially ingest biofilm-encrusted MPs because of their higher nutritional quality, most studies point to only adverse impacts of MPs on aquatic ecosystems.

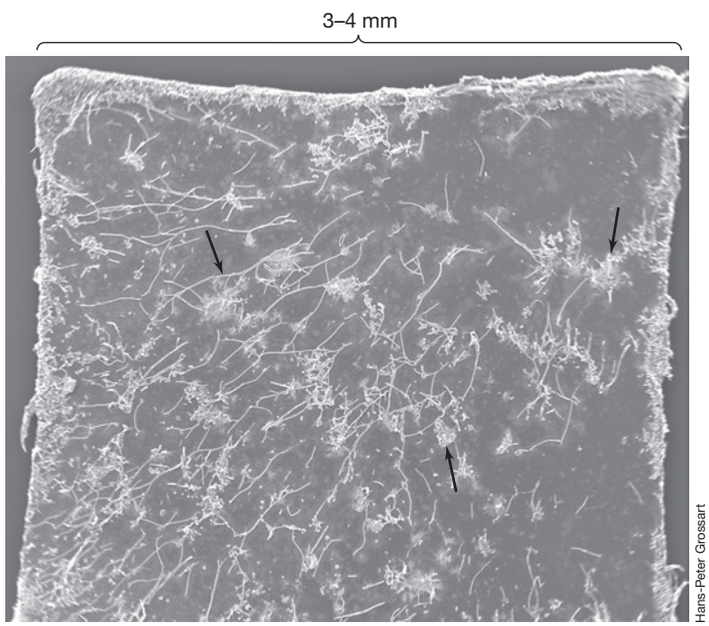


Figure 20.7 Biofilm formation on a microplastics particle. Microbial colonization and biofilm formation (arrows) on a polystyrene microplastics particle following immersion for three months in a bog in Germany. This microplastic particle is 3–4 mm in diameter and about 0.1 mm thick.

Problem Biofilms for Humans

In addition to aquatic systems, biofilms have significant implications in human medicine and commerce. In the body, bacterial cells within a biofilm are protected from attack by the immune system, and antibiotics and other antimicrobial agents often fail to penetrate the biofilm. One of the most clinically and industrially relevant properties of biofilm microbial communities is their inherent tolerance of antibiotics and other antimicrobial chemicals. A given species growing in a biofilm can be up to 1000 times more tolerant of an antimicrobial substance than planktonic cells of the same species. Reasons for this greater tolerance include slower growth rates in biofilms, reduced penetration of antimicrobial substances through the extracellular matrix, and the expression of genes that increase tolerance to stress. This tolerance of antimicrobial substances may explain why biofilms are responsible for many untreatable or difficult-to-treat chronic infections and are also hard to eradicate in industrial systems, such as wastewater plants, where surface growth (fouling) by microbes may impair important processes.

As we saw in earlier chapters, the bacterium *Pseudomonas aeruginosa* forms tenacious biofilms (◀ Sections 4.9 and 8.10 and Figure 4.16), and biofilms of this organism form in the lungs of humans suffering from the genetic disease *cystic fibrosis*. Once in the biofilm state, *P. aeruginosa* is difficult to treat with antibiotics and the biofilm helps the organism persist. *Burkholderia* (previously *Pseudomonas*) *cepacia* is also a threat to those with cystic fibrosis (◀ Section 16.2). Besides cystic fibrosis, biofilms have been implicated in several medical and dental conditions, including periodontal disease, chronic wounds, kidney stones, tuberculosis, Legionnaires' disease, and *Staphylococcus* infections (◀ Figure 4.17*b*). Medical implants are ideal surfaces for biofilm development. These include both short-term devices, such as urinary catheters, as well as long-term implants, such as artificial joints. It is estimated that 10 million people a year in the United States experience biofilm infections from implants or intrusive medical procedures. Biofilms also explain why routine oral hygiene is so important for maintaining dental health. Dental plaque is a typical biofilm and contains acid-producing bacteria responsible for dental caries (▶ Sections 24.3 and 25.2 and Figures 25.7, 25.8).

Biofilms affect commerce because they can slow the flow of water, oil, or other liquids through pipelines and can accelerate corrosion of the pipes themselves (◀ Figure 1.17). Biofilms also initiate the degradation of submerged objects, such as structural components of offshore oil platforms, boats, and shoreline installations. The safety

of drinking water may be compromised by biofilms that develop in water distribution pipes. These biofilms mostly contain harmless microbes, but if pathogens successfully colonize a biofilm, water purification practices (Chapter 22) may fail to kill them. Periodic releases of pathogenic cells can then lead to outbreaks of disease. For example, it is thought that *Vibrio cholerae*, the causative agent of cholera (▶ Section 33.3), may be propagated in this manner.

Biofilm control is big business, and thus far, only a limited number of tools exist to fight biofilms. Collectively, industries commit huge financial resources to treating pipes and other surfaces to keep them free of biofilms. New antimicrobial agents that can penetrate biofilms, as well as drugs that eliminate biofilm formation by interfering with intercellular communication, are being developed, but at this point, human battles with biofilms continue at a brisk pace.

Check Your Understanding

- Why might a biofilm be a good habitat for bacterial cells living in a flowing system?
- Give an example of a medically relevant biofilm that forms in virtually all healthy humans.
- How is it possible for both aerobes and obligate anaerobes to coexist in the same biofilm?

20.5 Microbial Mats

Microbial mats are among the most visibly conspicuous of microbial communities and can be thought of as extremely thick biofilms. Supported by phototrophic or chemolithotrophic bacteria, these layered microbial communities can be several centimeters thick (**Figure 20.8*a, b***). The layers are composed of species of different microbial guilds whose activities are governed by light availability and other resources (Table 20.1). The combination of microbial metabolism and nutrient transport controlled by diffusion results in steep concentration gradients of different microbial nutrients and metabolites, creating unique niches at different depth intervals in the mats. The most abundant and versatile phototrophic mat builders are filamentous cyanobacteria, which are oxygenic phototrophs and many of which tolerate extreme environmental conditions. For example, some species of cyanobacteria grow in waters as hot as 73°C or as cold as 0°C, and others tolerate salinities in excess of 12% and pH values as high as 10.

Cyanobacterial Mats

Cyanobacterial mats (Figure 20.8*a*, *b*) are complete microbial ecosystems, containing large numbers of **primary producers** (cyanobacteria and other phototrophic bacteria) that use light energy to synthesize new organic material from CO_2. These along with populations of consumers in the mat community mediate all key nutrient cycles.

Microbial mats have existed for over 3.5 billion years (stromatolites, ◀ Section 13.2 and Figure 13.6) but are found today only in aquatic environments where environmental stresses such as high temperatures or high salt concentrations restrict grazing by small animals and insects. Well-studied microbial mats are found in hypersaline solar evaporation basins; such basins have either formed naturally, such as Solar Lake (Sinai, Egypt), or have been constructed for the recovery of sea salt (Figure 20.8*a*). Layered phototrophic communities also form within the salt crusts of these evaporation basins (Figure 20.5). Because microbial mats are restricted to extreme environments, most are found in remote locations and many are not readily accessible to study. In contrast, however, the cyanobacterial mats that colonize the outflow channels of hot springs in Yellowstone National Park (USA), Iceland, and many other thermal regions in the world are easily accessible and have been widely studied (Figure 20.8*b*, *c*).

The chemical and biological structure of a microbial mat can change dramatically during a 24-h period (called a *diel cycle*) as a consequence of light to dark transitions. Using microsensors (◀ Section 19.9) it is possible to measure pH, H_2S, and O_2 repeatedly over a diel cycle in zones in the mat separated vertically by only a few micrometers. During the day, there is intense oxygen production in the cyanobacterial surface layer of microbial mats and active

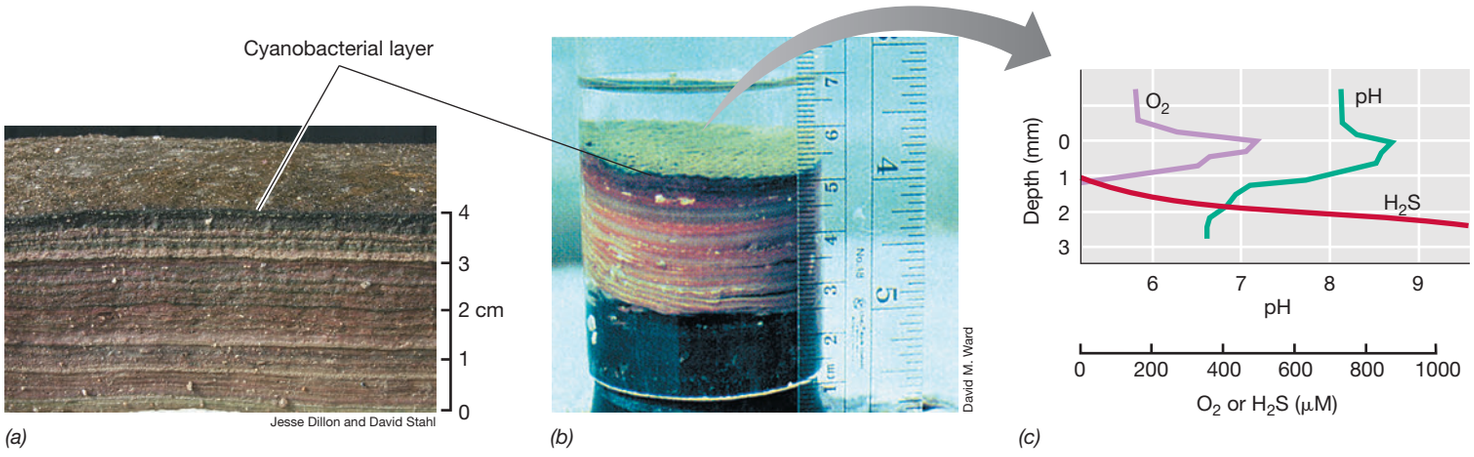


Figure 20.8 Microbial mats. *(a)* Mat specimen collected from the bottom of a hypersaline pond at Guerrero Negro, Baja California (Mexico). Most of the bottom of this shallow pond is covered with mats built by the major primary producer, the filamentous cyanobacterium *Microcoleus chthonoplastes*. *(b)* Microbial mat core from an alkaline Yellowstone National Park (USA) hot spring. The upper (green) layer contains mainly cyanobacteria, while the reddish layers contain anoxygenic phototrophic purple bacteria. *(c)* Daylight oxygen (O_2), H_2S, and pH profiles through a hot spring mat core such as that shown in part *b*.

sulfate reduction throughout the lower regions. Near the zone where O_2 and H_2S begin to mix, intense metabolic activity by phototrophic and chemolithotrophic sulfur bacteria may consume these substrates rapidly over very short vertical distances. Detecting the rate of these changes reveals the zones of greatest microbial activity (Figure 20.8*c*). These gradients disappear at night when photosynthesis stops, and the entire mat turns anoxic as H_2S accumulates. Some mat organisms rely on motility to follow the shifting chemical gradients. For example, sulfur-oxidizing filamentous phototrophic bacteria such as *Chloroflexus* and *Roseiflexus* (◀ Section 15.7) track the up-and-down movement of the O_2–H_2S interface on a diel basis.

Iron-Rich Microbial Mats

Microbial mats of oxidized iron also form in metal-rich, low-pH environments, such as occurs with the mining of metals (**Figure 20.9*a***; see acid mine drainage, Section 22.2) and in iron-rich hot springs, such as that of Echinus Geyser in Yellowstone National Park (Figure 20.9*b*). As Fe^{2+}-rich water is exposed to air, iron-oxidizing *Bacteria* and *Archaea* generate a thick mat of oxidized iron. Metagenomic analyses of the Echinus Geyser mat revealed a novel phylum of microaerophilic *Archaea*, provisionally named the *Marsarchaeota* (see Figure 20.16) after the resemblance of their red iron-oxide-rich habitat to the color of Mars. Enrichment cultures of *Marsarchaeota* show large cocci that can oxidize both organic carbon and ferrous iron. Their heterotrophic capabilities suggest that they receive organic carbon fixed by chemolithotrophs coexisting in these iron-rich mat communities. An example of such a chemolithotroph is *Metallosphaera*, a relative of the sulfur- and iron-oxidizing *Sulfolobus*, a genus of *Archaea* (◀ Section 17.10). We will see in Chapter 22 that acidophilic iron-oxidizing microbes in mining operations can cause considerable environmental damage to both flora and fauna when the acidity and toxic heavy metals their activities release enter aquatic systems.

J.M. Sánchez, J.J. deLope, and Ricardo Amils

(a)

W. Inskeep

(b)

Figure 20.9 Iron-oxidizer microbial mats. *(a)* A mat of iron-oxidizing bacteria attached to rocks in the Rio Tinto, Spain. As ferrous (Fe^{2+})-rich water from metal mining activities flows over and through the biofilm, the organisms oxidize Fe^{2+} to Fe^{3+}. *(b)* An iron-rich microbial mat in the acidic (pH 3–4) and hot (70–80°C) outflow of Echinus Geyser (Norris Geyser Basin, Yellowstone National Park, Wyoming, USA). The terraces in the outflow channel are formed by surface growth of ferrous-iron-oxidizing *Sulfolobales* (*Metallosphaera yellowstonensis*) (◀ Section 17.10) that form insoluble ferric oxides. The mat also contains significant populations of *Geoarchaeota* (a heterotrophic candidate archaeal phylum) and the phylum *Marsarchaeota*. Photo courtesy of William Inskeep, Research Permit YELL-2016-SCI-05068.

Chemolithotrophic Microbial Mats

Like iron-rich mats (Figure 20.9), which typically lack phototrophic components and are thus supported by the activities of iron-oxidizing chemolithotrophs, some microbial mats develop from the activities of sulfur-oxidizing chemolithotrophs. The most common of these are mats composed of filamentous sulfur bacteria such as *Beggiatoa* and *Thioploca* species that grow on marine sediment surfaces at the interface between O_2 supplied from oxic overlying waters and H_2S produced by sulfate-reducing bacteria residing in the anoxic sediments. In these dark habitats, photosynthesis cannot occur and so the bacteria oxidize H_2S to yield energy and support autotrophy (◀ Sections 14.7 and 15.12).

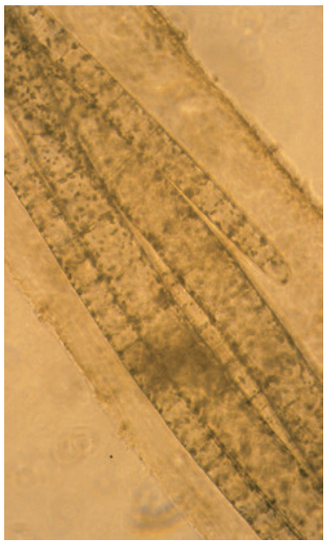
Chemolithotrophic mats composed of sulfur-oxidizing *Thioploca* species on sediments of the Chilean and Peruvian continental shelf are thought to be the most extensive microbial mats of any type on Earth (**Figure 20.10**). The bottom waters near the sediments in this region of the Pacific Ocean are oxygen-depleted (an oxygen minimum zone, Section 20.10) but contain significant levels (about 25 μM) of nitrate (NO_3^-). *Thioploca* (◀ Figure 15.32*b*) can oxidize sulfide anaerobically with nitrate as electron acceptor and has evolved a remarkable strategy to bridge these spatially separated resources—nitrate in the overlying water and sulfide in the sediments. *Thioploca* cells contain large internal vacuoles that can concentrate nitrate from the surrounding water to levels as high as 0.5 M. To do this, they migrate upward from the sediments by gliding motility (◀ Section 2.10) and charge their vacuoles with NO_3^- (Figure 20.10*a*, *b*). They then return into the anoxic sediment (gliding at speeds of 3–5 mm per hour) to use their stored NO_3^- as an electron acceptor for H_2S oxidation.

The physical and biological structures of both biofilms and microbial mats are determined by metabolic interactions among the microbes within them and the diffusion of nutrients. Thus, as biofilms form on a surface they become increasingly more complex, and in so doing generate new niches for organisms of differing physiologies. This diversity reaches a maximum in mature microbial mats (Figure 20.8*a*, *b*), as molecular community sampling (◀ Section 19.6) has shown these structures to be among the most complex microbial communities yet discovered.

Check Your Understanding

- What is a microbial mat, and what major nutrient changes occur in phototrophic mats during a diel cycle?
- How would motile aerobic bacteria in a microbial mat respond to changing O_2 concentrations over a diel cycle?
- What is the strategy used by *Thioploca* to oxidize sulfide in anoxic regions below the surface of marine sediments?

(a)



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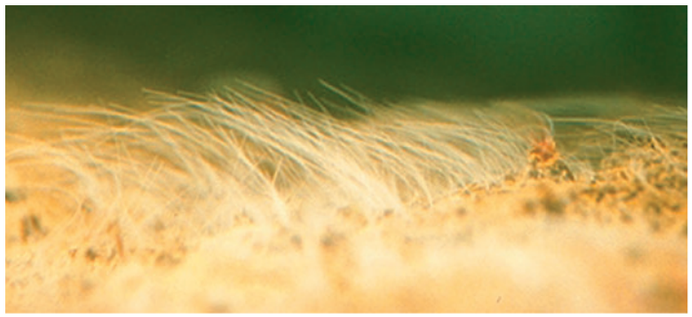


Andreas Teske and Markus Huettel

(c)

Figure 20.10 ***Thioploca* mats.** *(a, c)* Filaments of the large sulfur-oxidizing chemolithotroph *Thioploca* extend into the water above the sediment (87-m depth) in the Bay of Concepción off the Chilean coast. *(b) Thioploca* form bundles of 10 to 20 filaments (trichomes) held together by a gelatinous sheath, each bundle approximately 1.5 mm in diameter and 10–15 cm in length. Two species of *Thioploca* commonly inhabit the same bundle: *T. chileae*, about 20 μm in diameter, and *T. araucae*, about 40 μm in diameter. Individual trichomes glide independently within the sheaths and can extend up to 3 cm into the water.

III • Terrestrial Environments

Terrestrial environments range from wet to dry, warm to cold, alkaline to acidic, oxic to anoxic, and nutrient-rich to nutrient-poor. These characteristics control the diversity and activity of microorganisms that inhabit the terrestrial environments found on Earth.

Extensive microbial habitats exist in two terrestrial environments on Earth: surface soils and the deep subsurface. We cover these microbial habitats in the following three sections, the first two on soils and the third one on the subsurface. We consider the basic characteristics of each habitat, explore their microbial diversity, and discuss reasons why the microbes that are present in each habitat may be well suited to their environment.

20.6 Soils: General Properties

The word *soil* refers to the loose outer material of Earth's surface, a layer distinct from the bedrock that lies underneath (**Figure 20.11**). Soil develops over long periods through complex interactions among the parent geological materials (rock, sand, glacial drift materials, and so on), the topography, climate, and the presence and activities of living organisms.

Mastering Microbiology
Art Activity: Figure 20.9*a* Soil

Soils can be divided into two broad groups: *Mineral soils* are derived from the weathering of rock and other inorganic materials, and *organic soils* are derived from sedimentation in bogs and marshes. Most soils are a mixture of these two basic types. Although mineral soils, which are the primary focus of this section, predominate in most terrestrial environments, there is increasing interest in the role that organic soils play in carbon storage. A detailed understanding of carbon storage (sinks) and sources (such as release of CO_2) is of great relevance to the science of climate change. The carbon cycle is a major focus of Chapter 21.

Soil Composition

Vegetated soils have at least four components. These include (1) *inorganic mineral matter*, typically 40% or so of the soil volume; (2) *organic matter*, usually about 5%; (3) *air and water*, roughly 50%; and (4) *microorganisms and macroorganisms*, about 5%. Particles of various sizes are present in soil. Soil scientists classify soil particles on the basis of size: Those in the range of 0.1–2 mm in diameter are called *sand*, those between 0.002 and 0.1 mm *silt*, and those less than 0.002 mm *clay*. Different textural classes of soil are then given names such as "sandy clay" or "silty clay" based on the percentages of sand, silt, and clay they contain. A soil in which no one particle size dominates is called a *loam*.

Soils are categorized based on their depth in a soil profile, and the absolute depths vary from soil to soil. In a typical mineral soil the O horizon ("O" for "organic") (Figure 20.11*a*) is the uppermost layer and is dominated by decomposing organic matter; because of this, vast numbers (as high as 10^{10}/g) of microbial cells are often present here. The O horizon segues into the "A" horizon, the soil layer commonly referred to as "topsoil," which is also organic-rich and extends anywhere from a few centimeters to about 50 cm from the surface, depending on a soil's structural characteristics. The "B" horizon, called "subsoil," can extend over a meter below the A horizon and is rich in iron- and aluminum-containing clay minerals and thus red/brown in color (Figure 20.11*b*). Plant roots, especially tree roots, often extend into the B horizon. The "C" horizon, also called "parent material," transitions into underlying bedrock and can extend for several meters from the surface.

The organic content of a soil is typically inversely related to depth, with the organic-rich O–A horizons transitioning into the more organic carbon–depleted B and nearly organic carbon–free C horizons (Figure 20.11*a*). Organic matter content of a soil varies with the type, but in the soil shown in Figure 20.11*b* (a soil that the United States Department of Agriculture would classify as an "alfisol"), values of organic carbon as high as 5% can be present in the O–A horizons; values drop to less than 1% in the B horizon and less than 0.25% beneath this (Figure 20.11*a*). The nitrogen and phosphorus

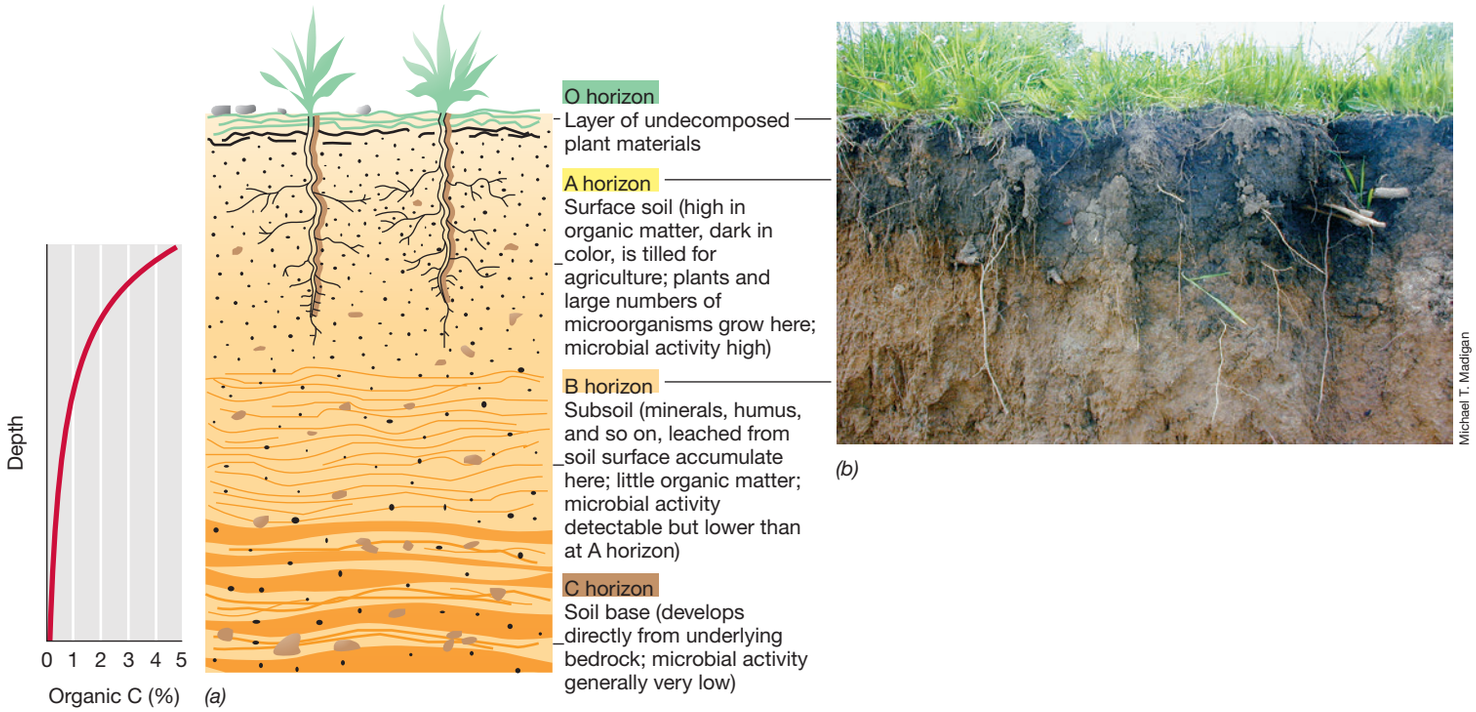


Figure 20.11 Soil. *(a)* Profile of a mature fertile soil. The soil horizons are zones defined by soil scientists, and the graph to the left shows approximate levels of organic carbon in different soil horizons. *(b)* Photo of a soil profile, showing O, A, and B horizons. This soil from Carbondale, Illinois (USA), is rich in clay and very compact. Such soils are not as well drained as those rich in sand. Note the clear color delineation between the organic-rich A horizon and the less-organic-rich B horizon.

content of soils follows a similar pattern to that of carbon, reflecting the fact that the bulk of these key nutrients are tied up in decaying plants and animals and in microbes, both dead and alive.

Soil Formation

Physical, chemical, and biological processes all contribute to the formation of soil. An examination of almost any exposed rock reveals the presence of algae, lichens, or mosses. These organisms are phototrophic and produce organic matter, which supports the growth of chemoorganotrophic bacteria and fungi. More complex chemoorganotrophic communities composed of *Bacteria*, *Archaea*, and eukaryotes then develop as the extent of the earlier colonizing organisms increases. Carbon dioxide produced during respiration becomes dissolved in water to form carbonic acid (H_2CO_3), which slowly dissolves the rock, especially rocks containing limestone ($CaCO_3$). In addition, many chemoorganotrophs excrete organic acids, which also promote the dissolution of rock into smaller particles.

Freezing, thawing, and other physical processes assist in soil formation by forming cracks in the rocks. As the particles generated combine with organic matter, a crude soil forms in these crevices, providing sites needed for pioneering plants to become established. The plant roots penetrate farther into the crevices, further fragmenting the rock; the excretions of the roots promote development in the **rhizosphere** (the soil that surrounds plant roots and receives plant secretions) of high microbial cell abundance (Figure 20.4*a*). When the plants die, their remains are added to the soil and become nutrients for more extensive microbial development. Minerals are rendered soluble, and as water percolates, it carries some of these substances deeper into the soil.

As weathering proceeds, the soil increases in depth and becomes able to support the development of larger plants and small trees. Soil animals such as earthworms colonize the soil and play an important role in keeping the upper layers of the soil mixed and aerated. Eventually, the movement of materials downward results in the *soil profile* (Figure 20.11). The rate of development of a typical soil profile depends on climatic and other factors, but it can take hundreds to thousands of years from bare rock to fertile soil.

Water Availability: Vegetated and Dryland Soils as Microbial Habitats

The limiting nutrients in soils are often inorganic, such as phosphorus and nitrogen compounds, key components of several classes of macromolecules. Another major factor affecting microbial activity in soil is the availability of water, and we have previously emphasized the importance of water for microbial growth (◀ Section 4.15).

Water is a highly variable component of soil, and a soil's water content depends on soil composition, rainfall, drainage, and plant cover. Water is held in the soil in two ways—by adsorption onto surfaces or as free water in thin sheets or films between soil particles (**Figure 20.12**). The water present in soils has materials dissolved in it, and the mixture is called the *soil solution*. In well-drained soils, air penetrates readily, and the oxygen concentration of the soil solution can be high, similar to that of the soil surface. In waterlogged soils, however, the only oxygen present is that dissolved in water, and this can be rapidly consumed by the resident microbiota. Such soils then become anoxic, and, as described for freshwater environments (Section 20.9), show profound changes in their biological activities.

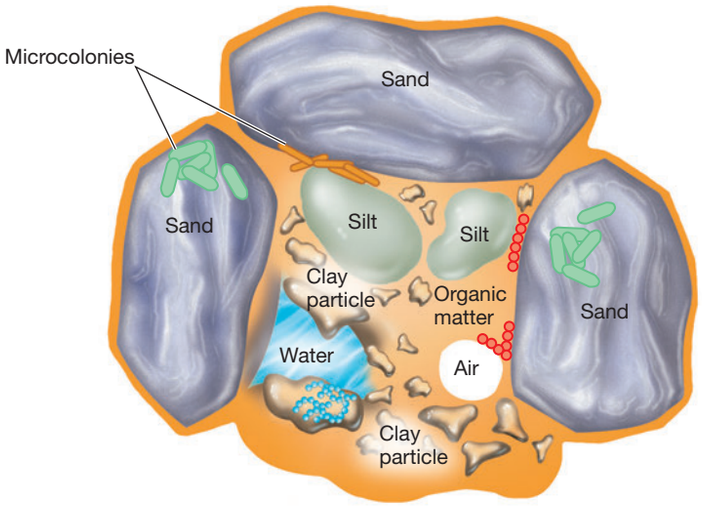


Figure 20.12 A soil microbial habitat. Very few microorganisms are free in the soil solution; most of them reside in microcolonies attached to the soil particles. Note the relative size differences among sand, clay, and silt particles.

There is also water in the larger channels in soil, where bulk flow is important for rapid transport of microorganisms and their substrates and products.

Arid Soils

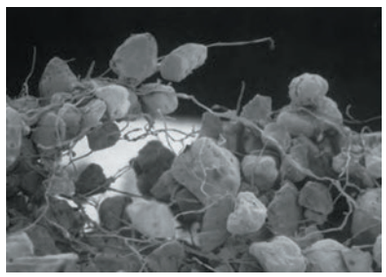
The greatest microbial activity in soils is in the organic-rich surface layers in and around the rhizosphere (Figure 20.4*a*). However, some soils are so dry that plant coverage is greatly limited, and only special microbial communities can thrive. These are *arid soils*, and approximately 35% of Earth's landmass is permanently or seasonally arid. Aridity can be defined by the *aridity index*, expressed as the ratio of precipitation to potential evapotranspiration (P/PET). Evapotranspiration is the sum of water loss through evaporation and plant transpiration. A region is deemed arid if there is a P/PET of less than 1; that is, water entering through precipitation (and fog and dew) is less than that lost through evapotranspiration.

Arid soils are among the most extreme environments on Earth, with temperature highs in excess of 60°C and lows of −24°C, high insolation (exposure to solar rays), and low water activity (◀ Section 4.15). Although arid regions are typically nearly devoid of leafy plants, they sustain important microbial communities that assemble in and stabilize soil near the surface and reside within and on the surfaces of rocks (◀ Section 19.8 and Figure 19.27). The dominant microorganisms present in these carbon-limited environments are cyanobacteria, with lesser numbers of green algae, fungi, heterotrophic bacteria, lichens, and mosses.

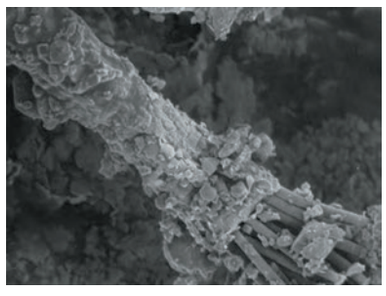
Dryland microbial habitats include *biological soil crusts* (BSCs) (**Figure 20.13**), ventral surfaces of translucent stones (*hypolithic* colonists), exposed rock surfaces (*epilithic* colonists), and the interior pore spaces, cracks, and fissures of rocks or minerals (*endolithic* colonists; ◀ Figure 18.35 and Figure 20.5). The soil crusts are dominated by the filamentous cyanobacterium *Microcoleus* (Figure 20.13*b*, *c*; ◀ Figure 19.27), whereas coccoid *Chroococcidiopsis* species are the predominant endolithic population. The rock colonists play an important role in weathering and soil formation; here we primarily consider the BSC communities.

The BSC functions in soil stabilization of desert ecosystems. Stabilization is critical because of the very slow rate of desert soil formation (<1 cm per 1000 years). Here, the *Microcoleus* and fungi provide soil cohesion, which is further stabilized aboveground by lichens and mosses when present. Importantly, this microbial

(a)



(b)



(c)

Figure 20.13 Biological soil crust (BSC). *(a)* BSC on the Colorado Plateau shown adjacent to lighter, disturbed soils. *(b, c)* Scanning electron micrographs of filamentous cyanobacteria (*Microcoleus* species) that bind sand grains together with their sheath material (◀ Figure 19.27). The sand grains in part *b* are about 100 μm in diameter and the filaments in part *c* about 5 μm in diameter.

network functions to virtually eliminate soil erosion from wind and water. The BSCs are major determinants of water infiltration and influence local hydrological cycles and water availability to vegetation. Remarkably, when moisture and temperature conditions are optimal, the photosynthetic rates of BSC are comparable to those of vascular plant leaves. In soil crusts dominated by *Microcoleus*, nitrogen-fixing bacteria (◀ Sections 8.9, 3.12, and 15.3) provide most of the nitrogen, and much of the fixed nitrogen is released immediately and made available to *Microcoleus* and other soil biota (◀ Section 19.8). To respond to variability in moisture conditions, desert soil microbial communities have higher relative abundances of genes encoding osmoregulation and dormancy functions; this reflects their adaptation to the extremes in water availability they experience, from rarely very wet to usually dry or even arid.

The disruption of BSCs is a major contributor to *desertification*, a process exacerbated by climate change and human activities. Dust storms resulting from BSC destruction reduce soil fertility, and when heavy dust is deposited on nearby snowfields it accelerates melt and evapotranspiration rates, thereby reducing freshwater inputs to rivers. Once compromised, soil crusts have recovery times varying from 15 to 50 years. Given the expansive terrestrial presence of BSCs, their importance to human and ecosystem function, and the projected increase in aridity associated with climate change (▶ Section 21.9), a better understanding of BSC formation and the rehabilitation of compromised BSCs is important for a healthy planet Earth. In this connection, important new understanding of the metabolic interactions among the microbes that sustain these critical communities is emerging from the new research area of metabolomics (◀ Section 19.8, subsection Utility of Metabolomics: An Environmental Case Study of Biocrusts). As we saw in this section, biocrusts contain both producer and consumer microbes, and the exchange of nutrients between these two groups is extensive (◀ Figure 19.28), bordering on what could be called a symbiosis.

With this brief background on soils, we next consider soil microbial diversity. Although some microbial eukaryotes reside in soils, our focus will be on prokaryotic cells because these are present in soils in highest numbers and greatest diversity.

Check Your Understanding

- What four components make up a typical vegetated soil?
- Which region of soil is the most microbially active? What are important sources and types of carbon in soil?
- Why is the cyanobacterium *Microcoleus* important in arid soils?

20.7 Prokaryotic Diversity in Soils

Mastering Microbiology
Art Activity: Figure 20.12 Soil bacterial and archaeal diversity

As we saw in Figure 20.3, even a single soil particle can contain many different microenvironments, and as a consequence, soils typically support the growth of several physiological types of microbes. To examine soil particles directly for microbes, fluorescence microscopes are often used, the organisms in the soil having been previously stained with a fluorescent dye. To visualize a specific microorganism or groups of related microorganisms in a soil particle, staining methods with fluorescent gene probes (including FISH), are used (◀ Section 19.5). Microorganisms can also be observed on soil surfaces directly by scanning electron microscopy (Figure 20.13*b*, *c*).

We learned in Chapter 19 that sequence analyses of 16S ribosomal RNA (rRNA) genes obtained from the environment are commonly used as a measure of bacterial and archaeal diversity (◀ Section 19.6). As yet, no natural communities have been so thoroughly characterized by these techniques that *all* resident species have been identified. However, within limits, the method is widely considered to be a valid measure of microbial diversity and avoids the more serious problems of enrichment bias that plague culture-dependent diversity studies (◀ Section 19.1).

Here and in later sections of this chapter we present "phylogenetic snapshots" of major microbial habitats, with the goal of emphasizing trends, patterns, and ecological rationales rather than comprehensive and exhaustive surveys. And importantly, it should be kept in mind when viewing these phylogenetic summaries that the proportions of each group shown in the diagrams reflect the *diversity* of that group (expressed as a fraction of the whole) and not necessarily the *abundance* of the group. Indeed, some environments contain highly diverse groups that are few in number, and vice versa.

Soil Bacterial and Archaeal Diversity

Molecular community sampling of a typical vegetated surface soil has shown typically *thousands* of different species of *Bacteria* and *Archaea* in a single gram of soil, likely reflecting the numerous microenvironments (Figure 20.3) present there. A "species" is defined here as a 16S rRNA gene sequence obtained from a microbial community that differs from all other sequences by more than 3% (◀ Section 13.12). Such an environmental sequence is called a *phylotype* (also commonly called an *operational taxonomic unit*, or OTU).

Besides very large species numbers, soil microbial diversity studies have also showed that diversity varies with soil type and geographical location. For example, analysis of an Alaska forest soil, an Oklahoma prairie soil, and a Minnesota farm soil (all sites in the USA) revealed approximately 5000, 3700, and 2000 different phylotypes, respectively. The Alaska and Minnesota soils showed similar distributions at the phylum level of taxonomy (for example, *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Planctomycetes*) but shared only about 20% of their species in common. This indicates that although the *proportions* of the dominant phyla in different soils are relatively constant, the *actual species present* within a phylum may vary considerably between different soils. In addition, lower bacterial diversity was observed in the farm soil than the Alaska soil, an indication that modern intensive agricultural practices that rely heavily on fertilization, low plant diversity, and the chemical suppression of unwanted plants and animals, reduce bacterial diversity.

Figure 20.14 shows a diversity snapshot of prokaryotic soil communities based on pooled 16S rRNA sequence data taken from several soils. As can be seen, *Proteobacteria* (Chapters 15 and 16) make up nearly half of the total phylotypes recovered, with all major subgroups except for *Epsilonproteobacteria* well represented. The dominance of *Proteobacteria* in soils is not surprising because the enormous phylogenetic diversity shown by this group is reflected in their enormous metabolic diversity. Phototrophy, chemolithotrophy, and a broad range of chemoorganotrophic metabolisms—both aerobic and anaerobic—are hallmarks of the *Proteobacteria* (Chapters 14–16). Many pseudomonads, for example, are genetically equipped to use any of over

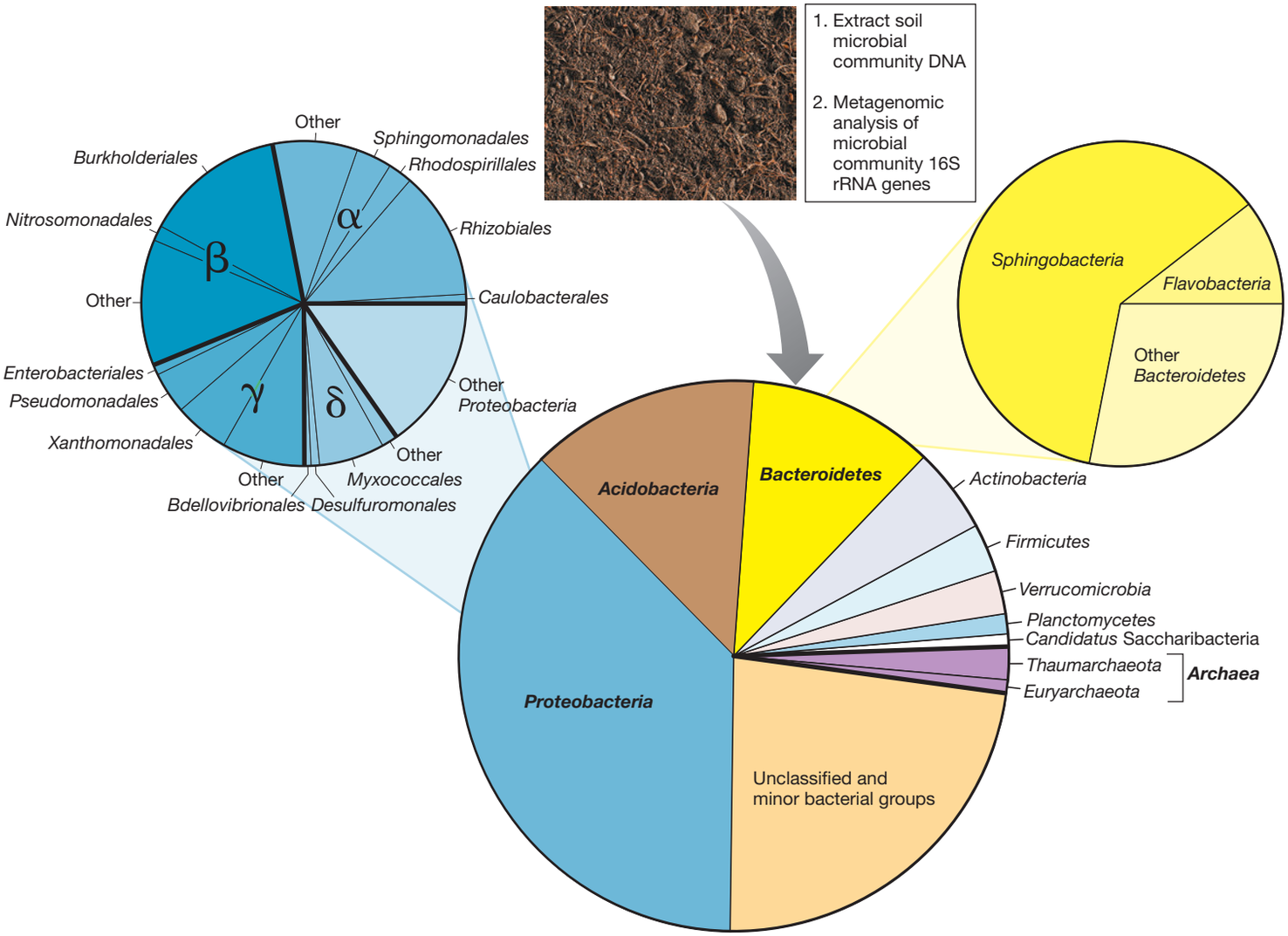


Figure 20.14 Soil bacterial and archaeal diversity. The results are pooled analyses from several studies of the 16S rRNA gene content of soil environments. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria* and *Bacteroidetes*, major subgroups are indicated. Note high species richness as indicated by the large proportion of the total community composed of unclassified and minor bacterial groups. Also note the relatively low proportion of the total prokaryotic soil diversity represented by *Archaea*. Data assembled and analyzed by Nicolas Pinel.

100 different organic compounds as carbon and energy sources, a logical metabolic strategy for thriving in soils where the timing and nature of organic inputs can be highly variable. Likewise, many species of *Rhizobiales* can fix N_2, others specialize in the use of C_1 compounds, and some are even chemolithotrophic or phototrophic, all metabolisms that can be supported in surface soils. In short, the diversity and abundance of *Proteobacteria* in soils is no surprise.

Acidobacteria (◀ Section 16.21) and *Bacteroidetes* (◀ Sections 16.13 and 16.14) are also diverse groups in soil (Figure 20.14), whereas *Actinobacteria* and *Firmicutes* are less so. Although relatively few species of *Acidobacteria* have been cultured, many that have are acid-tolerant and mostly aerobic chemoorganotrophs that collectively use a variety of organic compounds, including plant-derived aromatic compounds, sugars, and polymers common in soils, such as cellulose, xylan, and chitin. Metagenomic analyses (◀ Sections 10.7 and 19.8) are broadening our understanding of *Acidobacteria* metabolic diversity. Such studies suggest that in addition to a known capacity of some *Acidobacteria* to grow by anaerobic respiration with ferric iron (Fe^{3+}) as electron acceptor (◀ Section 14.13), other species can reduce nitrite, nitric oxide, sulfate, and sulfite (◀ Sections 14.10–14.12). Such capacities should serve these organisms well in soils transitioning from oxic to anoxic conditions. Similar to the *Acidobacteria*, the major groups of soil *Bacteroidetes* (*Sphingobacteria* and *Flavobacteria*) are chemoorganotrophs specialized in the use of complex soil polysaccharides (◀ Section 16.14). Their abilities to hydrolyze these polymers—which are common in soil—and use the monomers generated as electron donors in energy metabolism makes these groups ideally suited to a soil habitat.

In the case of soils, diversity also reflects abundance. *Proteobacteria* are the most abundant bacteria in most soils followed by *Acidobacteria* and *Actinobacteria*. *Bacteroidetes*, although a highly diverse group in most soils, is not very abundant, averaging about

the same number of cells per gram of soil as the *Chloroflexi*, a group much less diverse than the *Bacteroidetes* (Figure 20.14).

In contrast to *Bacteria*, the diversity of *Archaea* in soil is minimal, with relatively few sequences that group with only two major phyla—the *Euryarchaeota* and the *Thaumarchaeota*—commonly observed. But low diversity does not necessarily mean low cell numbers; an archaeal assemblage comprising a small slice of overall diversity can have major biogeochemical importance. For example, species of *Thaumarcheota*, comprised solely of ammonia-oxidizing *Archaea* (◀ Section 17.5), are a small part of overall soil diversity (Figure 20.14) but are present in relatively high abundance, greatly outnumbering the ammonia-oxidizing *Bacteria* in the *Nitrosomonadales* (*Betaproteobacteria*). Indeed, *Thaumarchaeota*, with their characteristic ability to oxidize ammonia present in trace amounts, are now thought to be responsible for the majority of nitrification in most soils (◀ Section 14.9). Nevertheless, when comparing entire domains, the abundance of *Bacteria* in most soils greatly exceeds that of *Archaea* by several orders of magnitude.

Polluted Soils

A similar study to that shown in Figure 20.14 but performed on hydrocarbon-polluted soil showed that the general taxonomic makeup of polluted and unpolluted soils is similar: *Proteobacteria* comprise the largest fraction in both soil types, followed by significant representation of *Acidobacteria, Bacteroidetes, Actinobacteria*, and *Firmicutes*. However, there was a significant shift in fractional representation of these taxa in the two soils. Polluted soils are enriched in *Actinobacteria, Gammaproteobacteria,* and *Euryarchaeota* but diminished in *Bacteroidetes, Acidobacteria*, and unclassified *Bacteria* relative to unpolluted soils. Hydrocarbon-polluted soils contained a single dominant *Bacteroidetes* phylotype, whereas unpolluted soils contained several phylotypes of *Bacteroidetes* (Figure 20.14). Notably, *Thaumarchaeota* are absent from all surveys of hydrocarbon-polluted soils, suggesting that hydrocarbon pollutants suppress ammonia-oxidizing *Thaumarchaeota*.

Although the *functional* significance of the observed diversity of microbial communities in polluted versus unpolluted soils is unknown, the shifts observed signal that the two soils will likely differ in their capacity to process carbon and nitrogen and to carry out other important nutrient cycling events. However, despite this lack of a functional connection, different 16S rRNA gene surveys of soils agree on two things: (1) undisturbed, unpolluted soils support very high prokaryotic diversity, and (2) soil perturbations trigger measurable shifts in community composition toward species that are more competitive in the disturbed soil and are accompanied by an overall reduction in diversity.

Climate and Disturbance Effects on Soil Microbes

The importance of understanding the diversity of microbes and microbial processes in soils has been highlighted by the concern about the impact climate change will have on soil systems, including the microbial response to higher temperatures, rainfall patterns, and the rapid changes taking place in land use. From what is known today of microbial and metabolic diversity, climate change will almost certainly impact biogeochemical cycles, and such functional understanding is essential for predicting whether soils will be a sink or a source of atmospheric CO_2.

Since cultured diversity from soil is still but a fraction of the diversity that is known to be there, metagenomic analyses combined with 16S rRNA surveys are relied upon to provide insight into the effects of climate and land use changes. For example, a recent metagenomic study of the consequences of deforestation in the Brazilian Amazon revealed distinct microbial community shifts between pasturelands and adjacent rain forests. *Acidobacteria* (Figure 20.14) were associated exclusively with pasture soils. In contrast, the candidate division *Rokubacteria* was almost exclusively associated with rain forest soils and displayed distinct metabolic properties, using carbon sources ranging from methane to complex hydrocarbons.

This and other examples of shifts in microbial diversity due to land use changes illustrate the fact that microbial diversity in any environment is a dynamic entity and will change—sometimes dramatically—in response to environmental changes in conditions and resources. Since large-scale changes in microbial activities typically follow large-scale shifts in diversity, future soil problems associated with climate change and land disturbances could be significant. And, since humans rely on soil one way or the other for the majority of their food, it is easy to see why studying the effect on microbes of climate and land changes is an urgent and important problem that will require more intensive cultivation and metagenomic surveys.

Check Your Understanding

- How might a microbial group having relatively minor representation in soil diversity still have major biogeochemical importance?
- Which three phyla of *Bacteria* dominate the bacterial diversity of soils? Which phylum of *Archaea* dominates and how does it obtain energy?
- Why should humans be concerned if a climate-induced major shift in soil microbial diversity occurred?

20.8 The Terrestrial Subsurface

Surface soils and subsurface terrestrial environments provide quite different habitats for microorganisms. Microbes in vegetated soils benefit from relatively high levels of nutrients because of their association with growing plants, periodic large inputs of nutrients from dead plants and animals, and inputs of moisture from rain events. By contrast, microbes living below the C horizon (Figure 20.11) have few if any of these advantages. Because of nutrient limitations, microbial diversity and abundance in the deepest terrestrial environments are typically extremely low.

Sampling deep subsurface or marine sediments is expensive and difficult, especially in extracting samples from great depths without contamination from upper subsurface zones. Nevertheless, a number of studies of subsurface soils, deep confined aquifers, and deep marine sediments have been done. Many have focused on the microbial diversity of easily accessible groundwater systems that are within a few hundred meters of the surface. More limited surveys, often requiring more specialized drilling equipment, have examined deep marine sediments and deep confined aquifers.

From research made possible by the development of improved drilling and aseptic sampling technology (**Figure 20.15**), it is now known that microbial life extends to at least 3000 meters (3 kilometers or 1.9 miles) below the Earth's surface. Results show that the microbial diversity of relatively shallow subsurface areas is similar to that of surface soils; *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and

(a)

(b)

Figure 20.15 Sampling the deep subsurface. *(a)* Drilling to 600 m in Allendale, South Carolina (USA), for the U.S. Department of Energy (DOE) Deep Subsurface Microbiology Program. Subsurface microbiology is both expensive and challenging from the standpoint of obtaining uncontaminated samples from deep underground. *(b)* Sampling hot (55°C) fissure water from a depth of 3000 m in the Tau Tona South African gold mine.

Firmicutes generally predominate (Figure 20.14), but their abundances decrease with depth, likely a result of decreasing organic carbon availability (Figure 20.11*a*). Less highly represented groups, such as *Acidobacteria*, *Verrucomicrobia*, and unclassified lineages, are also commonly encountered. Many unclassified *Bacteria* and *Archaea* affiliate with novel phyla and are extremely small and likely grow as symbionts of other microbes (see Figure 20.17). The only group that occasionally shows increases in relative abundance with depth are the *Firmicutes*, in particular, the *Clostridiales*. These are anaerobic bacteria capable of forming endospores (◀ Sections 2.8 and 16.8) and thus could be expected to survive long periods of dormancy.

Bacteria in the Deep Subsurface

Research on the deepest microbial biosphere has been facilitated by drilling of deep marine sediments and mining and drilling operations that expose aquifer waters in fractured granitic rock at great depths. For example, samples collected from a nearly 3-km-deep gold-mining operation in South Africa (Figure 20.15*b*) revealed phylotypes of species of *Bacteria* and *Archaea*. A common geochemical feature of deep granitic aquifers is the presence of significant sulfate at depths greater than 1000 meters. The possibility that sulfate respiration could contribute to a subsurface biosphere was confirmed by analysis of DNA extracted from fissure water deep in this mine, showing that a H_2-oxidizing, sulfate-reducing bacterium (◀ Sections 14.12 and 15.11) was virtually the only bacterium present. Genome analysis of the organism, as yet uncultured but given the provisional name *Desulforudis audaxviator*, indicated that it should be moderately thermophilic (the aquifer temperature at this depth was approximately 50°C) and should be capable of autotrophic growth using H_2 as the electron donor for anaerobic respiration and CO_2 fixation. In addition, the organism contained genes encoding nitrogen fixation (◀ Section 3.12), meaning that it should be able to live in an anoxic environment on a diet of a few minerals, CO_2, SO_4^{2-}, N_2, and H_2—a lean diet indeed!

D. audaxviator should be well suited to long-term isolation in the deep subsurface, as would other autotrophic and nitrogen-fixing bacteria that could use H_2 as electron donor. Possible subsurface sources of H_2 include the radiolysis of water by uranium, thorium, and other radioactive elements, and geochemical processes such as the release of H_2 from the oxidation of iron silicate minerals in aquifers. H_2 can satisfy the electron donor needs of bacteria that carry out many different anaerobic respirations, including sulfate reduction, acetogenesis, ferric iron reduction, and methanogenesis (Chapter 14), and examples of all these physiologies have been identified from metagenomic analyses of subsurface materials. Hence, *Bacteria* supported by these physiologies undoubtedly inhabit the subsurface microbial ecosystem along with *D. audaxviator*.

Archaea in the Deep Subsurface

As 16S rRNA sequencing is increasingly complemented by environmental genomics (◀ Sections 19.6 and 19.8), additional novel organisms and metabolisms almost certainly await discovery. Notably, although generally in lower abundance than *Bacteria*, many novel *Archaea* inhabit the terrestrial subsurface and marine sediments (Section 20.15). As one example, an archaeon affiliated with marine anaerobic methane oxidizers (◀ Section 14.16) is associated with sulfate-dependent methane oxidation in some deep aquifer communities. In addition to archaeal species from phyla having cultivated representatives (*Euryarchaeota*, *Crenarchaeota*, *Thaumarchaeota*, Chapter 17), several phyla so far identified only through PCR-based and metagenomic surveys include the *Bathyarchaeota*, the Asgard

group, and several others (**Figure 20.16**; ◀ Section 17.8). These surveys have also revealed a remarkable diversity of extremely small *Archaea* 0.15–1.2 μm in diameter (**Figure 20.17**) that contain small genomes (~500–1000 genes) and inhabit the subsurface and other nutrient-depleted environments (see Chapter 1 Explore the Microbial World, "Tiny Cells"). These minute *Archaea* form a single deep evolutionary divergence within the *Archaea* that encompasses multiple phyla called DPANN (a "superphylum," Figure 20.16). The first described member of DPANN was the hyperthermophile *Nanoarchaeum equitans*, a small parasitic species of limited metabolic capacity that grows in obligate physical association with a host archaeon, *Ignicoccus* (◀ Section 17.6). However, besides the parasitic lifestyle, metagenomics suggest that some DPANN species may be autotrophs and thus less metabolically dependent than *Nanoarchaeum*.

At least two major surprises have emerged from metagenomic studies of the *Bathyarchaeota*. First was the discovery of genomes from subsurface *Bathyarchaeota* (Figure 20.16) that contained genes homologous to those encoding methanogenesis and the acetyl-CoA pathway (the mechanisms of energy conservation and autotrophy, respectively, in methanogenic *Archaea*, ◀ Sections 14.14 and 14.15). Previously, methanogenesis was thought to be restricted to a single phylum of *Archaea*, the *Euryarchaeota*. Second, genomic analyses indicate that some *Bathyarchaeota* likely oxidize methane and others may oxidize longer-chain hydrocarbons, all under anoxic conditions (◀ Section 14.24). Metagenomics also tells us that some *Bathyarchaeota* are heterotrophs, hydrolyzing peptides as carbon and energy sources or degrading polymers such as lignin, a legacy of ancient buried plants.

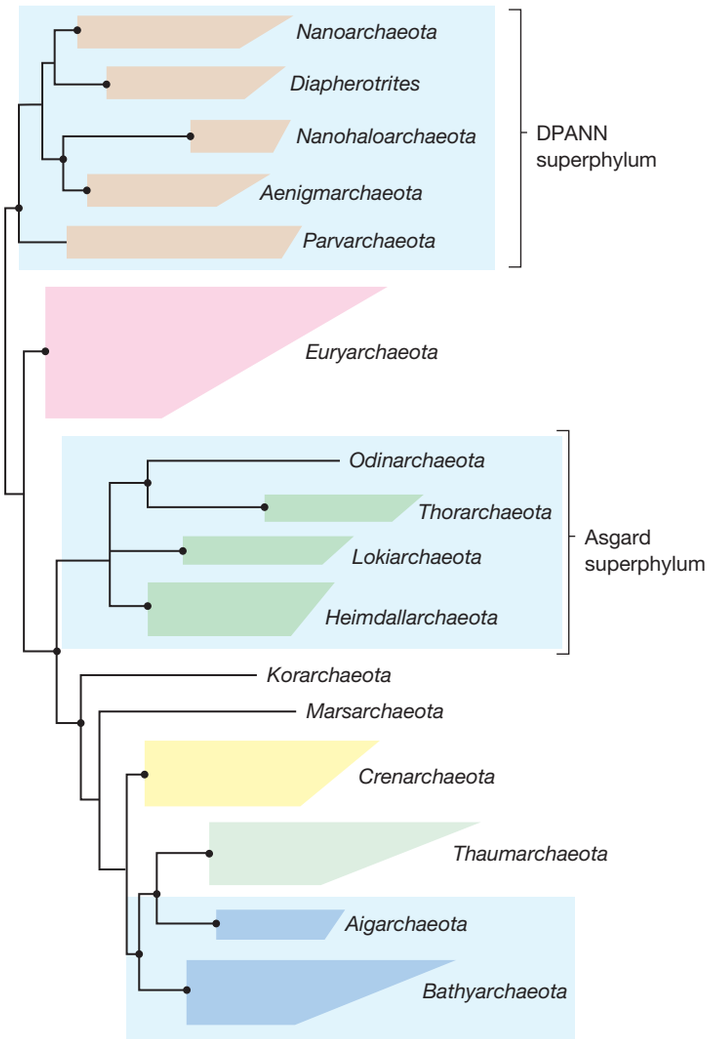


Figure 20.16 Diversity and abundance of subsurface *Archaea*. Phylogeny of archaeal clades found predominantly in the terrestrial and marine deep subsurfaces (this is a consensus tree based on 16S rRNA gene sequences and other conserved marker genes). The novel phyla previously unknown until studies of the deep subsurface are highlighted in light blue. Members of the Asgard superphylum are the closest known relatives of eukaryotic cells (◀ Section 13.4).

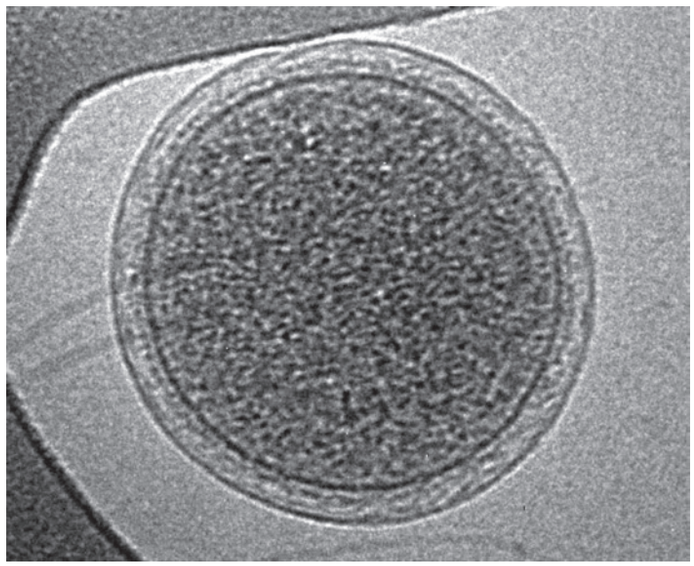


Figure 20.17 Small *Archaea*. Electron micrographic section of a cell of a small species of *Archaea* inhabiting acid mine drainage (▶ Section 22.2). The small *Archaea* found in this acidic environment is a member of the DPANN superphylum (Figure 20.16). The archaeal cell is approximately 0.4 μm in diameter.

Other unusual *Archaea*, such as the Asgard group (◀ Section 17.8; Figure 20.16), have emerged from deep marine subsurface metagenomic analyses. Species in this group appear to be heterotrophs and play a role in carbon degradation in anoxic sediments. Some seem genetically equipped to degrade methane—a large reservoir of which exists in the oceanic subsurface—and other short-chain hydrocarbons, such as butane. As discussed in Section 17.8, there is mounting evidence that the eukaryotic domain of cellular life emerged from within the *Archaea*, specifically the Asgard superphylum. This phylogenetic connection is supported by criteria other than just ribosomal RNA gene sequences, including the fact that Asgard genomes encode several eukaryotic-like proteins, such as actin (◀ Section 2.15). Thus, analysis of the deep subsurface biosphere has not only expanded the known phylogenetic diversity of *Archaea* (Figure 20.16) but has revealed new information about the origin of the eukaryotic cell.

Growth Rates and the Future of Subsurface Microbiology

Bacterial numbers in uncontaminated groundwater vary by several orders of magnitude (10^2–10^8 per ml), primarily as a function of dissolved organic carbon content. Measured and estimated generation times for deep subsurface bacteria also vary by orders of

magnitude, from days to centuries, as determined by the physicochemical environment, the physiology of the resident populations, and nutrient availability. Microbes in the deep subsurface likely originate from sedimentary accumulation of near-surface populations. Increasing nutrient limitation with depth of burial then results in selective death of starving populations, and remaining populations are sustained by organic carbon and other nutrients released from dead cells. Only in scattered regions of higher organic carbon content, such as in ancient coal deposits (Figure 20.41), can higher cell numbers be supported.

The many unanswered questions in subsurface microbiology have encouraged the establishment of permanent science laboratories at great depths in the earth. For example, the Sanford Underground Research Facility in Lead, South Dakota (USA) (2400 m deep), is supported by government and private agencies for research in physics, geology, and microbiology. The International Ocean Discovery Program has probed for microbial populations at great depths below the seafloor. Results thus far have shown *Archaea* and *Bacteria* at depths greater than 2000 m below the seafloor (Section 20.15) and in rocks more than 100 *million* years old. Although this may sound ancient, such ages are actually relatively young compared with viable bacteria that have been recovered from salt crystals dated to nearly a half *billion* years old. Obviously, microbial cells can remain viable for enormously long periods of time.

We leave soils and the subsurface now to explore the microbial diversity of aquatic environments in the final part of this chapter. Like terrestrial environments, aquatic environments vary considerably. On the one hand, aquatic environments can be nutrient-rich and highly dynamic ecosystems that support large and diverse microbial populations and a wealth of plants and animals. But on the other hand, some aquatic environments provide the same nutrient limitation problems for microbes as is characteristic of the deep subsurface.

Check Your Understanding

- What are possible sources of biologically available energy in the terrestrial subsurface?
- What is the major environmental factor that controls the abundance and type of cells in the deep subsurface?
- How have metagenomic analyses of the *Bathyarchaeota* and the Asgard group brought new metabolic and evolutionary concepts to the forefront?

IV • Aquatic Environments

Marine and freshwater environments host a tremendous diversity of microorganisms and viruses that infect them. Variations in light intensity, pressure, and nutrient availability, along with the effects of human pollution, greatly influence the activities, abundance, and diversity of aquatic microbes.

Freshwater and marine environments differ in many ways including salinity, average temperature, depth, and nutrient content, but both provide many excellent habitats for microorganisms. In this part of the chapter we focus first on freshwater microbial habitats. We then consider two marine environments: (1) coastal and open ocean waters, and (2) the deep sea. Much new information is emerging about marine microorganisms from studies using the molecular tools of microbial ecology, especially genetic stains, microbial community sampling, and metagenomics (Chapter 19).

20.9 Freshwaters

Freshwater environments vary significantly in the resources and conditions (Table 20.1) available for microbial growth because some lakes and streams are isolated and nearly pristine while others are highly polluted from agricultural, industrial, or residential runoff. Both oxygen-producing and oxygen-consuming organisms are present in aquatic environments, and the balance between photosynthesis and respiration (Figure 20.2) controls the natural cycles of oxygen, carbon, and other nutrients (nitrogen, phosphorus, metals).

Among microorganisms, oxygenic phototrophs include algae and cyanobacteria, both of which are *primary producers*. These organisms can be either *planktonic* (floating) and distributed throughout the water columns of lakes or *benthic*, meaning they are attached to the bottom or sides of a lake or stream. The activity and diversity of chemoorganotrophic aquatic microbial communities depend to a major extent on primary production, in particular its rates and temporal and spatial distributions. If primary production rates are very high, the resultant excessive organic matter production can lead to bottom-water O_2 depletion from respiration and the development of anoxic conditions. This in turn activates anaerobic metabolisms such as anaerobic respirations and fermentations (Chapter 14).

Oxygen Relationships in Freshwater Lakes

The biological and nutrient structure of lakes is greatly influenced by seasonal changes in physical gradients of temperature and salinity. In many lakes in temperate climates, the water column becomes

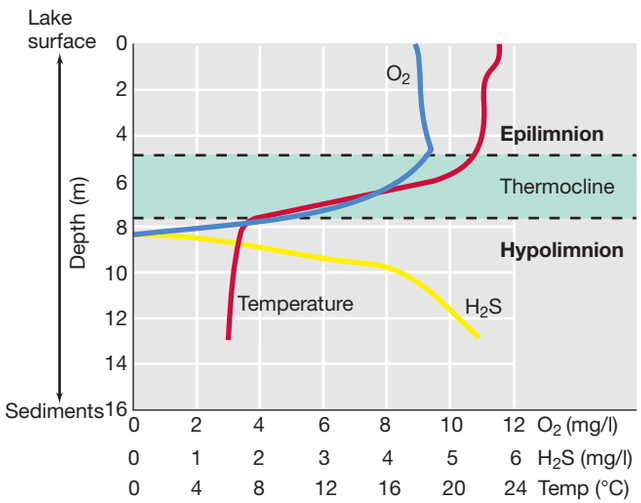


Figure 20.18 Development of anoxic conditions in waters of a temperate lake due to summer stratification. The colder bottom waters are more dense and contain H_2S from bacterial sulfate reduction. The thermocline is the zone of rapid temperature transition. As surface waters cool in the fall and early winter, they reach the temperature and density of hypolimnetic waters and sink, displacing bottom waters and effecting lake turnover. Data are from a small freshwater lake in northern Wisconsin (USA).

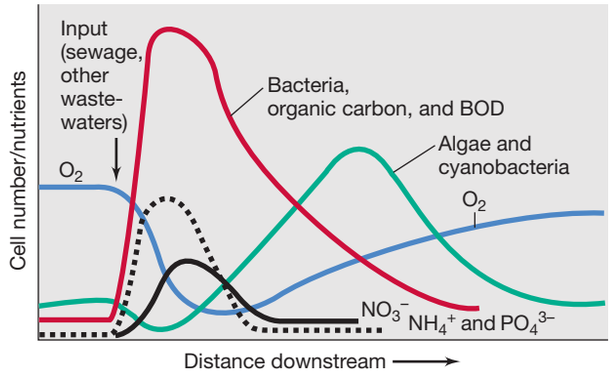
separated into layers of differing physical and chemical characteristics that constitute a **stratified water column**. During the summer, warmer and less dense surface layers, called the **epilimnion**, are separated from the colder and denser bottom layers (the **hypolimnion**). The *thermocline* is the transition zone from epilimnion to hypolimnion (**Figure 20.18**).

In the late fall and early winter, lake surface waters become colder and thus more dense than the bottom layers. This, combined with wind-driven mixing, causes the cooled surface water to sink and the lake to "turn over," mixing surface and bottom waters and their nutrients. The separation of a relatively well-mixed surface layer from a relatively static bottom layer limits the transfer of nutrients between layers until fall turnover once again mixes the water column.

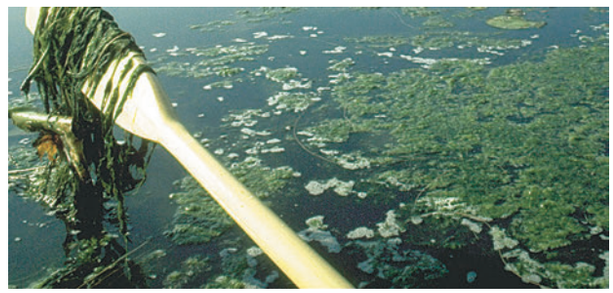
During periods of stratification, transfer between surface and bottom waters is controlled not by mixing but by the much slower process of diffusion. As a result, bottom waters can experience seasonal periods of either low or no dissolved O_2. Whether a lake actually becomes O_2-depleted depends on several factors, including the amount of organic matter present and the degree of mixing of the water column. Organic matter that is not consumed in surface layers sinks to the depths and is decomposed by anaerobes (Figure 20.2). Lakes may contain high levels of dissolved organic matter because inorganic nutrients that run off the surrounding land can trigger algal and cyanobacterial blooms; these organisms typically excrete various organic compounds and also release complex organic compounds when they die and decay. The combination of water body stratification during early summer, high organic loading, and limited O_2 transfer results in O_2 depletion of the bottom waters (Figure 20.18), making them unsuitable for aerobic organisms such as plants and animals.

The annual turnover cycle allows the bottom waters of a lake to pass from oxic to anoxic and back to oxic. Microbial activity and community composition is altered with these changes in oxygen content, but other factors that accompany fall turnover of the water column, especially changes in temperature and nutrient levels, govern microbial diversity and activity as well. If organic matter is sparse, as it is in unpolluted lakes or in the open ocean, there may be insufficient substrate available for chemoorganotrophs to consume all the oxygen. The microorganisms that dominate such environments are typically **oligotrophs**, organisms adapted to growth under very dilute conditions (Section 20.12). Alternatively, where currents are strong or there is turbulence because of wind mixing, the water column may be well mixed, and consequently oxygen may be transferred to the deeper layers.

Oxygen levels in rivers and streams are also of interest, especially those that receive inputs of organic matter from urban, agricultural, or industrial pollution. Rivers and streams are typically flowing systems and thus rarely become strongly stratified, but even in a river well mixed by water flow and turbulence, large organic inputs can lead to a marked oxygen deficit from bacterial respiration (**Figure 20.19*a***). As the water moves away from a point source input, for example, from an input of sewage, organic matter is gradually consumed, and the oxygen content returns to previous levels. As in lakes, nutrient inputs to rivers and streams from sewage or other pollutants can trigger massive blooms of cyanobacteria and algae (Figure 20.1) and aquatic plants (Figure 20.19*b*), thereby diminishing overall water quality and growth conditions for aquatic animals.



(a)



T.D. Brock

(b)

Figure 20.19 Effect of the input of organic-rich wastewaters into aquatic systems. *(a)* In a river, bacterial numbers increase and O_2 levels decrease with a spike of organic matter. The rise in algae and cyanobacteria is a response to inorganic nutrients, especially PO_4^{3-}. *(b)* Photo of a eutrophic (nutrient-rich) lake, Lake Mendota, Madison, Wisconsin (USA), showing algae, cyanobacteria, and aquatic plants that bloom in response to nutrients from agricultural runoff. (See also Figure 20.1).

Biochemical Oxygen Demand

The microbial oxygen-consuming capacity of a body of water is called its **biochemical oxygen demand (BOD)**. The BOD of water, such as that from a temperate lake, is determined by taking a sample, aerating it well to saturate the water with dissolved O_2, placing it in a sealed bottle, incubating it in the dark (to prevent photosynthetic oxygen production) for 5 days at 20°C, and determining the residual oxygen in the water at the end of incubation. A BOD determination gives a measure of the amount of organic material in the water that can be oxidized by the microbes present in the water. As a lake or river recovers from an input of organic matter or from excessive primary production, the initially high BOD becomes lower and is accompanied by a corresponding increase in dissolved oxygen in the ecosystem (Figure 20.19*a*).

In freshwaters, the oxygen and carbon cycles are linked, with the levels of organic carbon and oxygen being inversely related. In other words, high BOD leads to low dissolved oxygen levels. Anoxic aquatic environments, which are typically rich in organic material, are the end result of respiratory processes that remove dissolved oxygen from the ecosystem, leaving the remaining organic material to be mineralized by organisms employing the anaerobic energy metabolisms previously discussed (Chapter 14). It is also important to recognize the importance of storms, floods, and droughts in determining delivery, transport, and cycling of organic matter and inorganic nutrients in freshwater systems, including streams, rivers, lakes, and reservoirs.

These less predictable but nonetheless important changes also affect microbial productivity, diversity, distribution, and interactions.

A Phylogenetic Snapshot of Freshwater Prokaryotic Diversity

The importance of *Bacteria* and *Archaea* in lakes, streams, and rivers to the production, regeneration, and mobilization of nutrients is well recognized, and, as for soil (Section 20.7), 16S ribosomal RNA gene sequencing has been used as a culture-independent method to identify and quantify phylotypes in aquatic microbial habitats. Since most molecular studies of freshwater systems have explored the diversity in lakes, we focus on lakes here.

Figure 20.20 shows the major prokaryotic groups that inhabit lake surface samples (the epilimnion, Figure 20.18). Five major bacterial phyla are routinely observed: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and *Verrucomicrobia*. *Euryarchaeota*, *Crenarchaeota*, and *Thaumarchaeota* are the major *Archaea* present but collectively account for only a small fraction of the total prokaryotic abundance and diversity.

The phylum-level composition of *Bacteria* in lakes shares features in common with both surface soils and the oceans, environments where *Proteobacteria* and *Bacteroidetes* also comprise the greater part of diversity (Figure 20.14 and see Figure 20.31). However, compared with lakes and soils, where *Alpha-* and *Betaproteobacteria* show roughly equivalent diversity, we will see in Section 20.12 that in the oceans, *Alpha-* and *Gammaproteobacteria* predominate and *Betaproteobacteria* are only minor components. *Betaproteobacteria* tend to be fast-growing species that respond quickly to periodic pulses of organic nutrients (for example, leaf litter) that enter lake systems. By contrast, *Alphaproteobacteria* tend to dominate under the more nutrient-limiting conditions of the open ocean.

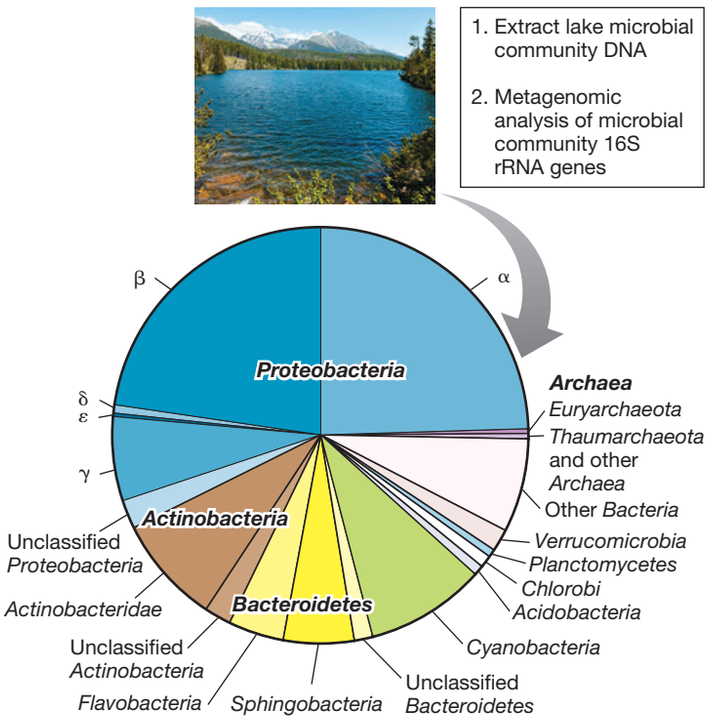


Figure 20.20 Freshwater lake bacterial and archaeal diversity. Distribution of 16S ribosomal RNA gene sequences by phylum determined from analysis of a collective dataset of 16S genes collected from the epilimnion of several freshwater lakes. Data assembled and analyzed by Nicolas Pinel.

In addition to leading in the diversity category, *Proteobacteria* are also the most abundant bacteria in most lake systems, with *Actinobacteria* and *Bacteroidetes* species being significant but in lesser abundance. Interestingly, *Acidobacteria*, major components of both diversity and abundance in soils (Figure 20.14), are but a tiny fraction of bacterial diversity and abundance in freshwater lakes.

With the exception of the *Acidobacteria*, it is likely that a similar rationale exists for why the major bacterial phyla found in soil (Figure 20.14) are also found in freshwater: metabolic diversity. *Proteobacteria* excel in this realm and *Bacteroidetes* are well known for their degradation of various biopolymers and aromatic compounds, such as humic materials. *Actinobacteria* are chemoorganotrophs that can also break down polymers, in particular nucleic acids and proteins. In addition, metagenomic analyses (◀ Section 19.8) have shown that at least some *Actinobacteria* contain genes that encode a bacteriorhodopsin-like protein, similar to that which converts light energy into ATP in extremely halophilic *Archaea* (◀ Section 17.1); thus, these *Actinobacteria* may be phototrophic. Moreover, many anoxygenic phototrophs are *Proteobacteria* (◀ Sections 15.4 and 15.5), and their competitive advantage in freshwaters would also rely on photosynthesis. A rationale for cyanobacteria in overall lake diversity (Figure 20.20) hardly needs mentioning, as oxygenic photosynthesis requires nothing but a few inorganic nutrients, CO_2, and plenty of sunshine. Cyanobacterial abundance can also be quite high in freshwater lakes, especially if the lake receives inputs of key inorganic nutrients such as phosphorus and nitrogen; these nutrients often trigger massive cyanobacterial blooms (Figures 20.1 and 20.19*b*).

Collectively, the high prokaryotic diversity observed in freshwater lakes (Figure 20.20) reflects the dynamic character of these habitats. Lakes typically receive seasonally variable inputs of endogenous and exogenous nutrients, a pattern that sustains a phylogenetically and metabolically complex community of *Bacteria* and a few groups of *Archaea*.

Check Your Understanding

- What is a primary producer? In a freshwater lake, would primary producers such as cyanobacteria more likely reside in the epilimnion or the hypolimnion, and why?
- Will addition of organic matter to a water sample increase or decrease its BOD?
- What factor(s) might account for the prokaryotic diversity of freshwater lakes?

20.10 Oxygen Relationships in the Marine Environment

With the exception of oxygen, nutrients in the open ocean (called the *pelagic zone*) are typically at very low levels compared with lakes and other freshwaters. This is especially true of key inorganic nutrients for phototrophic organisms, such as nitrogen, phosphorus, and iron. In addition, water temperatures in the oceans are cooler and more constant seasonally than those of most freshwater lakes. The activity of marine phototrophs is limited by these factors, and thus

total microbial cell numbers are typically about 10-fold lower in the oceans than in freshwater environments ($\sim 10^6$/ml versus 10^7/ml, respectively). These are average numbers, and studies of marine prokaryotic diversity are just beginning to reveal recurrent temporal patterns of diversity and abundance.

Tracking Seasonal Changes in Marine Microbial Composition

The Bermuda Atlantic Time-Series Study has a history of continuous biogeochemical monitoring of ocean waters since the mid-1950s, and since the dawn of the molecular ecology era, it has also incorporated diversity studies of microbial population structure. These studies have revealed three major microbial communities in ocean waters that follow a seasonal pattern: (1) the community corresponding to the spring surface water bloom, consisting of small eukaryotic algae, marine *Actinobacteria*, and two groups of *Alphaproteobacteria*; (2) the summertime community in the upper water column associated with water column stratification, consisting of *Pelagibacter, Puniceispirillum*, and two groups of *Gammaproteobacteria*; and (3) the more stable deep-water community, consisting of *Nitrosopumilus*, representatives of the SAR11 group with which the genus *Pelagibacter* (see Figure 20.30) affiliates, a group of *Deltaproteobacteria*, and species of two additional groups related to the *Chloroflexi* and *Fibrobacter*. Except for *Nitrosopumilus* (domain *Archaea*), all other deep-water microbes are *Bacteria* (Chapter 16). In Section 20.11, we will return to a more specific breakdown of prokaryotic cell diversity in the open oceans.

Most pelagic *Bacteria* and *Archaea* are very small cells, a characteristic often seen in organisms that inhabit nutrient-poor (oligotrophic) environments. Smallness is an adaptive feature for nutrient-limited microorganisms in that it requires less energy for cellular maintenance and yields a larger surface-to-volume ratio in the cell (◀ Section 1.3). As a consequence of their oligotrophic lifestyle, the genomes of pelagic microbes typically encode very high-affinity transport systems for acquiring the nutrients they need.

Microbial Activities in Marine Environments and Oxygen Minimum Zones

In pelagic waters there is a lower return of nutrients from the bottom waters to upper waters than in freshwater lakes because the waters do not completely turn over seasonally; thus, average primary productivity in the pelagic photic zone is less than in a lake. However, because the oceans are so large, the collective carbon dioxide sequestration and oxygen production from oceanic photosynthesis are major factors in Earth's carbon balance. Terrestrial runoff, retention of nutrients, and upwelling of nutrient-rich waters combine to support higher populations of phototrophic microorganisms in near-shore waters than in pelagic waters (**Figure 20.21**); these more productive near-shore waters in turn support higher densities of chemoorganotrophic bacteria and aquatic animals, such as fish and shellfish.

In shallow marine waters such as marine bays and inlets, eutrophication resulting from nutrient inputs can lead to the waters becoming intermittently anoxic from the removal of O_2 by respiration and the production of H_2S by sulfate-reducing bacteria. This can lead to **oxygen minimum zones (OMZs)**, regions of oxygen-depleted waters at intermediate depths, typically in waters between 100 and 1000 m, that extend over wide expanses of the open and

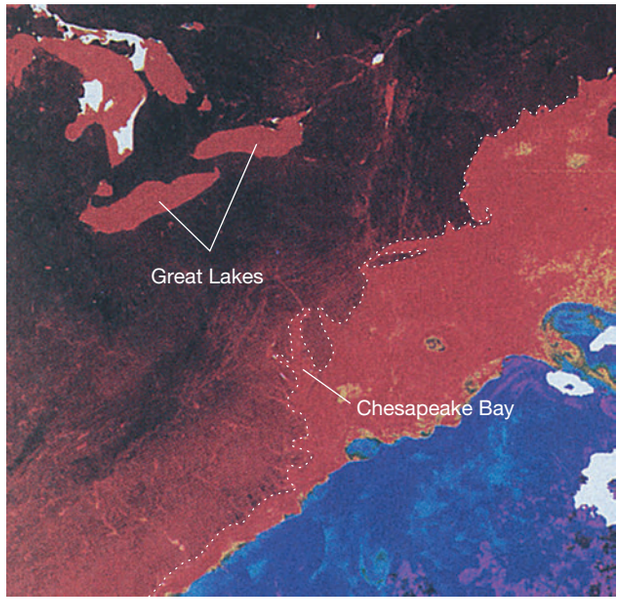


Figure 20.21 Distribution of chlorophyll in the western North Atlantic Ocean as recorded by satellite. The eastern coastline of the United States from the Carolinas to northern Maine is shown in dotted outline. Areas rich in phototrophic plankton are shown in red (chlorophyll concentrations of >1 mg/m^3); blue and purple areas have lower chlorophyll concentrations (<0.01 mg/m^3). Note the high primary productivity of coastal areas and the Great Lakes.

coastal ocean (**Figure 20.22**). These oxygen-depleted regions arise when the respiratory demand for oxygen exceeds oxygen availability, and they are associated with nutrient-rich, highly productive regions. In this way they are similar to the depletion of oxygen caused by agricultural runoff in coastal zones (Figure 20.22). However, OMZs predate human activity and originate naturally in regions of high surface production and little mixing with oxygen-rich water.

The oxygen saturation values of the largest of the OMZs in the eastern Pacific off the coast of Peru are less than 10% of that at the surface, but in some other pelagic waters the values actually reach zero. Because of this, OMZs have been recognized as significant sinks for the loss of fixed nitrogen through denitrification (◀ Section 14.11) and anammox (◀ Section 14.10), and as a significant source of nitrous oxide (N_2O), a potent greenhouse gas (▶ Section 21.9; Figure 20.22). Ongoing studies of OMZs have shown that these regions are expanding, and that their recent expansion is almost certainly associated with global warming. As the oceans absorb more heat, warming of the surface waters increases stratification of near-surface waters and reduces oxygen transfer through mixing to deeper regions. Expansion of the OMZs will favor anaerobic microbial processes at the expense of the aerobic processes that sustain critical oceanic food webs. Ultimately, these changes are expected to impact commercial fisheries.

Oxygen-Depleted Marine Dead Zones and the Deepwater Horizon Catastrophe

An extensive region (6000–8000 square miles) of seasonal oxygen depletion in the Gulf of Mexico is associated with high loads of nitrogen and phosphorus carried in by the Mississippi River from agricultural runoff in the Mississippi Valley. This input triggers

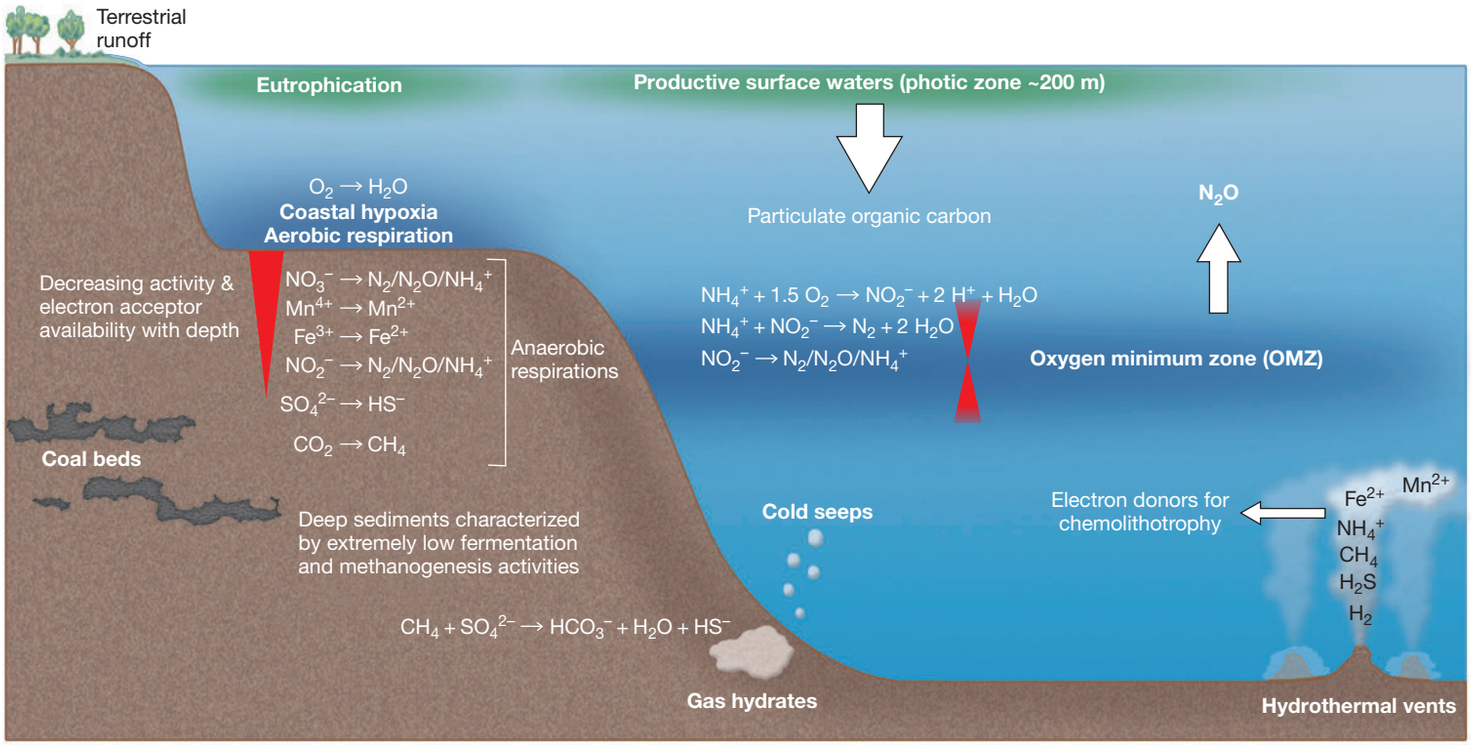


Figure 20.22 Diversity of marine systems and associated microbial metabolic processes. Decreasing electron acceptor availability with depth into the sediment or with increasing distance into an oxygen minimum zone is indicated by red wedges. Sulfate becomes limiting only at greater depths in marine sediments.The indicated metabolic diversity is described in Chapter 14.

excessive oxygen consumption from chemoorganotrophs and the formation of hypoxic (oxygen-depleted) water. This region, called the *Gulf of Mexico Dead Zone*, contributes to the loss and impairment of fish and benthic sea life that sustain major seafood industries in this region. In addition to the chronic degradation of the Gulf of Mexico ecology through agricultural runoff, increased offshore oil drilling also poses significant environmental risk. A major catastrophe for the Gulf of Mexico was the April 2010 explosion and sinking of the Deepwater Horizon offshore drilling platform; failure to control well pressure resulted in the rupture of the wellhead at a depth of 1.5 km and the release of over 4 million barrels of oil before the well was capped three months later (**Figure 20.23**).

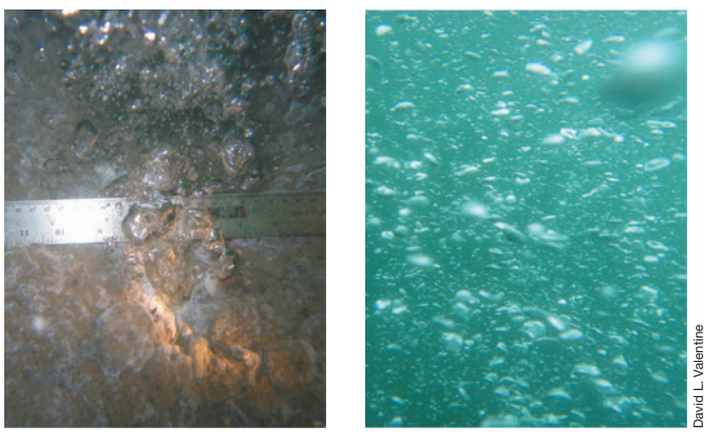
(a) (b)

Figure 20.23 The Deepwater Horizon oil spill in the Gulf of Mexico. *(a)* Inferno resulting from the wellhead blowout. *(b)* NASA Terra satellite image taken on May 24, 2010, of the Gulf of Mexico near New Orleans, Louisiana. A large plume of oil was released at about 1500-m depth, some of which reached the surface where sunlight reflects off of the oil slick (arrows).

This largest marine oil spill ever was unique in that most of the oil was released as a plume at great depths in the water column. Typically, marine oil spills contaminate surface waters, resulting in rapid volatilization and loss to the atmosphere of low-molecular-weight oil components such as naphthalene, ethylbenzene, toluene, and xylene. By contrast, the Deepwater Horizon spill released both low-molecular-weight components *and* natural gas (methane, ethane, propane) deep into the water column. These components comprised about 35% of the hydrocarbon plume that extended across many miles of the Gulf from the surface to depths greater than 800 m (Figure 20.23*b*) and offered an opportunity for microbiologists to measure the effect of a large input of oil to marine waters.

The microbial response to the Gulf spill was tracked over several months using both culture-based and molecular methods, including 16S ribosomal RNA gene and metagenomic sequencing, and GeoChip microarray analyses (◀ Sections 19.6–19.8). Hydrocarbon-degrading bacteria were already enriched in Gulf waters as a consequence of natural seepage of petroleum and natural gas from sediments in coastal regions rich in petroleum reserves (**Figure 20.24**). These were hydrocarbon-degrading *Gammaproteobacteria* species related to genera in the *Oceanospirillales* group, and of the genera *Colwellia* and *Cycloclasticus*. Microbial diversity and abundance measurements showed that the initial response to the oil spill in May and June of 2010 was a bloom of these very species, probably because of their ability to catabolize gaseous hydrocarbons released from natural seeps (Figure 20.24). This was confirmed with enrichment cultures (◀ Section 19.1) that grew rapidly when ethane or propane was the substrate. *Colwellia* species also contributed to the degradation of a variety of other hydrocarbons, as indicated by their growth in crude-oil enrichment cultures lacking natural gas and by stable isotope probing experiments (SIP, ◀ Section 19.10) that showed incorporation of ^{13}C benzene. Other oil-degrading *Bacteria* were detected in the Gulf spill as well. In studies of oil consumption on beach sand, a succession was observed in the microbial community during the course of oil consumption as lighter fractions were consumed and more refractory components remained for specialist organisms to consume.

Although there remains uncertainty about the fate of all the hydrocarbons released during the Deepwater Horizon spill, it appears that the early stimulation of hydrocarbon-degrading bacteria by the more soluble, low-molecular-weight gaseous components in the oil reduced the environmental impact of this immense oil spill. Nevertheless, billions of dollars in losses to commercial fisheries and other human activities in the Gulf of Mexico and significant losses of wildlife and wildlife habitat occurred because of the spill.



(a) (b)

Figure 20.24 Marine petroleum seep. *(a)* Methane and CO_2 are produced by microbial degradation of marine subsurface oil in the Coal Oil Point seep field and migrate through overlying geological strata to be released 21 m underwater in the Santa Barbara Channel off the California Coast. The bubbles also contain small amounts of additional hydrocarbons and H_2S from sulfate reduction. Continuous release of methane and trace hydrocarbons to the water maintain populations of hydrocarbon-degrading microbes capable of responding to accidental petroleum releases. *(b)* Approximately half the released methane dissolves into the water and becomes available to the microbial community.

Check Your Understanding

- Why are marine prokaryotic cells typically quite small?
- What is an oxygen minimum zone and why is expansion of these zones a problem for marine and global ecology?
- What did the Gulf oil spill tell us about how mixed hydrocarbons are degraded in nature?

20.11 Major Marine Phototrophs

The oceans contain large numbers of phototrophic microbes, including both prokaryotic and eukaryotic oxygenic phototrophs as well as significant numbers of a special group of purple (anoxygenic) phototrophs. We consider these organisms here as a prelude to exploring the marine prokaryotic world in general in Section 20.12.

Primary Productivity: *Prochlorococcus*

Much of the primary productivity in the open oceans, even at significant depths, comes from photosynthesis by **prochlorophytes**, tiny bacterial phototrophs that are phylogenetically cyanobacteria (◀ Section 15.3). However, unlike most cyanobacteria, prochlorophytes do not contain phycobilins, although they do contain chlorophylls *a* and *b*. The organism *Prochlorococcus* is a particularly important primary producer in the marine environment (**Figure 20.25**). Because *Prochlorococcus* lacks phycobilins, which are the accessory pigments of the cyanobacteria (◀ Section 14.4), dense suspensions of *Prochlorococcus* cells are olive green (as are green algae) rather than the blue-green color of cyanobacteria (compare Figures 20.1*c* and 20.25).

Prochlorococcus accounts for up to half of the photosynthetic biomass and primary production in the tropical and subtropical regions of the world's oceans, reaching cell densities of 10^5/ml. A number of strains of *Prochlorococcus* have now been identified in culture, and each inhabits its own depth range in pelagic waters. The different *Prochlorococcus* strains are considered distinct *ecotypes*, genetic variants of a species that differ physiologically and therefore occupy slightly different niches. For example, different *Prochlorococcus* ecotypes photosynthesize at different light intensities (high-light versus low-light ecotypes) and use different inorganic and organic nitrogen and phosphorus sources. *Prochlorococcus* is thus distributed in both surface waters and deeper waters to depths of 200 m, and when an oxygen minimum zone (Section 20.10) is present, *Prochlorococcus* extends into the upper regions of this zone (Figure 20.22). This is

Figure 20.25 ***Prochlorococcus*, the most abundant oxygenic phototroph in the oceans.** A bottle of *Prochlorococcus* showing the olive-green color of the cells containing chlorophylls *a* and *b*. Inset: FISH-stained cells (◀ Section 19.5) of *Prochlorococcus* in a marine water sample.

near the bottom of the photic zone where light intensities are very low (see Figure 20.29).

Genome sequences of about a dozen cultured *Prochlorococcus* strains revealed that although each contains about 2000 genes, only about 1100 genes (the core genome, ◀ Section 13.10) are shared by all strains. Each presumptive ecotype contains approximately 200 unique genes, which likely have adaptive significance for growth in the realized niche of that ecotype. This was illustrated in Chapter 19 where we compared the genome of a single cultured *Prochlorococcus* ecotype to metagenome sequences obtained from pelagic waters (◀ Section 19.8 and Figure 19.24).

Single-cell genomics (◀ Section 10.11) of natural *Prochlorococcus* populations have refined our understanding of genetic diversity and the relationship between environmental conditions and the fitness of individual genotypes. These analyses revealed a high degree of fine-scale genetic diversity within the high-light (HL) and low-light (LL) ecotypes. Each ecotype is actually composed of hundreds of subpopulations, each of which is united by a common set of core genes encoding functions that control the interaction of the organism with its environment (e.g., transport functions, oxidative stress responses, and cell surface structure) (**Figure 20.26*b***). However, each subpopulation also contains a small set of genes termed "flexible" genes (ranging from 4 to 14 genes per characterized genome) that vary within that subpopulation. The flexible genes are packaged as "cassettes" localized to highly variable regions (genomic islands) within the *Prochlorococcus* genome (◀ Sections 13.10, 19.8, and Figure 19.24). Variation in flexible gene content contributes to exceptionally high *microdiversity* within natural populations of *Prochlorococcus*, pointing to tremendous versatility in their adaptive response to new niche opportunities. The importance of genotypic variability in sustaining large numbers of this major marine phototroph is shown by seasonal shifts in different genotypes in response to changing light intensity, nutrient availability, and predator populations (Figure 20.26*a*).

Mastering Microbiology
Art Activity: Figure 20.23 Seasonal variation of *Prochlorococcus* ecotypes in marine surface waters

Other Pelagic Oxygenic Phototrophs

In tropical and subtropical oceans, the planktonic filamentous marine cyanobacterium *Trichodesmium* (**Figure 20.27*a***) is a widespread and occasionally abundant phototroph. Cells of *Trichodesmium* form colonies of filaments called puffs. Each puff can contain many hundreds of individual filaments, each filament composed of 20–200

UNIT 5

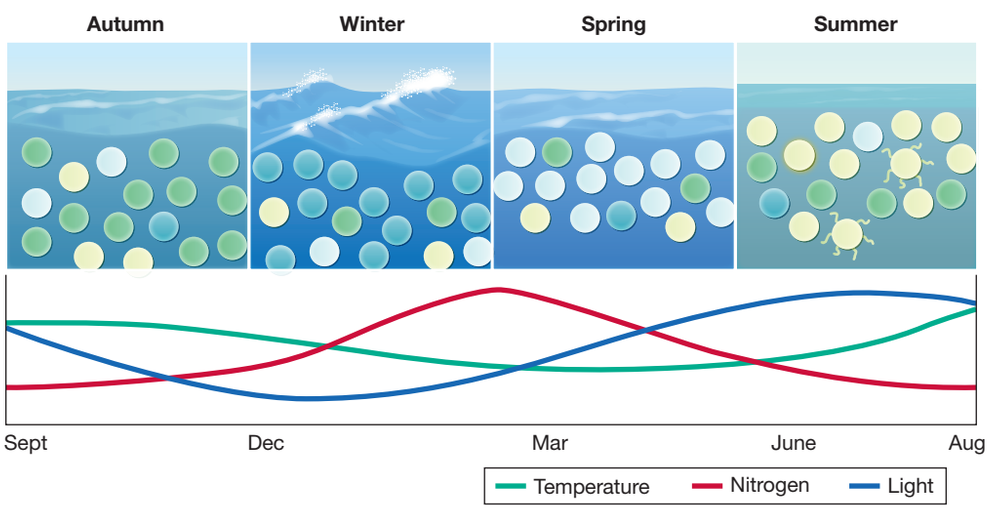
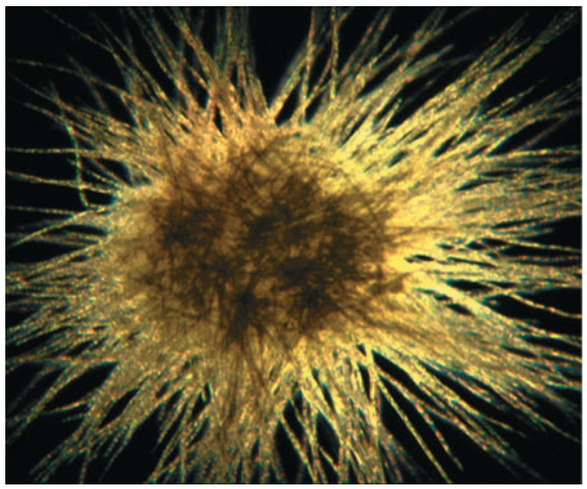


Figure 20.26 **Seasonal variation of *Prochlorococcus* ecotypes in marine surface waters.** *(a)* Single-cell genome sequencing (◀ Section 10.11) has shown that the abundance of different genotypes, presumptive ecotypes (represented by cell color and surface features), correlate with seasonal changes in temperature, light, nutrients, and predators (grazers and bacteriophages). *(b)* Adaptations to different physical conditions (light and temperature) partition at higher taxonomic levels than do adaptations to different nutrient conditions (e.g., nitrogen availability) and resistance to specific predators, which show closer genetic relationships among ecotypes (see also Figure 19.24).



(a)

(b)

Figure 20.27 ***Trichodesmium*** **and** ***Ostreococcus.*** *(a)* Light photomicrograph of a puff of cells of the nitrogen-fixing cyanobacterium *Trichodesmium*. The filaments in the puff are chains of cells, each of which is about 6 μm in diameter. *(b)* Transmission electron micrograph of a cell of *Ostreococcus*, a small green alga found primarily in marine coastal waters. The arrow points to the chloroplast. An *Ostreococcus* cell is about 0.7 μm in diameter.

cells. In the Caribbean Sea surface waters, colonies of *Trichodesmium* can approach $100/m^3$. *Trichodesmium* is a nitrogen-fixing cyanobacterium, and the production of fixed nitrogen by this organism is thought to be an important link in the marine nitrogen cycle. *Trichodesmium* contains phycobilins, absent from prochlorophytes, and thus differs from these organisms in its absorption properties (◀ Section 14.4).

Very small *eukaryotic* phototrophs also inhabit coastal and pelagic waters, and some of these are among the smallest eukaryotic cells known. Three common genera—*Bathycoccus*, *Micromonas*, and *Ostreococcus*—are so small that they contain only one mitochondrion and one chloroplast per cell (a human cell can have hundreds or even thousands of mitochondria). These genera belong to the family *Prasinophyceae*, a group of green algae that diverged early from other lineages of green algae (◀ Section 18.16). Cells of *Ostreococcus* are cocci that measure only about 0.7 μm in diameter (Figure 20.27*b*), which is even smaller than a cell of *Escherichia coli*.

Although cells of the eukaryotic *Ostreococcus* and the prokaryotic *Prochlorococcus* are of roughly the same dimensions and they are both oxygenic phototrophs, their genomes are distinct. The genome of *Ostreococcus* is 12.6 Mbp (distributed over 20 chromosomes), which is more than seven times the size of the *Prochlorococcus* genome. Even though this is large relative to cyanobacteria, the *Ostreococcus* genome is unusual for that of a eukaryote in being very gene dense, containing about 8000 genes, and is thought to be near the minimum genome size of a free-living phototrophic eukaryote. By comparison, the genome of a common plant, Japanese rice (*Oryza sativa* subsp. *japonica*), is 420 Mbp and contains about 50,000 genes.

In many marine waters, other small eukaryotic cells are present at about 10^4/ml. Although many of these are *Ostreococcus* or relatives, some are chemoorganotrophs and some are phototrophs unrelated to *Ostreococcus* that incorporate small amounts of organic matter to supplement their primarily phototrophic lifestyle.

Aerobic Anoxygenic Phototrophs

Besides *oxygenic* phototrophs, *anoxygenic* phototrophs are also present in coastal and pelagic marine waters. Like purple anoxygenic phototrophs, these organisms contain bacteriochlorophyll *a* (◀ Sections 14.3, 14.5, 15.4, and 15.5). However, unlike classical purple bacteria that carry out photosynthesis only under *anoxic* conditions, these anoxygenic phototrophs carry out photosynthetic light reactions only under *oxic* conditions.

Aerobic anoxygenic phototrophs include bacteria such as *Erythrobacter*, *Roseobacter*, and *Citromicrobium* (**Figure 20.28**), all genera of *Alphaproteobacteria* (◀ Section 16.1). Aerobic anoxygenic phototrophs synthesize ATP by photophosphorylation when oxygen is present (which is all of the time in oxic pelagic waters), but they are unable to grow autotrophically and thus rely on organic carbon for their carbon sources (a nutritional condition called *photoheterotrophy*). These organisms thus use the ATP produced by photophosphorylation to supplement their otherwise chemoorganotrophic metabolism.

Surveys have shown that a great diversity of aerobic anoxygenic phototrophs exist in marine waters, especially near-shore waters. Oligotrophic and highly oxic freshwater lakes are also habitats for these interesting phototrophic bacteria. The physiology of aerobic

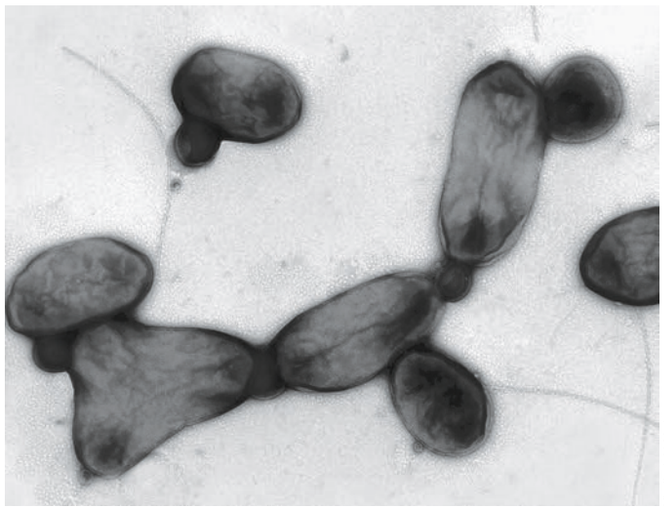


Figure 20.28 **Aerobic anoxygenic phototrophic bacteria.** Transmission electron micrograph of negatively stained cells of *Citromicrobium*. Cells of this marine, aerobic anoxygenic phototroph produce bacteriochlorophyll *a* only under oxic conditions and divide by both budding and binary fission, yielding morphologically unusual and irregular-shaped cells.

anoxygenic phototrophs is thus ideal for their illuminated and highly oxic habitats.

Check Your Understanding

- How does *Ostreococcus* differ from *Prochlorococcus*? What do they have in common?
- How does the organism *Prochlorococcus* contribute to both the carbon and oxygen cycles in the oceans?
- How does *Roseobacter* differ from *Prochlorococcus*?

20.12 Pelagic *Bacteria* and *Archaea*

Despite vanishingly low nutrient levels, significant numbers of *Bacteria* and *Archaea* live a planktonic existence in pelagic marine waters. Of these, one species in particular has garnered significant attention, a bacterium named *Pelagibacter*.

Distribution and Activity of *Archaea* and *Bacteria* in Pelagic Waters

The abundance of prokaryotic cells in the open oceans decreases with depth. In pelagic waters, the photic zone extends to a depth of about 200 m, and within this region cell numbers are the highest. In surface waters, cell numbers average about 10^6/ml. Below 1000 m, however, total cell numbers fall to between 10^3 and 10^5/ml. The distribution of *Bacteria* and *Archaea* with depth has been tracked in pelagic waters using fluorescence in situ hybridization (FISH) technology (◀ Section 19.5). Species of *Bacteria* tend to predominate in waters above 1000 m, although cells of *Bacteria* and *Archaea* are found in near-equal abundance in deeper waters (**Figure 20.29**). Deep-water *Archaea* are almost exclusively species of *Thaumarchaeota* (◀ Section 17.5), and many, perhaps even most, are ammonia-oxidizing chemolithotrophs (◀ Sections 14.9 and 17.5) that play an important role in coupling the marine carbon and nitrogen cycles (Chapter 21). Extrapolating to a global basis using the data in Figure 20.29, it is estimated that 1.3×10^{28} and 3.1×10^{28} cells of *Archaea* and *Bacteria*, respectively, exist in the world's oceans. This means that the oceans contain the largest microbial biomass on Earth's surface.

Pelagic *Bacteria* and *Archaea* are ecologically important because they consume dissolved organic carbon in the oceans, one of the largest pools of usable organic carbon on Earth. These small and free-living planktonic microbes consume about half the total oceanic organic carbon produced from photosynthesis and are responsible for about half of all marine respiration and nutrient regeneration. Planktonic marine microbes thus return organic matter to the marine food web that would otherwise be lost because of the inability of larger marine organisms to take up such diluted organic nutrients. This so-called "secondary production" (primary production being that from photosynthesis) is balanced by cell losses from grazing protists and from virus attack (see Figures 20.32, 20.33, and 20.34). This leads to a near-steady state in which the abundance of prokaryotic cells in the open oceans remains roughly constant over time. But importantly, secondary production allows some of the dissolved organic carbon in seawater to reach larger organisms, including fish, because microbes are eventually passed up the food web by the feeding activities of larger organisms.

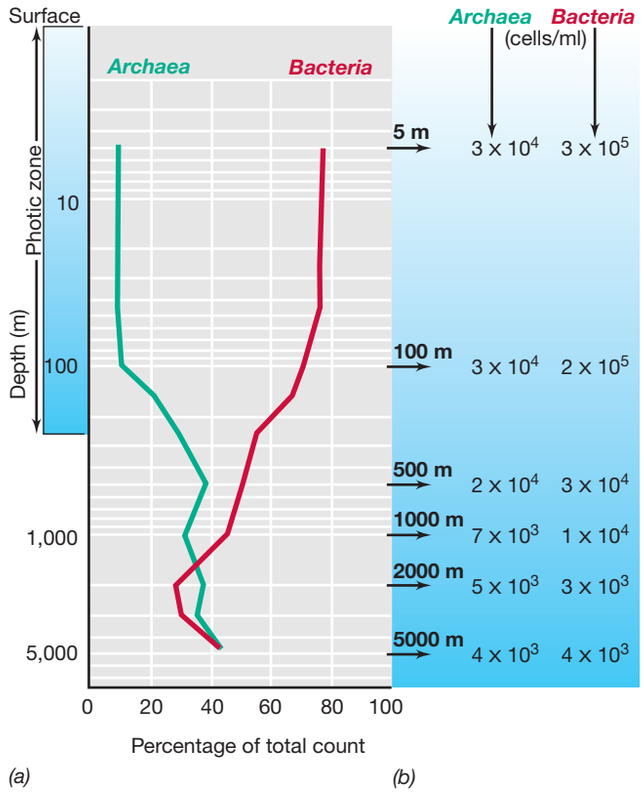


Figure 20.29 Distribution of *Archaea* and *Bacteria* in North Pacific Ocean water. *(a)* Percentage of *Archaea* and *Bacteria* with depth. *(b)* Approximate absolute numbers of *Archaea* and *Bacteria* per milliliter with depth in the open ocean.

Pelagibacter: The Most Abundant Bacterium

Small planktonic chemoorganotrophic bacteria inhabit pelagic marine waters in numbers of 10^5–10^6 cells/ml. The most abundant of these are members of the "SAR11 group" within the *Alphaproteobacteria* (◀ Section 16.1) that includes the genus *Pelagibacter*. Environmental metagenomic studies (◀ Sections 10.7 and 19.8) and cell counts done using FISH (◀ Section 19.5) have revealed a great abundance of SAR11 group organisms in pelagic waters. The total oceanic population of this group is estimated to be about 2.4×10^{28} cells—about three-quarters of all *Bacteria*—making it the most successful microbial group, as reflected by abundance, on the planet. *Pelagibacter* is an oligotroph, an organism that grows best at very low concentrations of nutrients and grows in laboratory culture only up to the densities it is found in nature.

What makes *Pelagibacter* so successful in the open oceans? In part, its success is related to small size. Cells of *Pelagibacter* are small rods with a diameter of only 0.2–0.5 μm and a volume of just 0.01 μm^3, making cells difficult to resolve with the light microscope but not the electron microscope (**Figure 20.30**). The resulting high surface-to-volume ratio (◀ Section 1.3 and Figure 1.7) facilitates nutrient transport, increasing substrate concentration and processing rates within the cell. Proteomic analyses (◀ Section 10.9) of *Pelagibacter* have also revealed a high abundance of periplasmic binding proteins (◀ Section 2.2) for soluble nutrients such as phosphate, amino acids, and sugars. Another adaptive feature of *Pelagibacter* is its fairly small genome (1.3 Mbp). Consistent with the proteome analysis,

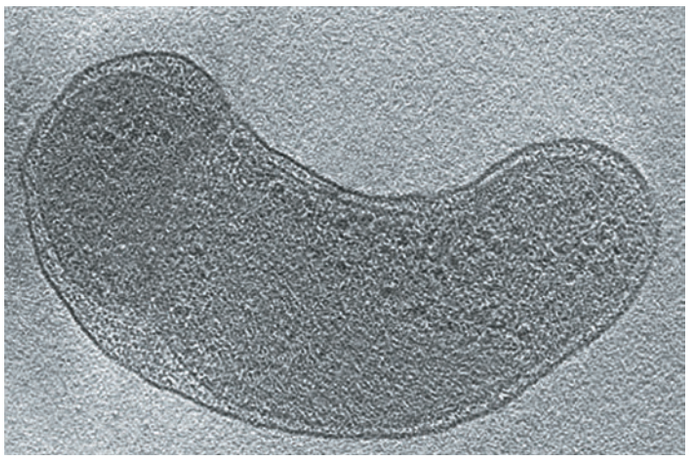


Figure 20.30 ***Pelagibacter*, the most abundant bacterium in the ocean.** Electron micrograph taken by electron tomography, a technique that introduces a three-dimensional effect. A single cell of *Pelagibacter* is about 0.2 μm in diameter.

the genome encodes an unusually high number of ABC-type transport systems—transporters that have an extremely high affinity for their substrates (◀ Section 2.2)—and other enzymes useful for an oligotrophic lifestyle. The *Pelagibacter* genome is also highly "streamlined," with intergenic spacings averaging only 3 base pairs; such a highly compact genome reduces the cost of replication.

Rhodopsins in Marine Microbes

The *Pelagibacter* genome also contains genes encoding a form of the visual pigment rhodopsin that can convert light energy into ATP. In Section 17.1 we discussed the now well-studied molecule *bacteriorhodopsin*, a light-activated protein complex present in the extreme halophile *Halobacterium* (*Archaea*). Bacteriorhodopsin functions in ATP synthesis as a simple light-driven proton pump; protons pumped across the membrane by this system fuel ATPase in the membrane, generating ATP (◀ Figure 17.4). The form of rhodopsin in *Pelagibacter* and other pelagic *Bacteria* is structurally similar to bacteriorhodopsin and has been called **proteorhodopsin** ("proteo" referring to *Proteobacteria*). Although proteorhodopsin was first discovered in species of *Proteobacteria*, it is actually fairly widely distributed in *Bacteria*; the system is found in many *Gamma-* and *Alphaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* (all covered in Chapter 16), and also in nonhalophilic species of *Archaea*, such as species of the marine *Euryarchaeota* that inhabit the photic zone. The different variants of proteorhodopsins in marine microbes have absorption properties that reflect changing spectral properties of light at increasing depths in the water column, with near-surface variants absorbing green light and those at greater depths absorbing blue light.

Experiments have shown that proteorhodopsin-containing marine bacteria survive starvation better in the light than in the dark. This indicates that energy-starved cells use light-mediated ATP production to compensate for energy unavailable from carbon respiration when organic carbon levels are low. Proteorhodopsins are thought to exist in ~80% of bacteria in some marine waters and are thus a widespread strategy to supplement the energy metabolism of marine microbes such that they need not rely solely on scarce organic carbon for their energy needs. Metagenomics has shown that a variant of proteorhodopsin called *heliorhodopsin* is widely distributed among marine *Archaea*, *Bacteria*, and *Eukarya*, many of which were previously unsuspected to sense light. However, unlike the energy-generating proteorhodopsins, heliorhodopsins appear to function only as a photosensor, most likely assisting in cellular positioning at an optimal location in a light field that would benefit light-mediated energy-generating systems, such as rhodopsins or chlorophylls.

A Phylogenetic Snapshot of Marine Bacterial and Archaeal Diversity

Several studies have documented the diversity of planktonic marine *Bacteria* and *Archaea* from sequences of 16S ribosomal RNA genes obtained from seawater. The existence of abundant alphaproteobacterial populations with which *Pelagibacter* is affiliated was first revealed by such methods. Mesophilic *Archaea* subsequently shown to be related to ammonia-oxidizing *Nitrosopumilus maritimus* (◀ Section 17.5) were also first observed using this approach.

Major bacterial groups now recognized as abundant in the ocean include *Alpha-* and *Gammaproteobacteria*, *Cyanobacteria*, *Bacteroidetes*, and to a lesser extent *Marinimicrobia* and *Actinobacteria*; *Firmicutes* are only minor components. Long-term studies of oceanic bacterial and archaeal population dynamics using molecular methods (◀ Section 19.6) have provided an unprecedented view of microbial diversity in marine systems. A six-year seasonal sampling study at a coastal site (near Plymouth, England) is one such example (**Figure 20.31**). Notably, *Cyanobacteria*, which include the ecologically important and relatively abundant phototroph *Prochlorococcus*, comprise only about 1% of diversity in these coastal waters because of competition from eukaryotic phytoplankton (including diatoms and green algae). In contrast, *Cyanobacteria*—and *Prochlorococcus* in particular—dominate the nutrient-limited open ocean.

Proteobacteria, in particular the alpha and gamma classes, clearly dominate the microbial diversity of the oceans. Many marine *Alpha-* and *Gammaproteobacteria* are chemoorganotrophs that metabolize dimethylsulfoniopropionate (DMSP; ◀ Table 4.6), including the alphaproteobacterium *Ruegeria pomeroyi* and species of the gammaproteobacterial genus *Marinomonas*. DMSP is a soluble organic-sulfur compound produced by marine phytoplankton (microscopic eukaryotic phototrophs) that functions primarily as a compatible solute to control osmotic tension in the cell (◀ Section 4.15). However, much of this DMSP gets excreted, and bacteria degrade it, forming the volatile compound dimethyl sulfide (DMS). This gas has a significant effect on Earth's climate. As the single most important natural source of sulfur released to the atmosphere, DMS becomes photooxidized to sulfuric acid in the atmosphere, and this promotes water condensation and the formation of clouds. The latter deflect sunlight back into space and help cool our planet (▶ Sections 21.4 and 21.9).

R. pomeroyi is a nonphototrophic member of the *Roseobacter* lineage within the *Rhodobacterales* (◀ Section 16.1), an assemblage that also includes many aerobic purple bacteria (◀ Section 15.5) and composes up to a third of the bacterioplankton in coastal waters and a few percent in open ocean surface waters. *R. pomeroyi* and relatives are often the dominant taxa to develop during blooms of

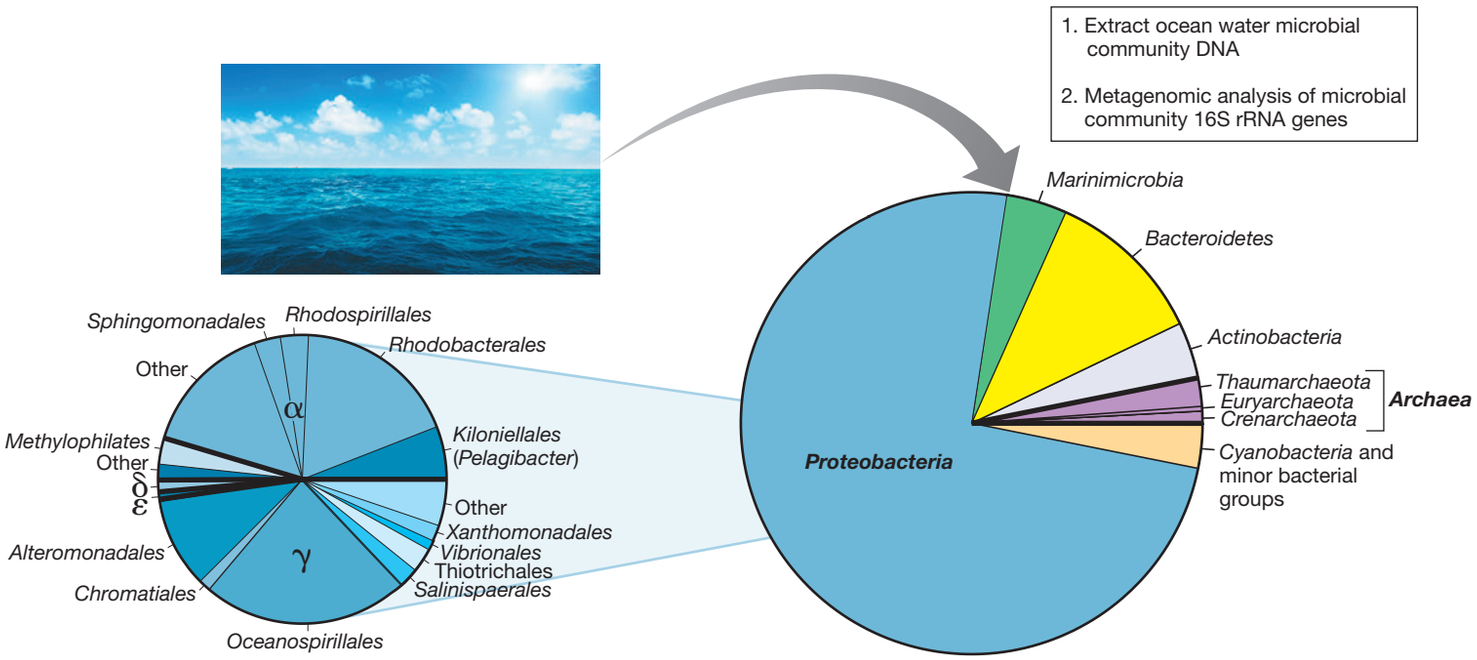


Figure 20.31 Coastal ocean bacterial and archaeal diversity. The results are pooled analyses of nearly one million 16S ribosomal RNA sequences from seasonal sampling near the coast of Plymouth (United Kingdom) over a six-year period. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of *Alphaproteobacteria* and *Gammaproteobacteria* sequences. Data assembled and analyzed by Nicolas Pinel. Compare the prokaryotic diversity of seawater with that of freshwater shown in Figure 20.20.

DMSP-producing phytoplankton, pointing to the importance of this bacterium in generating DMS and influencing atmospheric chemistry.

As is the case for soil microbial diversity (Figure 20.14), a large group of *Bacteroidetes* species and unclassified and minor bacterial groups are also present in seawater. A major group of marine *Gammaproteobacteria* is the yet-to-be-cultured "SAR86 group." Although accounting for approximately 10% of the total prokaryotic community in the open oceans' surface layer, this group is only poorly represented in higher-nutrient coastal waters (Figure 20.31). The candidate bacterial phylum *Marinimicrobia* is widely distributed in the ocean, including coastal and pelagic surface waters, oxygen minimum zones (Figure 20.22), and depths greater than 6000 meters. Metagenomic analysis of this phylum has revealed tremendous flexibility in the use of electron acceptors other than oxygen to support anaerobic respirations, including sulfoxides, nitrous oxide, polysulfides, and nitrate, and some species can oxidize thiosulfate as an electron donor. With the exception of the cyanobacteria, most marine *Bacteria* are likely chemoorganotrophs adapted to extremely low nutrient availability in the open ocean, with some augmenting energy conservation through proteorhodopsin or aerobic anoxygenic phototrophy (Section 20.11).

Archaea in pelagic waters comprise a rather restricted diversity of *Euryarchaeota*, *Crenarchaeota*, and *Thaumarchaeota*, most of which have not yet been brought into laboratory culture. The isolation of *Nitrosopumilus maritimus*, affiliated with the *Thaumarchaeota*, revealed that, although not highly diverse (Figure 20.31), ammonia-oxidizing chemolithotrophic *Archaea* are also among the most abundant marine microbes, comprising 20–40% of all marine prokaryotic cells beneath the photic zone. Although species of *Euryarchaeota* can also be abundant in the upper ocean, comprising 4–20% of the prokaryotic community in surface waters, none have thus far been cultured. However, based on metagenomic analyses, most encode proteorhodopsin, suggesting they grow primarily as photoheterotrophs.

"Dilution culture" and single cell isolation methods employing very dilute culture media have successfully brought some pelagic microbes into culture (◀ Sections 19.2 and 19.3). It appears that most of these organisms have evolved to grow only at very low nutrient concentrations and very low cell densities (10^5–10^6/ml), so it is either difficult or impossible to generate large amounts of cell material in the laboratory. This limitation renders many of the common tools for measuring cell growth (turbidity or microscopic counts) useless on samples that are not first concentrated. Nevertheless, there have been notable successes with dilution culturing of marine bacteria, and the aforementioned bacterium *Pelagibacter* is perhaps the best example of this (◀ Figure 19.6).

Check Your Understanding

- What is proteorhodopsin and why is it so named? Why might proteorhodopsin make a bacterium such as *Pelagibacter* more competitive in its habitat? How might heliorhodopsin be helpful to a phototrophic microbe?
- What important role does the bacterium *Ruegeria pomeroyi* play in the health of our planet?
- Why are dilute culture media used for isolating pelagic microbes?

20.13 Pelagic Marine Viruses

Viruses are found everywhere on and in Earth where cellular life is present (including on and in plants and animals) and are present in some environments in enormous numbers. The number of bacterial and archaeal cells on Earth is far greater than the total number of eukaryotic cells; estimates of total prokaryotic cell numbers are on the order of 10^{29}. However, the number of viruses is even greater than this, an estimated 10^{30}. Thus, one might expect that, despite their small size, viruses would play a major ecological role in nature. Soil, freshwater, Earth's deep subsurface, and microbial mats, among many other habitats, are teeming with microbes and the viruses that feed on them. We focus here on seawater to give a feel for the numbers involved and how viruses interact with their prokaryotic hosts.

Bacteriophages and Archaeal Viruses in Seawater

There are about 10^6 prokaryotic cells/ml of seawater and approximately ten times as many viruses. Although viruses account for most of the total microbes present in seawater in terms of numbers, because of their very small sizes they constitute only about 5% of the total microbial biomass. However, using special fluorescence microscopy techniques, viruses can be visualized in seawater (**Figure 20.32**). It has been estimated that at least 5% and as many as 50% of the *Bacteria* in seawater are killed by bacteriophages each day, and most of the others are eaten by protozoa. For example, Syn5 is a bacteriophage that attacks and lyses *Synechococcus* species (**Figure 20.33**), who, along with their relative *Prochlorococcus*, are the major primary producers of the ocean and account for over 30% of the CO_2 fixed globally (Section 20.11).

The two primary mechanisms of bacterial mortality, grazing by protists and lysis by bacteriophages, contribute about equally to cell death but differ greatly in their impact on marine food webs. Grazing moves carbon up through the food chain when bacteria are consumed by protozoa, which are then consumed by zooplankton, which in turn sustain higher trophic levels up to fish and larger organisms. In contrast, the cytoplasm released as dissolved organic matter (DOM) by viral attack on *Prochlorococcus* (Figure 20.33) and other marine microbes—estimated to be as much as 3 gigatons of carbon per year—decreases the transfer of carbon to higher trophic levels. Although providing a significant amount of organic matter for other microbes, this released carbon amounts to about 25% of primary production that could otherwise be used by grazing populations.

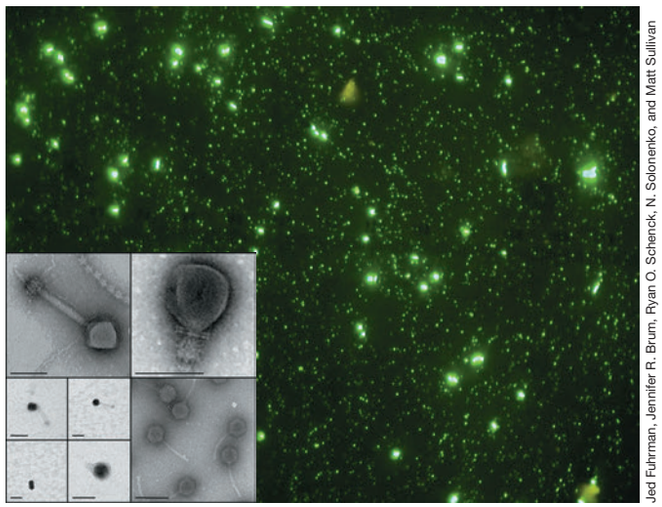

Jed Fuhrman, Jennifer R. Brum, Ryan O. Schenck, N. Solonenko, and Matt Sullivan

Figure 20.32 Viruses in seawater. A water sample collected on a 0.02-μm (20-nm) pore size filter is stained with SYBR Green and viewed by epifluorescence microscopy. The tiny green dots are viruses while the larger, brighter dots are prokaryotic cells about 0.5 μm in diameter. Viruses are typically 10 times more abundant than the total of prokaryotic cells in seawater. Inset transmission electron micrographs show various marine bacterial viruses (scale bars, 100 nm in all images).

Most described marine bacteriophages are head-and-tail bacteriophages containing double-stranded DNA genomes (Baltimore class I, ◀ Figure 11.2); by contrast, described RNA-containing phages and non-tailed doubled-stranded DNA viruses are relatively rare. However, electron microscopic observations of marine water samples have revealed that non-tailed viruses appear to dominate in the global ocean, even though this group is almost completely missing from marine virus isolates and sequence collections. This mystery was at least partially solved by the discovery of an entirely new lineage of non-tailed double-stranded DNA bacteriophage called the *Autolykiviridae* (the group is named for Autolykos of Greek

Figure 20.33 Cyanophage infection of *Synechococcus*. Phase contrast–electron cryotomography sections of cells of *Synechococcus* in various stages of infection with phage Syn5. *(a)* Early. *(b)* Intermediate. *(c)* Late. Arrows point to phage particles.

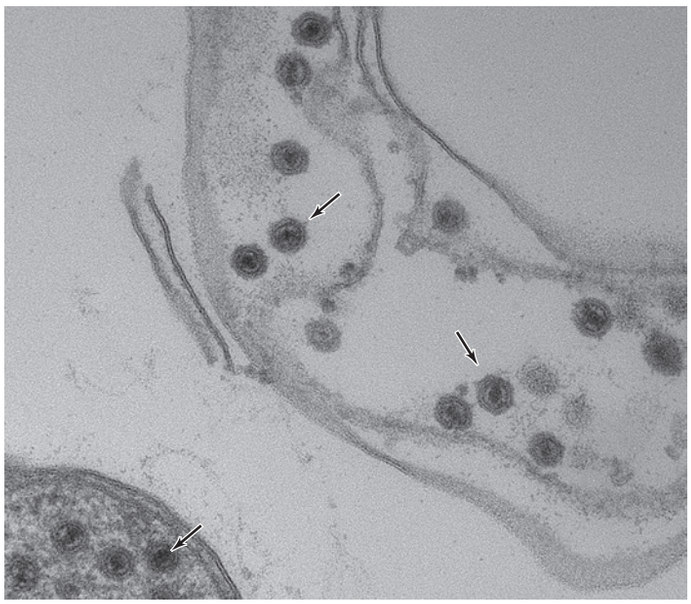


Figure 20.34 ***Autolykiviridae.*** Thin-section electron micrograph of lysed marine *Vibrio* cells containing virions of *Autolykiviridae*, a family of non-tailed dsDNA viruses. The viruses contain a lipid-bilayer membrane with an outer wall surrounding the virus core. A single virion is approximately 30 nm in diameter. Arrows point to individual virions.

mythology, a shape-changing thief notable for being difficult to catch). The virion of the virus has a more-or-less icosahedral morphology (**Figure 20.34**). The *Autolykiviridae* were recovered from plaque assays (◀ Section 5.3 and Figure 5.10) using marine *Vibrio* cells as host. When genome sequences of isolated *Autolykiviridae* were used to screen marine metagenomic and genomic libraries for related viruses, sequences homologous to the gene encoding the capsid protein of the *Autolykiviridae* were identified in 13 bacterial and archaeal phyla, including two of the most abundant marine groups, the *Alphaproteobacteria* and the *Thaumarchaeota*.

The discovery of this previously unrecognized virus family of very broad host range (infecting both *Bacteria* and *Archaea*) has filled a major gap in our understanding of environmental virology. It is likely that this approach to discovery—combining classic methods of bacteriophage isolation in laboratory culture and comparative metagenomic sequence analysis—will continue to expand our knowledge of the marine virosphere. For example, recognition that bacteriophages infecting *Pelagibacter* species (Figure 20.30) are widespread in the marine environment was also dependent on first describing the bacteriophage infecting laboratory cultures of the host bacterium.

Marine Viral Diversity

Although laboratory characterization remains the definitive approach to virus description, additional understanding of viral diversity is being provided by *viral metagenomics*. Since viruses lack universal marker genes that can be used for targeted surveys (such as rRNA genes in cellular microbes), metagenomic approaches are used to conduct diversity surveys. Viral metagenomics combines virus particle purification and sequencing of DNA isolated from the virus particles. The viral metagenome is the sum total of all the virus genes in a particular environment. When combined with next-generation sequencing technologies, this approach has shown that a large part of genetic diversity on Earth resides with bacteriophages.

Several viral metagenomic studies have been undertaken, and they invariably show that immense viral diversity exists on Earth. For example, approximately 75% of the gene sequences found in viral metagenomic studies show no similarity to any other genes known, either viral or cellular. By comparison, surveys of bacterial metagenomes typically reveal approximately 10% unknown genes. However, viral metagenomics alone is generally not sufficient to identify the host(s) infected by a specific virus. Methods in development to improve host assignment include matching of CRISPR spacers (antiviral defenses of *Bacteria* and *Archaea*, ◀ Section 9.12) to environmental viral sequences and identifying patterns of co-occurrence between viral populations and putative host bacteria. Thus, most viruses still await discovery and most viral genes have unknown functions. This makes the study of the virosphere and viral diversity one of the most exciting areas of microbiology today. A rough estimate thus far of marine viral diversity indicates that at least 200,000 different kinds of viruses exist in the world's oceans, harboring an enormous number of novel genes.

Viruses as Agents of Genetic Transfer in the Marine Environment

As we have seen, lysogenic bacteriophages can integrate into the genomes of their bacterial hosts (◀ Section 5.6), and when they do, they can confer new properties on the cell. Moreover, some lytic phages facilitate the transfer of bacterial genes from one cell to another through the process of transduction, a major means of horizontal gene transfer in which a virus carries host genes between cells, for example by picking up host DNA and becoming a nonlytic *transducing particle* (◀ Section 9.7). As agents of transduction, bacteriophages are thought to have a major influence on bacterial evolution by conferring new metabolic or other beneficial properties on recipient cells that allow them to successfully colonize new habitats.

A good example of bacteriophage gene transfer in marine systems are the "cyanophages," phages that can transfer certain photosynthesis genes among strains of *Synechococcus* and *Prochlorococcus*. When these phages are released from their lysed host cells (Figure 20.33), some of them incorporate host genes that encode photosystem (PS) II, one of the key components of oxygenic photosynthesis (◀ Section 14.6). When such a phage infects a new host cell, it provides the cell with genes encoding a modified PSII. It is hypothesized that a more diverse complement of PSII proteins improves both cell and phage fitness by allowing the host cell to better adapt to changing environmental conditions—for example, changes in light intensity or light quality—and in the process, producing more cyanophage.

When host-derived genes were first found in temperate (nonlytic) bacteriophages, they were called lysogenic conversion factors. However, recognition that hostlike genes are widely distributed in both temperate and lytic viruses required the introduction of the much broader term **auxiliary metabolic genes (AMGs)**. During lytic infection, expression of AMGs alters host metabolism toward pathways that maximize production of new viral particles. For example, in the cyanobacteria–cyanophage system just described, the AMGs encode proteins that supplement photosynthetic electron transport

and redirect energy from carbon fixation to the pentose phosphate pathway (◀ Section 3.13) for increased production of ribose 5-phosphate, a nucleotide precursor needed to support virus replication. Similarly, cyanophages isolated from marine regions of low phosphate are more likely to have AMGs involved in phosphorus acquisition, such as genes for phosphate-binding protein (*pstS*) and alkaline phosphatase (*phoA*); this ensures that the host will have sufficient phosphate to produce cyanophage DNA.

Marine viruses infecting eukaryotes also encode AMGs. For example, an AMG of a virus infecting the calcareous phytoplankton *Emiliania huxleyi* (▶ Figure 21.20*a* and ◀ Section 18.7) alters host lipid biosynthesis pathways to produce lipids needed for viral particle assembly. Although viral metagenomics has shown that one way or the other, the most common AMGs affect nucleotide biosynthesis, viral AMGs can influence many different pathways of microbial metabolism, most often to the benefit of the virus and not the host.

Auxiliary Metabolic Genes in *Thaumarchaeota*

Archaea are abundant in the oceans, and a major group of marine *Archaea* of ecological importance is the *Thaumarchaeota* (◀ Section 17.5). These ammonia-oxidizing chemolithotrophs consume the vanishingly low levels of ammonia present in pelagic waters. Although lytic archaeal viruses have yet to be isolated for this group, several species of the archaeon *Nitrosopumilus* have been shown to harbor viral genomes within their chromosome (that is, the cells contain a *provirus*, ◀ Sections 5.6 and 5.7) whose genes suggest that the infecting virus was either of the head-and-tail dsDNA bacteriophage type (◀ Section 11.4) or icosahedral-shaped, similar to the herpesviruses (◀ Section 11.7). The discovery that these *Thaumarchaeota* proviruses contain an AMG homologous to the gene encoding a subunit of ammonia monooxygenase, the enzyme that catalyzes the first step in ammonia oxidation, was used to screen marine metagenomes for related viruses. From these results, 32 new thaumarchaeal viruses encompassing 15 putative viral species were identified in a variety of marine habitats. It is thus likely that at least some, and perhaps even many, of the viruses in seawater infect marine *Archaea* instead of marine *Bacteria* (Figure 20.31). This is bolstered by the observation that virtually all known archaeal viruses contain double-stranded DNA genomes (◀ Section 11.5 and Figures 11.3*a* and 11.13), and these are the most commonly observed viral genomes in the oceans.

Survival Strategies of Viruses in Nature

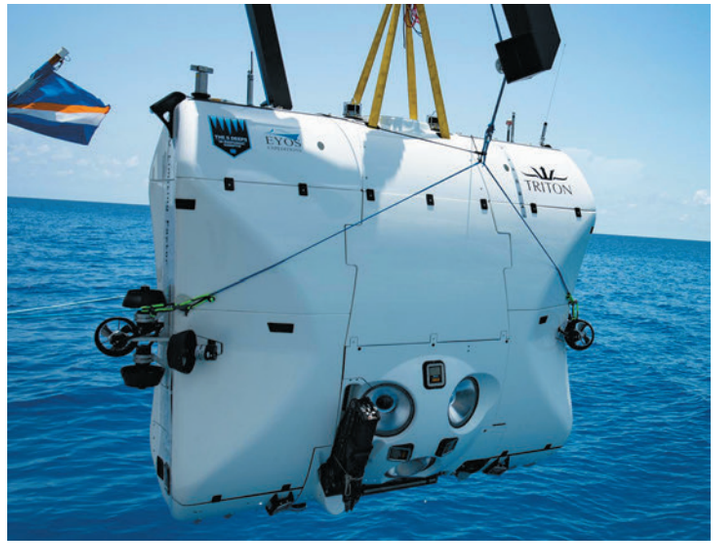
When hosts are plentiful in nature, it is thought that bacteriophages adopt the lytic lifestyle and thus large numbers of host cells are killed. By contrast, when host numbers are low, it may be difficult for viruses to find a new host cell, and under such circumstances, lysogeny would be favored if the virus is lysogenic (◀ Section 5.6). Under these conditions, the virus would survive as a prophage until host numbers increased and a lytic lifestyle could once again be supported. This hypothesis is consistent with the observation that in the depths of the ocean where bacterial numbers are lower than in surface waters, around half the bacteria examined have been found to contain one or more lysogenic viruses. As far as is known, no single-stranded DNA viruses and no RNA viruses can enter a lysogenic state, and so how these viruses might survive periods of low host numbers is unknown.

Check Your Understanding

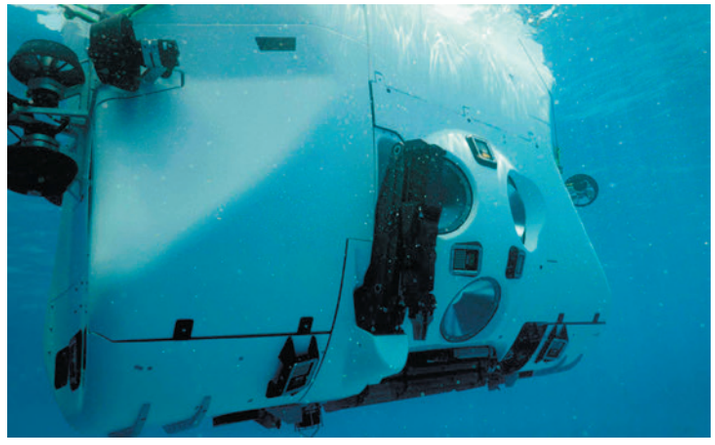
- What type of bacteriophages are most common in the oceans, based on what evidence?
- How can bacteriophages affect bacterial evolution?
- What does the viral metagenome suggest about our understanding of viral diversity?
- What is an AMG, and how do AMGs benefit marine microbes?

20.14 The Deep Sea

Light penetrates no farther than about 250 m in pelagic waters; as mentioned, this illuminated region is called the *photic zone* (Figure 20.29). Beneath the photic zone, down to a depth of about 1000 m, there is still considerable biological activity. However, water at depths greater than 1000 m is, by comparison, much less biologically active and is known as the *deep sea*. Greater than 75% of all



(a)



(b)

Figure 20.35 **The deep-diving submersible *Limiting Factor*.** *(a)* Deployment of the vessel. *(b)* Beginning a dive. This two-person vessel is capable of reaching depths of 11,000 meters with an operational dive time of 16 hours.

ocean water is deep-sea water, lying primarily at depths between 1000 and 6000 m. The deepest waters in the oceans lie below 10,000 m. However, because depressions this deep are very rare, the waters in them make up only a very small proportion of all pelagic waters.

The biology of the deepest ocean regions, the *abyssal zone* (below 4000 m) and the *hadal zone* (below 6000 m), has stirred the imagination for centuries. These regions remain among the least explored of Earth's biosphere because their study requires very specialized equipment. Only a few remotely operated vehicles (ROVs), autonomous underwater vehicles, and human-occupied submersibles can dive to the deepest marine waters, which exceed depths of 10,000 m; pressures at such depths are around 16,000 pounds per square inch (110,000 kilopascals). Among the newest generation of human-occupied submersibles is the *Limiting Factor* (**Figure 20.35**). This two-person vessel is capable of diving to 11,000 meters, allowing humans to directly visit and explore all regions of the ocean floor, including the very deepest—the Challenger Deep, which lies within the Mariana Trench (Pacific Ocean, depth of 10,900 m).

Conditions in the Deep Sea

Organisms that inhabit the deep sea face three major environmental extremes: (1) low temperature, (2) high pressure, and (3) low nutrient levels. In addition, deep-sea waters are completely dark such that photosynthesis relying on sunlight is impossible. Thus, microbes that inhabit the deep sea must be chemotrophic and able to grow under high pressure and oligotrophic conditions in the cold.

Below depths of about 1000 m, ocean water temperatures stay constant at 2–3°C. We discussed the responses of microorganisms to changes in temperature in Sections 4.11–4.13. As would be expected, many bacteria isolated from marine waters below 1000 m are psychrophilic (cold-loving) or at least psychrotolerant. However, since mesophilic microorganisms from warmer and more productive surface waters are constantly sinking through the water column, a variety of mesophiles can also be isolated from water collected at great depths. Deep-sea microbes must also be able to withstand the enormous hydrostatic pressures associated with great depths.

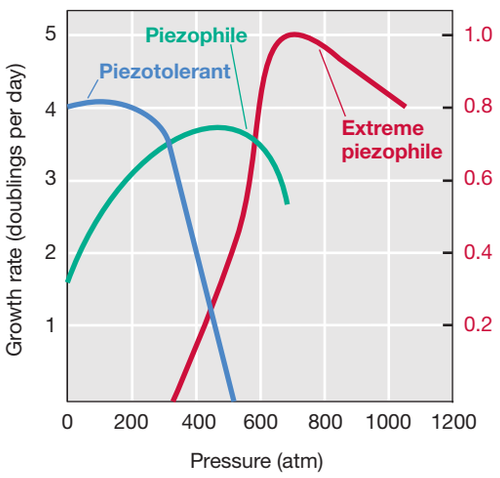


Figure 20.36 Growth of piezotolerant, piezophilic, and extremely piezophilic bacteria. Compare the slower growth rate of the extreme piezophile (right ordinate) with the growth rate of the piezotolerant and piezophilic bacteria (left ordinate) and note the inability of the extreme piezophile to grow at low pressures.

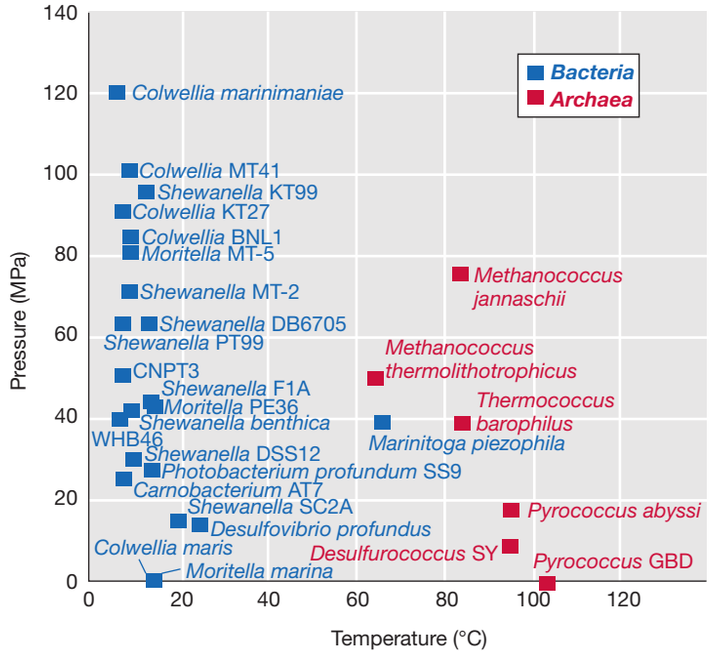


Figure 20.37 Pressure and temperature optima for cultured bacterial and archaeal piezophiles. Pressure is in pascals (Pa), the SI unit for pressure. One megapascal (MPa) corresponds to approximately 10 atmospheres. Note that different species of the same genus (for example, *Moritella*) can have vastly different pressure optima. Data assembled by Doug Bartlett.

Pressure increases by 1 atm for every 10 m of depth in a water column. Thus, an organism growing at a depth of 5000 m must be able to withstand pressures of 500 atm. We will see that microorganisms in general are remarkably tolerant of high hydrostatic pressures; many species can withstand pressures of 500 atm, and some species can withstand far more than this. Moreover, from studies of deep-sea microbial diversity performed thus far, some unusual microbes call this extreme environment home.

Piezotolerant and Piezophilic *Bacteria* and *Archaea*

Different physiological responses to pressure are observed in different deep-sea microorganisms. Some organisms simply tolerate high hydrostatic pressure, but do not grow optimally under such pressure; these organisms are **piezotolerant** (**Figure 20.36**). By contrast, others actually *grow best* under elevated hydrostatic pressure; these are called **piezophiles**. Organisms isolated from surface waters down to about 3000 m are typically piezotolerant. In piezotolerant organisms, higher metabolic rates are observed at 1 atm than at 300 atm, although growth rates at the two pressures may be similar (Figure 20.36). However, piezotolerant isolates typically do not grow at pressures greater than about 500 atm (Figure 20.36).

By contrast, cultures derived from samples taken at greater depths, 4000–6000 m, are typically piezophilic, growing optimally at pressures of around 300–400 atm. However, although piezophiles grow best under high pressure, they can still grow at 1 atm (Figure 20.36). In even deeper waters (for example, 10,000 m), **extreme piezophiles** (also called *hyperpiezophiles*) are present. These organisms require very high pressure for growth (**Figure 20.37**). For example, the extreme piezophile *Moritella* MT-5 isolated from the Mariana Trench

UNIT 5

Mastering Microbiology
Art Activity: Figure 20.30 Growth of piezotolerant, piezophilic, and extremely piezophilic bacteria

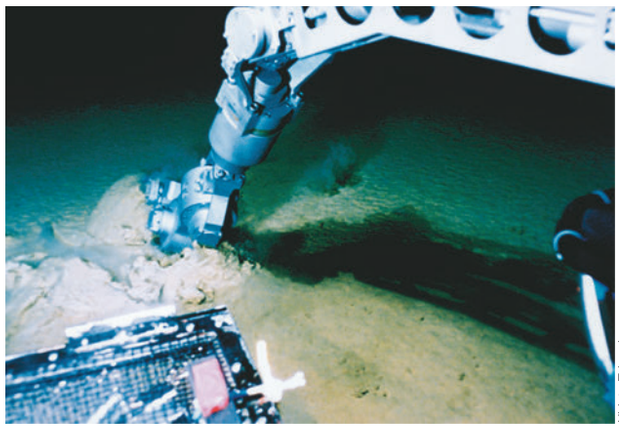


Figure 20.38 Sampling the deep sea. The unmanned Japanese submersible *Kaiko* collecting a sediment sample on the seafloor of the Mariana Trench off the Philippines at a depth of 10,897 m. The tubes of sediment are used for enrichment and isolation of piezophilic bacteria, such as the extreme piezophile (*Moritella*) isolated from this seafloor.

(Pacific Ocean, >10,000-m depth) (**Figure 20.38**) grows optimally at a pressure of 700–800 atm (Figure 20.37) and grows nearly as well at 1035 atm, the pressure it experiences in its natural habitat. The current pressure champion, *Colwellia marinimaniae*, was isolated from the Challenger Deep. This obligately hyperpiezophilic psychrophile is a member of the *Gammaproteobacteria* and grows at pressures between 80 and 140 MPa, with an optimum growth pressure of 120 MPa and optimum growth temperature of 6°C.

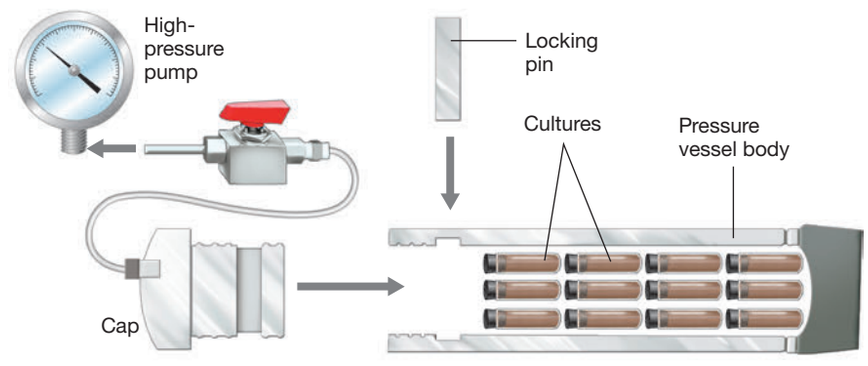
Molecular Effects of High Pressure

High pressure affects cellular physiology and biochemistry in many ways. In general, pressure decreases the ability of the subunits of multi-subunit proteins to interact. Thus, large protein complexes in extreme piezophiles must interact in such a way as to minimize pressure-related effects. Protein synthesis, DNA synthesis, and nutrient transport are sensitive to high pressure. Piezophilic bacteria grown under high pressure have a higher proportion of unsaturated fatty acids in their cytoplasmic membranes than when grown at 1 atm. Unsaturated fatty acids allow membranes to remain functional and keep from gelling at high pressures or at low temperatures. The rather slow growth rates of extreme piezophiles compared with other marine bacteria (Figure 20.36) are likely due to the combined effects of pressure and low temperature; low temperature slows down the reaction rates of enzymes, directly affecting cell growth (◀ Section 4.11).

Studies of gene expression and adaptive features contributing to growth at high pressure require special pressurized incubation devices (**Figure 20.39**). These studies have shown that when a gram-negative piezophile is grown under high pressure, a specific outer membrane protein called OmpH (*o*uter *m*embrane *p*rotein H) is present that is absent from cells grown at 1 atm. OmpH is a type of porin. Porins are proteins that form channels through which molecules diffuse into the periplasm (◀ Section 2.4 and Figure 2.12*c*). Presumably, the porin made by cells grown at 1 atm cannot function properly at high pressure and thus a different porin must be synthesized. Interestingly, pressure controls transcription of *ompH*, the gene encoding OmpH. In characterized gram-negative piezophiles, a pressure-sensitive membrane protein complex is present that monitors pressure and triggers transcription of *ompH* only when conditions of high pressure warrant it. Transcriptomic analyses (◀ Section 10.8) indicate that even relatively modest changes in hydrostatic pressure alter the expression of a large number of genes in piezophiles, so it is likely that many other pressure-monitoring proteins exist in these organisms.

There remains much to explore in the abyssal and hadal zones of the ocean. As we discussed in Chapter 19, recent advances in metagenomics and high-throughput sequencing have revolutionized studies of deep-sea microbes and their adaptive strategies, such as alterations in membrane and protein structure that allow growth under such extreme conditions. Diversity studies have shown that both free-living and particle-attached archaeal and bacterial piezophiles are abundant, and that most have not yet been cultured. For example, *Bacteria* related to the phyla *Marinimicrobia* (Figure 20.31) and *Gemmatimonadetes* are enriched at these great depths. Since no member of the *Marinimicrobia* has been isolated and the few *Gemmatimonadetes* available are from soils or wastewater, a more complete understanding of the unique properties of these hadal *Bacteria* awaits their future cultivation. Ammonia-oxidizing *Archaea* related

(a)



(b)

Figure 20.39 Pressure cells for growing piezophiles under elevated pressure. *(a)* Photo of several pressure cells incubating in a cold room (4°C). *(b)* Schematic design of a pressure cell. These vessels are designed to maintain pressures of 1000 atm. Illustration based on drawing by Doug Bartlett.

to *Nitrosopumilus* (*Thaumarchaeota*, ◀ Section 17.5), an organism common in the upper marine water column, are also among the most abundant free-living *Archaea* in hadal waters, highlighting their global dominance in both marine and terrestrial environments. Thus, presence of these novel *Archaea* and *Bacteria* at great depths points to a unique hadal microbiology that we are only now beginning to more fully explore.

Check Your Understanding

- How does pressure change with depth in a water column?
- What molecular adaptations are found in piezophiles that allow them to grow optimally under high pressure?

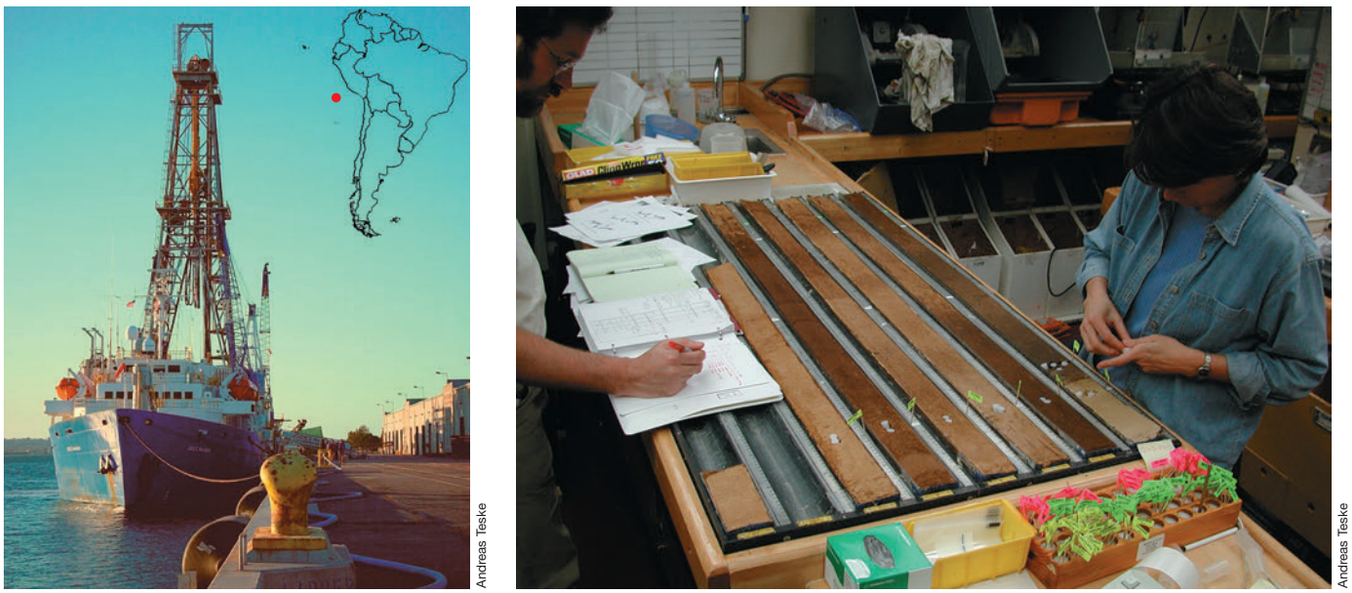
20.15 Deep-Sea Sediments

In addition to deep-sea *waters*, another vast and mostly unexplored microbial biosphere exists below the seafloor in the deep-sea *sediments*. Drilling expeditions to explore far below the ocean seafloor have revealed both archaeal and bacterial populations in sediment cores taken at depths greater than 2000 m (**Figure 20.40**). Most studies thus far have focused on relatively organic-rich sediments along continental margins. Here, cell numbers typically decrease from about 10^9 cells/g in surface sediment to about 10^6 cells/g at depths as great as 1000 m below the seafloor. In these coastal sediments, sulfate-reducing bacteria and other anaerobes deplete sulfate and other electron acceptors within a few meters of the sediment–water interface (Figure 20.22). This depletion of electron acceptors and organic matter with increasing depth constrains energy available to the deep subsurface microbial communities, accounting for the major decrease in cell numbers with depth (Figure 21.29).

Cell Numbers in Deep-Sea Sediments

The better-studied continental margins and shelf sediments are not representative of most of the ocean floor, about 90% of which is at greater than 2000-m depth and associated with marine waters of low productivity and therefore of much lower organic matter content (Figure 20.22). In the absence of significant transport of organic material to the sediment surface, sulfate and other electron acceptors may permeate all the way through the sediments to the underlying bedrock. However, because of the dearth of organic matter, cell numbers in these sediments are several orders of magnitude lower than in organic-rich sediments, ranging from about 10^6 cells/gram at the surface to fewer than 10^3 cells/gram at depths of a few hundred meters (**Figure 20.41**). Cell counts in all sediments generally decline significantly with depth, reflecting lower organic carbon availability and catabolic quality in older deep sediment material. Despite these low numbers, because deep-sea sediments are so vast, it has been estimated that a total of $\sim 5.4 \times 10^{29}$ prokaryotic cells exist in deep-sea sediments ($\sim 4 \times 10^{15}$ g), a number similar to the sum total in all the oceans.

Figure 20.41 also shows that cell numbers can deviate from the global regression as a function of spotty distributions of resources with depth at any particular drill site. In the example shown in Figure 20.41, cell numbers in coastal plain sediments are elevated near methane (cold) seeps (Figure 20.22), a region associated with active anaerobic methane oxidation (◀ Section 14.16), and in coal bed deposits (Figures 20.22 and 20.41), where, in addition to coal, associated organic materials boost electron donor availability. Other than these nutrient "oases," nutrient limitation in deep sea sediments greatly restricts the size of indigenous microbial communities.



(a) (b)

Figure 20.40 Drilling deep-sea sediments. *(a)* Deep-sea drilling vessel the JOIDES *Resolution*. Inset: Red dot indicates the location of sediment sampling in the Peru Basin. *(b)* Sediment cores recovered from the Peru Basin at 4800-m depth. Cores were split lengthwise to allow subsampling for molecular characterization. See Section 20.5 and Figure 20.10 for discussion of sulfide-oxidizing microbial mats that develop on the sediment surface off the Chilean and Peruvian coasts.

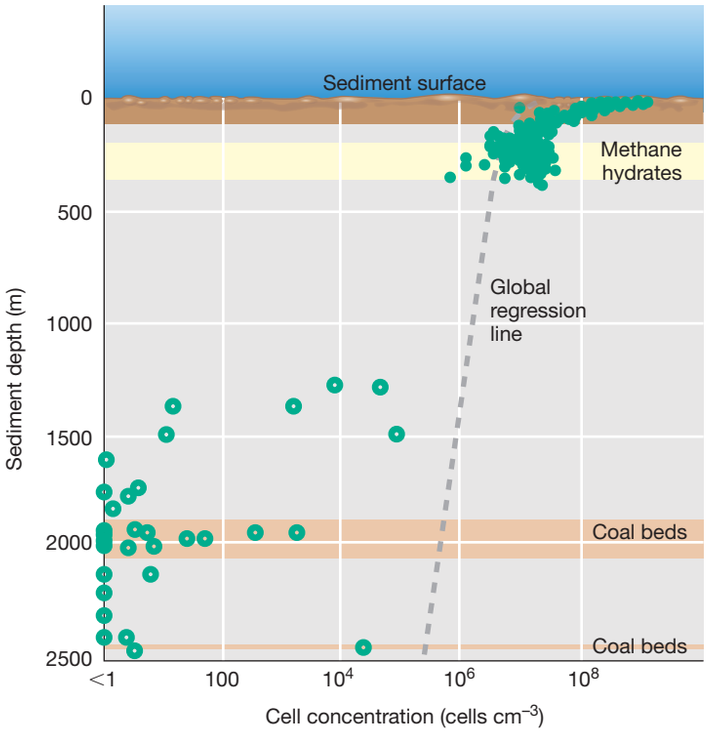


Figure 20.41 Microbial cell numbers in deep-sea sediments. Depth-related cell abundance from the analysis of one coastal sediment (filled circles) and the global average for all sampled ocean sediments (dashed regression line) based on cell count data.

A Phylogenetic Snapshot of Marine Sediment Prokaryotic Diversity

Marine sediment communities have been explored only to a limited extent because of the difficulty and expense of obtaining uncontaminated drilling cores from great depths (Figure 20.40). However, analyses of 16S ribosomal RNA gene sequences obtained by using PCR methods (◀ Section 19.6) on deep core samples have clearly established that novel *Archaea* make up a large fraction of the archaeal diversity and that bacterial diversity in both deep and shallow marine sediments is dominated by *Proteobacteria* (**Figure 20.42**). This abundance of *Proteobacteria* is also true of all the other habitats explored by culture-independent techniques (Figures 20.14, 20.20, 20.31, and see Figure 20.47).

Within marine sediment *Proteobacteria*, phylotypes associated with sulfate-reducing bacteria such as the *Desulfobacterales* are quite common (Figure 20.42), which is consistent with the fact that sulfate reduction (◀ Sections 14.12 and 15.11) is the major form of anaerobic respiration in marine sediments. *Bacteroidetes* and the unclassified/minor groups are also well represented in shallow marine sediments. Although abundant in marine waters, cyanobacteria compose just a tiny proportion of the total cell population in the permanently dark and anoxic sediments and probably represent cells that have reached the sediments after attaching to a particle or dead animal that eventually sank.

In addition to a variety of *Bacteria*, novel phyla of *Archaea* unrelated to cultured representatives are widespread in the deep subsurface. Genome sequences of uncultured *Bathyarchaeota* recovered from both terrestrial and marine sediments (Figure 20.16) revealed a physiological capacity to degrade and assimilate protein and a capacity for some species to degrade carbohydrates. Other *Bathyarchaeota* appear to carry out methanogenesis and are autotrophs, likely existing on the small amounts of H_2 produced from geochemical reactions (Section 20.8) in deep sediments.

Energy Limitation and Microbial Life Below the Seafloor

How microbes in the deepest marine sediments survive in their nutrient-depleted environment remains unclear, but it is likely that they employ many of the strategies we have seen in marine pelagic microbes including small cell size (Figure 20.17) and small, compact genomes. Sequencing of 16S ribosomal RNA genes selectively amplified by PCR (◀ Section 19.6) using DNA extracted from deep drilling cores, as well as from more limited metagenomic surveys, has identified relatively few sequences related to the classical sulfate-reducing bacteria (◀ Section 15.11) or methanogenic and known methane-oxidizing *Archaea* (◀ Sections 14.16, 15.15, and 17.2), organisms that by contrast are quite common in surface sediments. Thus, from a nutritional standpoint, how do buried cells thrive under conditions of extreme electron donor limitation?

In the marine environment, easily degraded organic material is removed by microbial respiration in the water column and anaerobic respiration in surficial sediments (the sediments at and near the sediment surface), leaving behind a dilute pool of less readily degradable organic material that slowly trickles down into deeper sediments. Microbes that inhabit the deep sediments presumably utilize this low-quality organic material along with organic carbon released from dead cells as electron donors in energy metabolism. However, since representative organisms have yet to be isolated from deep sediments, our understanding of the supporting energy-conserving metabolisms is sketchy and can only be inferred from a few partial genome sequences assembled from metagenomes (◀ Sections 10.7 and 19.8) or obtained from single cells isolated directly from sediments, using single-cell genomic technology (◀ Section 10.11). Various known and yet-to-be-discovered forms of anaerobic respiration and fermentation are the most likely candidates for deep-sea sediment metabolisms, perhaps along with other metabolic options not obvious from the conditions known to exist in deep marine sediments. Also, with growth rates of deep sediment microbes likely to be near the slowest of any on Earth, tiny amounts of organic material may be all that these cell numbers need to survive in small populations for extended periods.

Since sediment microorganisms largely control the fate of carbon in this vast subsurface reservoir of organic matter, the discovery of novel *Archaea* (Figure 20.16) living at the thermodynamic edge of life has given microbial ecologists a new perspective on carbon cycling in marine sediments. However, the extent to which abiotic processes may also contribute energy sources to these buried microbes is unclear but could be significant. For example, the higher temperatures found at greater depths may promote the alteration of organic material, releasing methane and other hydrocarbons, H_2, acetate, and CO_2 that may diffuse upwards to nourish microbes inhabiting the deep sediment biosphere. There is clearly much more to learn about the microbial ecology of Earth's deep subsurface, both in terrestrial and marine sediment contexts.

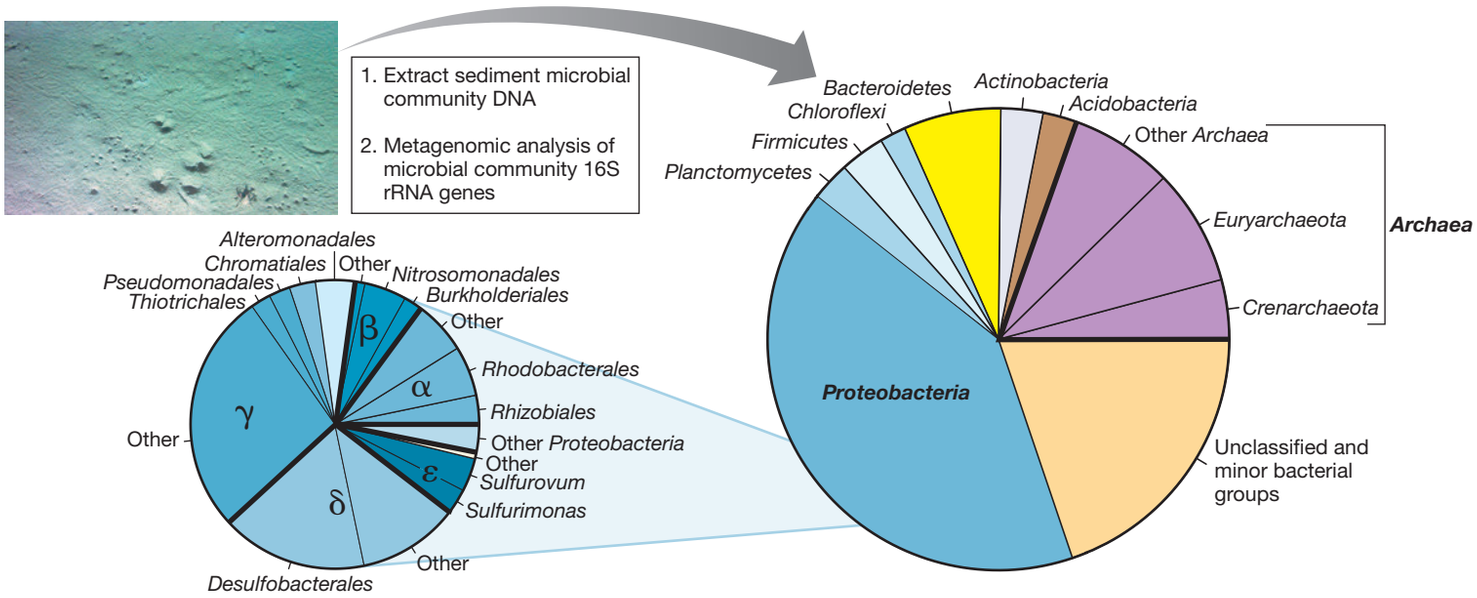


Figure 20.42 **Marine sediment bacterial and archaeal diversity.** The results are pooled analyses of 13,360 16S ribosomal RNA gene sequences from several studies of shallow and deep marine sediments. Many of the groups indicated are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of archaeal sequences and of *Gamma-*, *Delta-*, and *Epsilonproteobacteria*. Data assembled and analyzed by Nicolas Pinel. Compare the prokaryotic diversity of marine sediments with that of open ocean water shown in Figure 20.31.

Check Your Understanding

- Give two reasons why sulfate-reducing bacteria are common in shallow marine sediments.
- How and why do the numbers of bacterial and archaeal cells vary with depth in marine deep sediments?
- What alternative sources of energy are suggested to nourish microorganisms in extremely deep marine sediments?

20.16 Hydrothermal Vents

Although we have thus far described the deep sea and deep-sea sediments as a remote, low-temperature, high-pressure, oligotrophic environment suitable only for slow-growing piezotolerant and piezophilic microorganisms (Section 20.14), there are some amazing exceptions. Thriving animal and microbial communities cluster in and around deep-sea hot springs throughout the world. These hot springs are located at depths from less than 1000 m to greater than 4000 m in regions of the seafloor where volcanic magma and hot rock have caused the floor to split open at crustal spreading centers (Figure 20.22 and **Figure 20.43**), or where iron and magnesium minerals associated with ancient rocks react with seawater and generate heat. Seawater seeping into these dynamic cracking regions of the crust reacts with hot rock, resulting in hot springs saturated with inorganic chemicals and dissolved gases. Collectively, these types of underwater hot springs are called **hydrothermal vents**. We discuss several remarkable symbiotic associations between hydrothermal vent–associated animals and microorganisms in Chapter 23. Here we consider the vent environment as a habitat for free-living microbes.

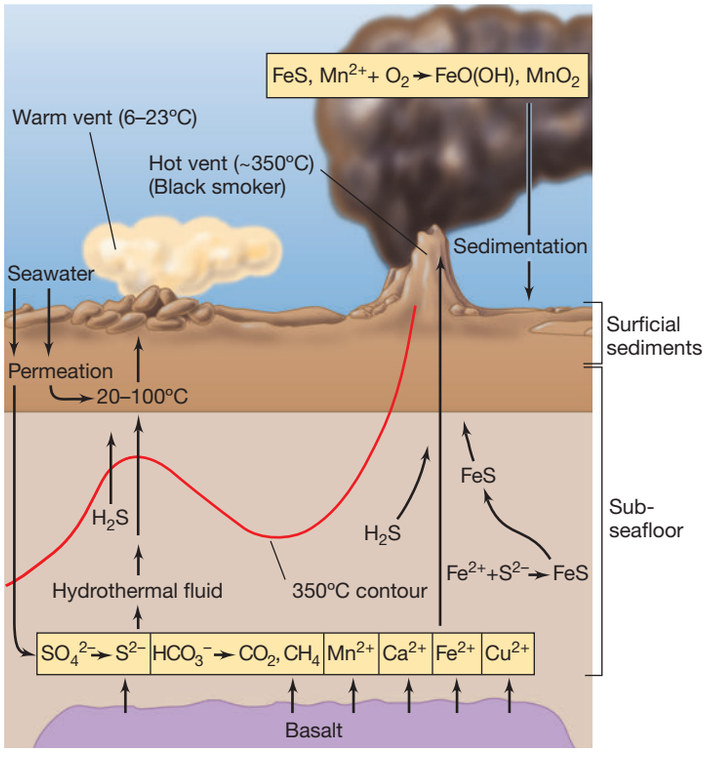


Figure 20.43 **Hydrothermal vents.** Schematic showing geological formations and major inorganic chemicals and minerals that are emitted from warm vents and black smokers. In warm vents, the hot hydrothermal fluid is cooled by cold 2–3°C seawater permeating the sediments. In black smokers, hot hydrothermal fluid near 350°C reaches the seafloor directly. The term "surficial" is a geological term meaning surface, in this case, the top layers of sediment.

UNIT 5

Mastering Microbiology
Art Activity: Figure 20.37 Hydrothermal vents

Types of Vents

Volcanic hydrothermal systems are typically either warm (~5 to >50°C), diffuse vents or very hot vents that emit hydrothermal fluids at 270 to >400°C. The gently flowing, warm, diffuse fluids are emitted from cracks in the seafloor and the exterior walls of hydrothermal chimneys. The fluids originate from the mixing of cold seawater with hot hydrothermal fluids in subsurface regions of the sediments. Hot vents, called *black smokers*, form upright sulfide edifices called *chimneys* that can be less than 1 m to over 30 m in height. Chimneys form when acidic hydrothermal fluids rich in dissolved metals and magmatic gases are suddenly mixed with cold, oxygenated seawater. The rapid mixing causes fine-grained metal sulfide minerals such as pyrite and sphalerite to precipitate out, forming dark, buoyant plumes that rise above the seafloor (**Figure 20.44**).

A quite different type of hydrothermal vent environment is the "Lost City" formation located in the mid-Atlantic Ocean. Lost City is formed from the exposure of minerals associated with ocean crust 1–2 million years old that was once deep beneath the seafloor. Geological faults in these slow-spreading systems exposed magnesium and iron-rich rocks called *peridotites* at the seafloor. Chemical reactions of seawater and newly exposed peridotite are highly exothermic, generating heat and also driving the pH up to as high as pH 11. Extremely high levels of H_2, CH_4, and other low-molecular-weight hydrocarbons are also present in the hot (200°C) hydrothermal fluids. In contrast to the acidic volcanic black smoker systems (Figure 20.44), which are relatively transient, mixing of these alkaline fluids with seawater results in the formation of calcium carbonate (limestone) chimneys (**Figure 20.45**) that can reach up to 60 m in height and be active for 100,000 years or more.

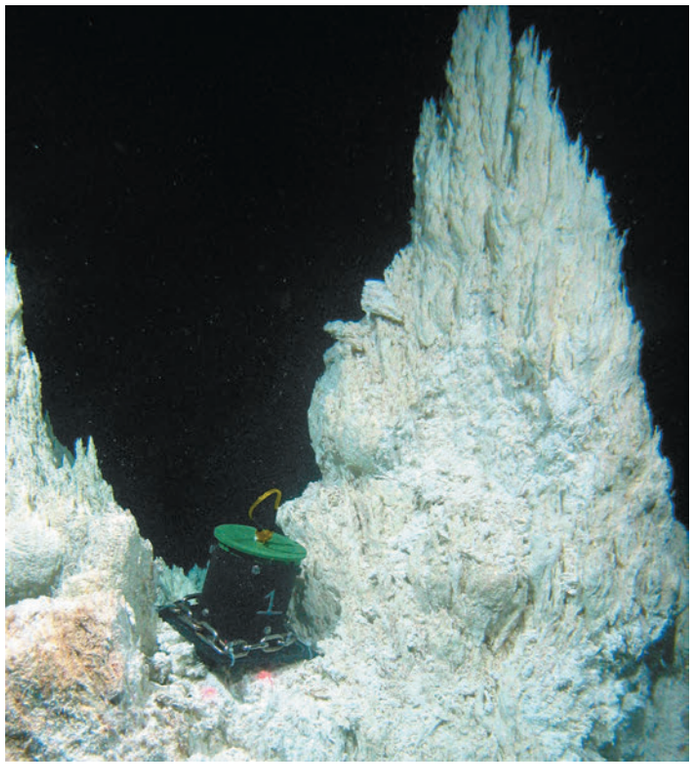


Figure 20.45 Massive carbonate chimney formation at Lost City peridotite-hosted vent system. Microbial colonization of freshly exposed mineral surfaces was studied by placing sterile mineral fragments in the green-topped device positioned over an actively venting area of the chimney. The diameter of the cylindrical collection device is approximately 10 cm. In contrast to the highly sulfidic vents (Figure 20.44), Lost City hydrothermal fluids are alkaline and lack sulfide but are rich in H_2 and CH_4.

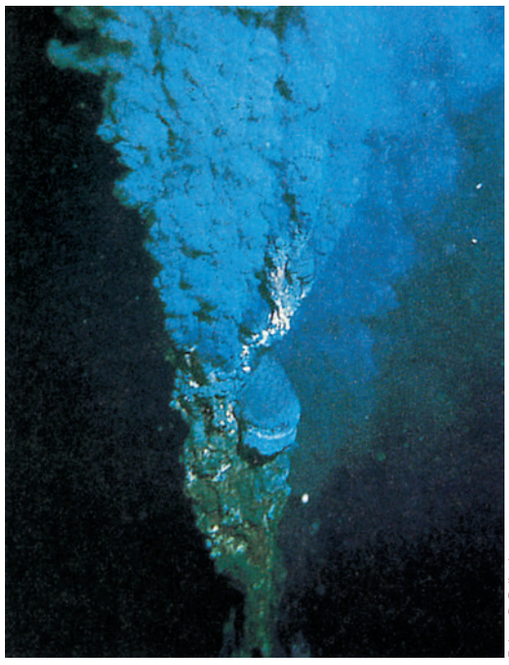


Figure 20.44 A hydrothermal vent black smoker emitting sulfide- and mineral-rich water at temperatures of 350°C. The walls of the black smoker chimneys display a steep temperature gradient and contain several types of *Bacteria* and *Archaea*.

Bacteria and *Archaea* in Hydrothermal Vents

Chemolithotrophic metabolisms (◀ Table 14.1) dominate hydrothermal vent microbial ecosystems (Figure 20.22). Sulfidic vents support sulfur-oxidizing bacteria, whereas vents that emit other inorganic electron donors support nitrifying, hydrogen-oxidizing, iron- and manganese-oxidizing, or methylotrophic bacteria, the latter presumably growing on the CH_4 and carbon monoxide (CO) emitted from the vents. **Table 20.2** summarizes the inorganic electron donors and electron acceptors that are thought to play a role in chemolithotrophic metabolisms at hydrothermal vents.

Although microbes cannot survive in the superheated hydrothermal fluids of black smokers, thermophilic and hyperthermophilic organisms do thrive in the gradients that form as the superheated water mixes with cold seawater. For example, within the walls of smoker chimneys can be found hyperthermophiles such as *Methanopyrus*, a species of *Archaea* that oxidizes H_2 and makes CH_4 (◀ Section 17.2). In fact, phylogenetic FISH staining (◀ Section 19.5) has detected cells of both *Bacteria* and *Archaea* in smoker chimney walls (**Figure 20.46**). The most thermophilic of all known sulfur-reducing microbes, species of *Pyrolobus* and *Pyrodictium* (Chapter 17), were isolated from black smoker chimney walls. In contrast to the significant microbial diversity in volcanic vent chimney walls, the carbonate chimney walls of the Lost City vents are primarily

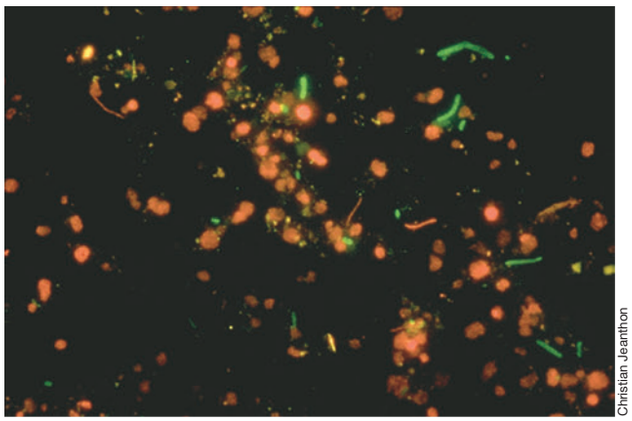


Figure 20.46 Phylogenetic FISH staining of sulfidic black smoker chimney material. Taken from the Snake Pit vent field in the Mid-Atlantic Ridge, depth of 3500 m. A green fluorescing dye was conjugated to a probe that reacts with the 16S rRNA of all *Bacteria* and a red dye to a 16S rRNA probe for *Archaea*. The hydrothermal fluid going through the center of this chimney was at 300°C.

populated by methanogens of the genus *Methanosarcina* and are nourished by the H_2-rich fluids that permeate the porous, CO_2-rich chimney walls.

When smokers plug up from mineral debris, hyperthermophiles presumably drift away to colonize active smokers and somehow become integrated into the growing chimney wall. Surprisingly, although they require very high temperatures for growth, hyperthermophiles are quite tolerant of cold temperatures and oxygen. Thus, transport of cells from one vent site to another in cold oxic seawater apparently is not a problem.

TABLE 20.2 Chemolithotrophic *Bacteria* and *Archaea* present near deep-sea hydrothermal vents[a]

Chemolithotroph	Electron donor	Electron acceptor	Product from donor
Sulfur-oxidizing	HS^-, S^0, $S_2O_3^{2-}$	O_2, NO_3^-	S^0, SO_4^{2-}
Nitrifying	NH_4^+, NO_2^-	O_2	NO_2^-, NO_3^-
Sulfate-reducing	H_2	S^0, SO_4^{2-}	H_2S
Methanogenic	H_2	CO_2	CH_4
Hydrogen-oxidizing	H_2	O_2, NO_3^-	H_2O
Iron- and manganese-oxidizing	Fe^{2+}, Mn^{2+}	O_2	Fe^{3+}, Mn^{4+}
Methylotrophic	CH_4, CO	O_2	CO_2

[a]See Chapter 14 for detailed discussions of these metabolisms and Chapters 15–17 for further coverage of each group of organisms.

A Phylogenetic Snapshot of Hydrothermal Vent Prokaryotic Diversity

Using a variety of molecular methods (◀ Section 19.6–19.8), studies of the prokaryotic diversity present near volcanic hydrothermal vents have revealed an enormous diversity of *Bacteria*. These 16S rRNA gene sequence surveys include waters near both warm and hot vents. Hydrothermal vent microbial communities are dominated by *Proteobacteria*, in particular *Epsilonproteobacteria* (◀ Section 16.5; **Figure 20.47**). *Alpha-*, *Delta-*, and *Gammaproteobacteria* are also abundant, whereas *Betaproteobacteria* are much less so.

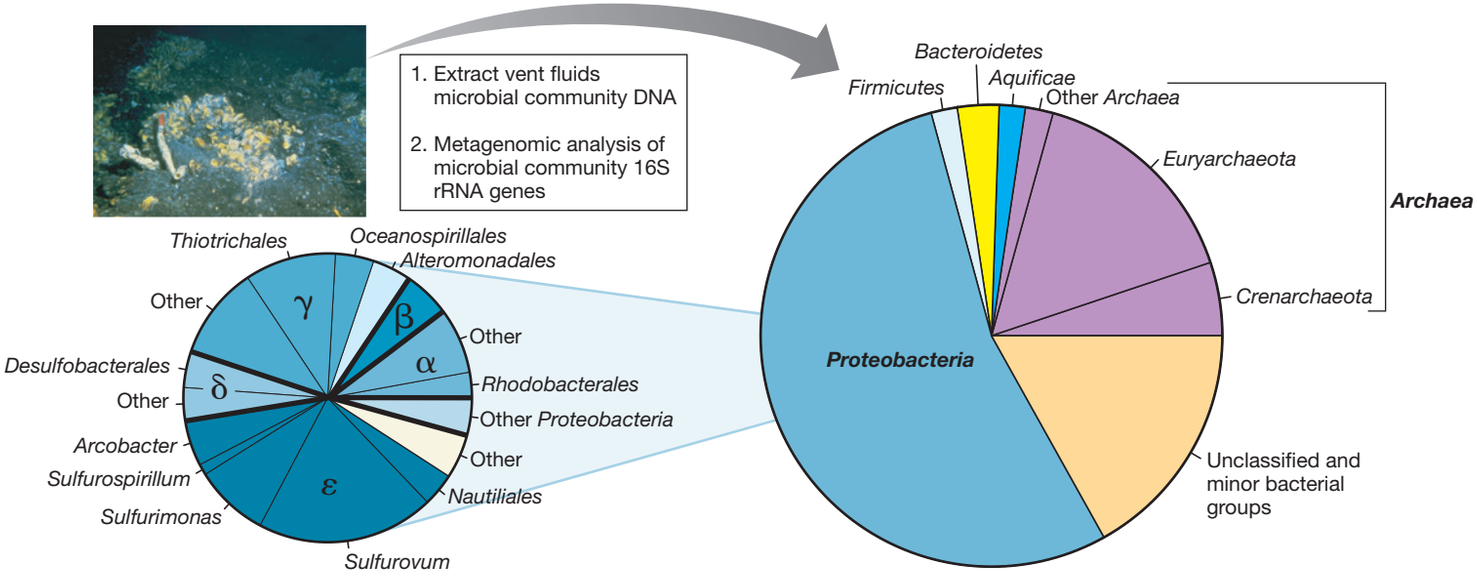


Figure 20.47 Hydrothermal vent bacterial and archaeal diversity. The results are pooled analyses from several studies of the 16S rRNA gene content of warm and hot hydrothermal vents. Many of these groups are covered in Chapters 15 and 16 (*Bacteria*) or 17 (*Archaea*). For *Proteobacteria*, major subgroups are indicated. Note the high proportion of *Archaea* and of *Epsilonproteobacteria*. The physiology of many of these organisms is summarized in Table 20.2. Most of the chemolithotrophs listed in Table 20.2 grow with CO_2 as carbon source (autotrophic growth). Data assembled and analyzed by Nicolas Pinel.

Many *Epsilon-* and *Gammaproteobacteria* oxidize sulfide and sulfur as electron donors with either O_2 or nitrate (NO_3^-) as electron acceptors (Chapter 16). As shown in the detailed diagram of *Proteobacteria* in Figure 20.47, vent *Epsilonproteobacteria* phylotypes most closely match those of chemolithotrophic sulfur bacteria such as *Sulfurimonas*, *Arcobacter*, *Sulfurovum*, and *Sulfurospirillum*. These bacteria oxidize reduced sulfur compounds as electron donors (◀ Sections 14.7 and 15.12), and such a physiology is consistent with their presence near vent fluids charged with sulfur and sulfide. In addition, most *Deltaproteobacteria* specialize in anaerobic metabolisms using oxidized sulfur compounds as electron acceptors.

In contrast to *Bacteria*, the diversity of volcanic hydrothermal vent *Archaea* is more limited. Estimates of the number of unique phylotypes indicate that the diversity of *Bacteria* near hydrothermal vents is about 10 times that of *Archaea*. However, *Archaea* prevail in samples recovered from the walls of hot vent chimneys (Figure 20.46). Most of the *Archaea* detected near hydrothermal vents are either methanogens (◀ Section 17.2) or species of marine *Crenarchaeota* and *Euryarchaeota* (Chapter 17). With the exception of the ammonia-oxidizing *Nitrosopumilus* (*Thaumarchaeota*, ◀ Section 17.5), organisms in these groups remain uncultured and their physiologies poorly understood.

As we close this chapter dedicated to microbial ecosystems, it should be clear that any environment capable of supporting microbial life will be colonized with one or more microorganisms. The microbes present in each environment are typically a reflection of those whose metabolisms are most compatible with the conditions and resources available. But whether an ecosystem is nutrient robust or nearly devoid of nutrients, microbial communities will develop, and populations within a community will rise or fall in response to the dynamics of their environment.

Check Your Understanding

- How does a warm hydrothermal vent differ from a black smoker, both chemically and physically?
- Why is 350°C water emitted from a black smoker not boiling?
- Which phylum of *Bacteria* and which subgroups of this phylum dominate hydrothermal vent ecosystems, and why?

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CHAPTER REVIEW

I • Microbial Ecology

20.1 Ecosystems consist of organisms, their environments, and all of the interactions among the organisms and environments. The organisms are members of populations and communities and are adapted to habitats. Species richness and abundance are aspects of species diversity in a community and an ecosystem.

Q Explain the difference between an ecosystem and a habitat. Why are some microbial habitats unsuitable for plant and animal life?

20.2 Microbial communities consist of guilds of metabolically similar organisms. Microorganisms play major roles in energy transformations and biogeochemical processes that result in the recycling of elements essential to living systems.

Q Describe two ways in which the diversity of microbial species in a community can be expressed.

II • The Microbial Environment

20.3 The niche for a microorganism consists of the specific assortment of biotic and abiotic factors within a microenvironment in which that microorganism can be competitive. Microorganisms in nature often live a feast-or-famine existence such that only the best-adapted species reach high population densities in a given niche. Cooperation among microorganisms is also important in many microbial interrelationships.

Q Why are extended periods of exponential microbial growth in nature rare and often slower than rates recorded in laboratory settings?

20.4 When surfaces are available, bacteria grow in attached masses of cells called biofilms. Biofilm formation confers several protective advantages on cells. Biofilms can have significant medical and economic effects on humans when unwanted biofilms develop on inert as well as living surfaces.

Q What property of biofilm microbial communities makes them clinically and industrially relevant? Why do biofilms possess this property? How can this cause problems in many instances?

20.5 Microbial mats can be phototrophic or chemolithotrophic. Phototrophic cyanobacterial mats are thick biofilms consisting of microbial cells and trapped particulate materials and are widespread in hypersaline or thermal waters. Mats of iron-oxidizing microorganisms are common in low-pH and metals-rich waters of geothermal springs and mining waste waters. Sulfur-oxidizing chemolithotrophs form extensive mat communities on marine sediment surfaces below the photic zone at the oxygen–sulfide interface.

Q What is the importance of gliding motility in the formation of marine chemolithotrophic mat systems?

III • Terrestrial Environments

20.6 Soils are complex microbial habitats consisting of sand, silt, and clay and contain numerous microenvironments and niches. Microorganisms are present in the soil primarily attached to soil particles or plant roots. The most important factors influencing microbial activity in soil are the availability of water and nutrients. However, in very arid soils microorganisms play important roles in stabilizing soil structure.

Q In what soil horizon are microbial numbers and activities the highest, and why?

20.7 *Proteobacteria*, *Acidobacteria*, and *Bacteroidetes* are the most diverse groups of *Bacteria* in soil because their metabolisms fit best with the available resources. Among *Archaea*, only *Euryarchaetoa* and *Thaumarchaeota* are commonly present in soils. Polluted soils contain different proportions of the same major groups of *Bacteria* but are also enriched in *Actinobacteria* and nearly devoid of *Thaumarchaeota*. Changes in soil microbial diversity due to pollution and climate change threaten global food supplies.

Q Based on their metabolism, why are *Thaumarchaeota* well suited to inhabit soils?

20.8 The deep subsurface is a significant microbial habitat that sustains chemolithotrophic populations whose resource demands are few and inorganic, such as CO_2, SO_4^{2-}, N_2, and H_2. Novel phyla of small-celled *Archaea* are present in the deep subsurface of both terrestrial and marine sediment environments. The discovery of the Asgard superphylum has provided new understanding of the origin of eukaryotes.

Q Studies of the deep subsurface have identified populations of chemoorganotrophs. What types and sources of nutrients are available to those organisms?

IV • Aquatic Environments

20.9 In freshwater aquatic ecosystems, phototrophic microorganisms are the main primary producers. Most of the organic matter produced is consumed by bacteria, which can lead to depletion of oxygen in the environment. The BOD of a body of water indicates its relative content of organic matter that can be biologically oxidized.

Q How and in what way does an input of organic matter, such as sewage, affect the oxygen content of a river or stream?

20.10 Pelagic marine waters are more nutrient deficient than most freshwaters, yet substantial numbers of microbes inhabit the oceans. However, in some highly productive and expansive oceanic regions, oxygen can be drawn down to low or undetectable levels at depths between 100 and 1000 m; oxygen-depleted waters at these depths are called oxygen minimum zones (OMZs). Bacteria specialized in the biodegradation of petroleum are enriched in regions of natural seepage of petroleum and natural gas.

Q How is climate change related to the ongoing increase in size of OMZs?

20.11 The major oxygenic microbial phototrophs in the open oceans are the cyanobacterium *Prochlorococcus* and the alga *Ostreococcus*. Marine anoxygenic phototrophs include *Roseobacter* and its relatives, the aerobic phototrophic purple bacteria. Unlike other anoxygenic phototrophs, aerobic purple bacteria carry out photosynthesis only under aerobic conditions and thus are well suited to open ocean redox conditions.

Q How has single-cell genomics contributed to new understanding of factors controlling the diversity of *Prochlorococcus* in the ocean?

20.12 Species of *Bacteria* tend to predominate in marine surface waters, whereas in deeper waters *Archaea* and *Bacteria* make up similar shares of the microbial community. Most pelagic *Bacteria* are specialized to grow at very low nutrient concentrations, and many found in the photic zone use light to make ATP by rhodopsin-driven proton pumps. *Thaumarchaeota* and *Euryarchaeota* are the major *Archaea* inhabiting the oceans.

Q Many pelagic *Bacteria* can use light energy but are not considered "phototrophs" in the same sense as cyanobacteria or purple bacteria. Explain.

20.13 Marine viruses far outnumber total cell numbers in the ocean, greatly impact marine food webs, and confer new properties to their hosts as gene transfer agents. Phage-encoded auxiliary metabolic genes typically function to subvert host metabolism for increased virus production.

Q Describe two types of viral auxiliary metabolic genes and how they benefit the virus.

20.14 The deep sea is a cold, dark habitat where hydrostatic pressure is high and nutrient levels are low. Piezophiles grow best under pressure but do not require pressure, whereas extreme piezophiles require high pressure, typically several hundred atmospheres, for growth. Ammonia-oxidizing *Archaea* are among the most abundant free-living microbes in the deepest regions of the ocean.

Q What property is shared by piezotolerant, piezophilic, and extremely piezophilic microorganisms?

20.15 Deep-sea sediments show decreasing levels of nutrients with depth, and thus the microbes that reside there experience constant near-starvation conditions. Although not yet cultured, novel phyla of *Archaea* inhabit deep marine sediments (as well as the terrestrial deep subsurface), and these organisms likely survive by scavenging the trace levels of both organic matter and electron acceptors present there.

Q What sources of organic matter in deep-sea sediments could be used to support growth?

20.16 Hydrothermal vents are deep-sea hot springs where either volcanic activity or unusual chemistry generates fluids containing large amounts of inorganic electron donors (such as sulfide, ammonia, and hydrogen) that can be used by various chemolithotrophic bacteria.

Q Would you expect to find the same types of microorganisms associated with black smoker and carbonate vent systems? Explain.

APPLICATION QUESTIONS

1. Imagine a sewage plant that is releasing sewage containing high levels of ammonia and phosphate and very low levels of organic carbon. Which types of microbial blooms might be triggered by this sewage? How would the graphs of oxygen near and beyond the plant's release point differ from the graph shown in Figure 20.19*a*?
2. Keeping in mind that the open-ocean waters are highly oxic, predict the possible metabolic lifestyles of open-ocean *Archaea* and *Bacteria*. How might rhodopsin-like pigments be more abundant in one group of organisms than in the other?
3. Warming associated with climate change has been suggested to result in reduced transfer of oxygen to deeper waters in the ocean. How might such warming also result in reduced nutrient availability to planktonic species in marine surface waters?

CHAPTER GLOSSARY

Auxiliary metabolic genes (AMGs) virus-encoded genes that alter host metabolism toward pathways that maximize production of new virus particles

Biochemical oxygen demand (BOD) the relative amount of dissolved oxygen consumed by microorganisms for complete oxidation of bioavailable organic and inorganic material in a water sample

Biofilm colonies of microbial cells encased in a porous organic matrix and attached to a surface

Biogeochemistry the study of biologically mediated chemical transformations in the environment

Community two or more cell populations coexisting in a certain area at a given time

Ecosystem a dynamic complex of organisms and their physical environment interacting as a functional unit

Epilimnion the warmer and less dense surface waters of a stratified lake

Extreme piezophile an organism requiring several hundred atmospheres of pressure for growth

Guild metabolically similar microbial populations that exploit the same resources in a similar way

Habitat a part of the ecosystem best suited to one or a few populations

Hydrothermal vent a deep-sea hot spring emitting warm (~20°C) to superheated (>300°C) water

Hypolimnion the colder, denser, and often anoxic bottom waters of a stratified lake

Microbial mat a thick, layered, diverse community nourished either by light in a hypersaline or an extremely hot aquatic environment, in which cyanobacteria are essential; or by chemolithotrophs growing on the surface of sulfide-rich marine sediments

Microenvironment a micrometer-scale space surrounding a microbial cell or group of cells

Niche in ecological theory, the biotic and abiotic characteristics of the microenvironment that contribute to an organism's competitive success

Oligotroph an organism that grows only or grows best at very low levels of nutrients

Oxygen minimum zone (OMZ) an oxygen-depleted region of intermediate depth in the marine water column

Piezophile an organism that grows best under a hydrostatic pressure greater than 1 atm

Piezotolerant able to grow under elevated hydrostatic pressures but growing best at 1 atm

Population a group of organisms of the same species in the same place at the same time

Primary producer an organism that synthesizes new organic material from CO_2 and obtains energy from light or from oxidation of inorganic compounds

Prochlorophyte a bacterial oxygenic phototroph that contains chlorophylls *a* and *b* but lacks phycobiliproteins

Proteorhodopsin a light-sensitive protein present in some pelagic *Bacteria* that fuels a proton pump that yields ATP

Rhizosphere the region immediately adjacent to plant roots

Species abundance the proportion of each species in a community

Species richness the total number of different species present in a community

Stratified water column a body of water separated into layers having distinct physical and chemical characteristics

Nutrient Cycles 21

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III Humans and Nutrient Cycling 746

MICROBIOLOGY**NOW**

An Uncertain Future for Coral Reef Ecosystems

Carbon dioxide (CO_2) released from the burning of fossil fuels is contributing to global warming and ocean acidification. Although all ecosystems will ultimately be impacted by this, coral reef ecosystems are particularly sensitive. About a quarter of the oceans' coral reefs have already been lost to thermal stress and pollution, and ongoing acidification is impairing their formation of calcium carbonate skeletons. Although occupying less than 1% of the ocean floor, coral reefs support about one-quarter of all marine species that provide food for millions of people. Thus it is essential that we quantify current rates of coral reef loss and predict potential future losses.

In an experimental study of adding CO_2 to ocean waters bathing a natural coral reef ecosystem, it was clear that even a minor reduction in pH greatly reduces the rate of biogenic calcification. This study, performed at a section of the Great Barrier Reef (Australia), used tidal flow from an upper to a lower lagoon to adjust the pH of water bathing the lower section of reef. A 4000-gallon floating tank of seawater was first acidified by bubbling with CO_2 and then pumped from the edge of the upper lagoon during tidal change, reducing the pH in the lower lagoon from ambient pH (8.13) to a treatment level (7.98). A nonreactive red dye was added before release to follow the dilution of the acidified plume as it mixed with seawater (photo).

In this experiment, calcification rates were quantified by measuring changes in alkalinity (due to carbonate production) over a one-hour period in the pH-adjusted water compared with seawater maintained at the original pH. Remarkably, net calcification of this natural reef community was reduced 34% by this relatively small change in pH, showing that these remarkable and very sensitive ecosystems are in jeopardy unless a rapid reduction in global CO_2 emissions is initiated.

Source: Rebecca Albright, Yuichiro Takeshita, and David A. Koweek, et al. 2018. Carbon dioxide addition to coral reef waters suppresses net community calcification. *Nature* 555: 516.

I • Carbon, Nitrogen, and Sulfur Cycles

The microbial cycling of carbon, nitrogen, and sulfur compounds provides essential nutrients for autotrophs. The organic matter these organisms produce is oxidized by heterotrophs and by human activities to CO_2, an excess of which contributes to the ongoing problem of global warming.

The key nutrients for life are cycled by both microorganisms and macroorganisms, but for each nutrient it is microbial activities that dominate. Understanding how microbial nutrient cycles work is important because the cycles and their many feedback loops are essential for plant agriculture and the overall health of a sustainable planet.

We begin our coverage of nutrient cycles with the carbon cycle. Major areas of interest here are the magnitude of carbon reservoirs on Earth, the rates of carbon cycling within and between reservoirs, and the coupling of the carbon cycle to other nutrient cycles. We emphasize the gases *carbon dioxide* (CO_2) and *methane* (CH_4) because they are major components of the carbon cycle and have major impacts on the global ecosystem. We return to the carbon cycle in Part III of this chapter to consider how human activities are affecting this critical nutrient cycle.

21.1 The Carbon Cycle

Mastering Microbiology
Art Activity: Figure 21.1 The carbon cycle

On a global basis, carbon (C) travels as CO_2 through all of Earth's major carbon reservoirs: the atmosphere, the land, the oceans, freshwaters, sediments and rocks, and biomass (**Figure 21.1**). As we have already seen for freshwater environments, the carbon and oxygen cycles are intimately linked (◀ Section 20.9). All nutrient cycles link in some way to the carbon cycle, but the nitrogen (N) cycle links particularly strongly because, other than water (H_2O), C and N make up the bulk of living organisms (◀ Section 4.1 and see Figure 21.6).

Carbon Reservoirs

By far the largest C reservoir on Earth is the sediments and rocks of Earth's crust (Figure 21.1), but the rate at which sediments and rocks decompose and carbon is removed as CO_2 is so slow that flux out of this reservoir is insignificant on a human time scale. A large amount of C is found in land plants. This is the organic C of forests, grasslands, and agricultural crops—the major sites of phototrophic CO_2 fixation. However, more C is present in dead organic material, called **humus**, than in living organisms. Humus is a complex mixture of organic materials that have resisted rapid decomposition and is derived primarily from dead plants and microorganisms. Some humic substances are quite recalcitrant, with a decomposition time of several decades, but certain other humic components decompose much more rapidly.

The most rapid means of transfer of C is via the atmosphere. Carbon dioxide is removed from the atmosphere primarily by photosynthesis of land plants and marine microorganisms and is returned to the atmosphere by respiration of animals and chemoorganotrophic microbes (Figure 21.1). The single most important contribution of CO_2 to the atmosphere is by microbial decomposition of dead organic material. However, since the Industrial Revolution, human activities have increased atmospheric CO_2 levels by nearly 40%, primarily from the combustion of fossil fuels. This rise in CO_2, a major *greenhouse gas*, has triggered global changes in climate, the most notable feature of which is steadily increasing global temperatures, a condition called **global warming** (see Section 21.9 and Figure 21.24). That global warming will affect microbial nutrient cycling is a virtual certainty because everything we know about the physiology of microorganisms tells us that microbial activities change in response to temperature. Whether these changes will be favorable or unfavorable to higher organisms (including humans) is a major area of active research today (Section 21.9).

Major Carbon Reservoirs on Earth

Reservoir	Percent of Total[a]
Rocks and sediments	99.5[b]
Oceans	0.05
Methane hydrates	0.014
Fossil fuels	0.006
Terrestrial biosphere	0.003
Aquatic biosphere	0.000002

[a]Total carbon, 76×10^{15} tons
[b]80% inorganic

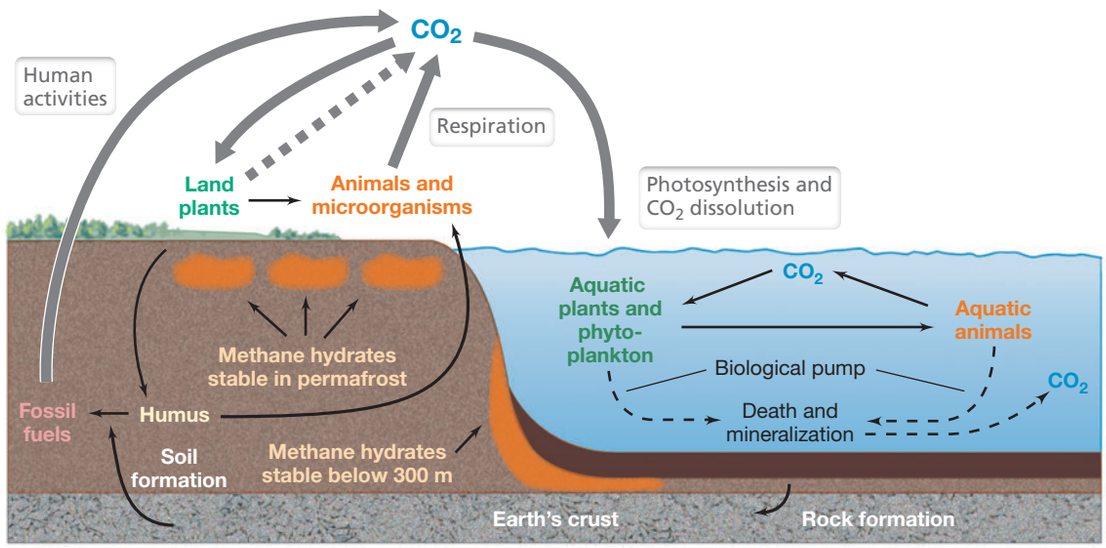


Figure 21.1 The carbon cycle. The carbon and oxygen cycles are closely connected, as oxygenic photosynthesis both removes CO_2 and produces O_2, and respiration both produces CO_2 and removes O_2. The accompanying table shows that the greatest reservoir of carbon on Earth is in rocks and sediments, and most of this is in inorganic form as carbonates.

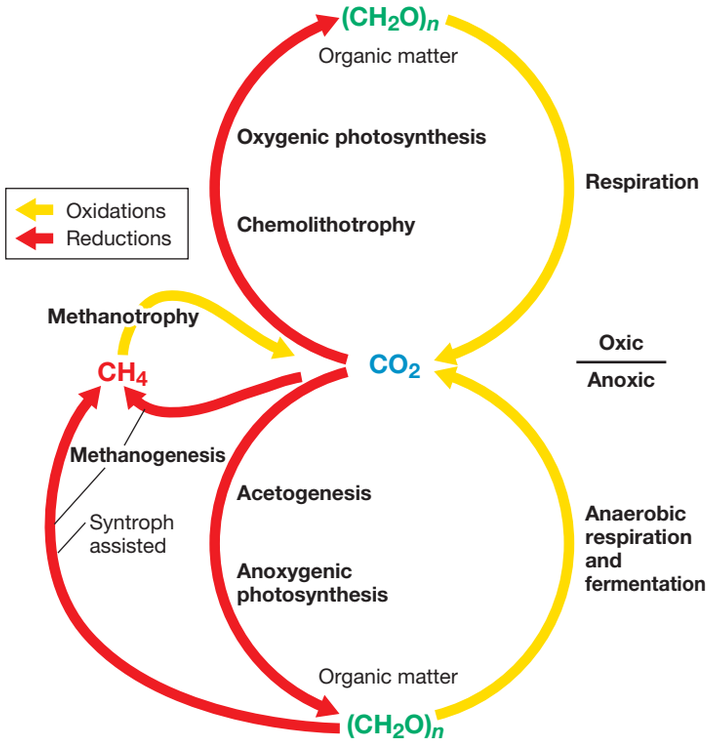


Figure 21.2 Redox cycle for carbon. The diagram contrasts autotrophic processes (CO_2 → organic compounds) and heterotrophic processes (organic compounds → CO_2).

Photosynthesis and Decomposition

New organic compounds are biologically synthesized on Earth only by CO_2 fixation by autotrophs—the phototrophs and chemolithotrophs. Most organic compounds originate in photosynthesis and thus phototrophic organisms are the foundation of the carbon cycle (Figure 21.1). However, phototrophic organisms are abundant in nature only in habitats where light is available. The deep sea, deep terrestrial subsurface, and other permanently dark habitats are devoid of indigenous phototrophs. There are two groups of oxygenic phototrophic organisms: *plants* and *microorganisms*. Plants are the dominant phototrophic organisms of terrestrial environments, whereas phototrophic microorganisms dominate in aquatic environments.

The redox cycle for C (**Figure 21.2**) begins with photosynthetic CO_2 fixation, driven by the energy of light (◀ Sections 3.11, 3.12, 14.5, and 14.6):

$$CO_2 + H_2O \rightarrow (CH_2O) + O_2$$

CH_2O represents organic matter at the oxidation–reduction level of cell material. Phototrophic organisms also carry out respiration, both in the light and the dark. The overall equation for respiration is the reverse of oxygenic photosynthesis:

$$(CH_2O) + O_2 \rightarrow CO_2 + H_2O$$

For organic matter to accumulate, the rate of photosynthesis must exceed the rate of respiration. In this way, autotrophic organisms build biomass from CO_2, and then this biomass in one way or another supplies the C heterotrophic organisms need. Anoxygenic phototrophs and chemolithotrophs also produce excess organic compounds, but in most environments the contributions of these organisms to the net accumulation of organic matter are minor compared to the inputs of oxygenic phototrophs. This is because the reductant used by oxygenic phototrophs, H_2O (◀ Section 14.3), is in virtually unlimited supply.

Organic compounds are degraded biologically to CH_4 and CO_2 (Figure 21.2). Carbon dioxide, most of which is of microbial origin, is produced by aerobic and anaerobic respirations (◀ Sections 3.8 and 3.9). Methane is produced in anoxic environments by *methanogens* from the reduction of CO_2 with hydrogen (H_2) or from the splitting of acetate into CH_4 and CO_2 (◀ Section 14.15). However, any naturally occurring organic compound can eventually be converted to CH_4 from the cooperative activities of methanogens and various fermentative bacteria, as we will see in Section 21.2. Methane produced in anoxic habitats is insoluble and most often diffuses rapidly to oxic environments where it is either released to the atmosphere or oxidized to CO_2 by *methanotrophs* (◀ Sections 14.16 and 15.15; Figure 21.2). Hence, most of the C in organic compounds eventually returns to CO_2, and the links in the carbon cycle are closed.

Methane Hydrates

Although present in the atmosphere at lower levels than CO_2, CH_4 is a potent greenhouse gas that is over 20 times more effective in trapping heat than is CO_2. Some CH_4 enters the atmosphere from methanogenic production, but not all biologically produced CH_4 is immediately consumed or released to the atmosphere. Huge amounts of CH_4 derived primarily from past microbial activities are trapped underground or under marine sediments as *methane hydrates* (Figure 21.1 and **Figure 21.3**), molecules of frozen CH_4. Methane hydrates form when sufficient CH_4 is present in environments of high pressure and low temperature, such as beneath the permafrost in the Arctic and in marine sediments (Figure 21.1). These deposits can be up to several hundred meters thick and are estimated to

Figure 21.3 Burning methane hydrate. Frozen methane ice retrieved from marine sediments is ignited.

UNIT 5

Mastering Microbiology
Art Activity: Figure 21.2 Redox cycle for carbon

contain 700–10,000 petagrams (1 petagram × 10^{15} g) of CH_4. This exceeds other known CH_4 reserves on Earth by several orders of magnitude.

Methane hydrates are highly dynamic, absorbing and releasing CH_4 in response to changes in pressure, temperature (**Figure 21.4**), and fluid movement. Methane hydrates also fuel deep-water ecosystems, called either *cold seeps* or *methane seeps* (◀ Figure 20.24). Here, the slow release of CH_4 from seafloor hydrates nourishes not only anaerobic methane-oxidizing *Archaea* (◀ Section 14.16), but also animal communities that contain aerobic methane-oxidizing endosymbionts that oxidize CH_4 and release organic matter to the animals (▶ Section 23.11). Anaerobic oxidation of CH_4 is coupled to the reduction of sulfate (SO_4^{2-}), nitrate (NO_3^-), and iron and manganese oxides, reducing the amount of free methane released. Hydrogen sulfide (H_2S), produced by the oxidation of methane coupled to sulfate reduction (◀ Section 14.16), can nourish dense microbial mats of aerobic filamentous sulfur-oxidizing bacteria (◀ Section 14.7) on the seafloor overlaying methane hydrates (**Figure 21.5**; see also Section 20.5 and Figure 20.10).

Although deep oceanic methane hydrates are stabilized by high pressure, hydrates in shallower coastal sediments are much more sensitive to small changes in temperature (Figure 21.4). Hydrates in shallow sediments are at the margin of what is called the *gas hydrate stability zone* (GHSZ). In these marginal regions, relatively small changes in temperature can destabilize the hydrates. For example, on-site monitoring of hydrate deposits in a marine coastal plain showed them to be sensitive to 1–2°C seasonal changes in bottom-water temperature. During periods of seasonally elevated water temperature, methane bubbles freely (a phenomenon called methane flares) from the sediments (Figure 21.4). In addition to the release of methane from marine and terrestrial hydrates, as permafrost melts, its huge reserve of organic matter could be catabolized by microbes, leading eventually to the production of yet more methane (Section 21.9).

Carbon Balances and Coupled Cycles

Although it is convenient to consider carbon cycling as a series of reactions separate from those in other nutrient cycles, in reality, all nutrient cycles are *coupled cycles*; major changes in one cycle affect the functioning of others. But certain cycles, such as the carbon and nitrogen cycles (**Figure 21.6**), are extremely closely coupled because of the large amount of C and N in living organisms. The rate of primary productivity (CO_2 fixation) is controlled by several factors, in particular by the magnitude of photosynthetic biomass and by available N, often a limiting nutrient. Thus, large-scale reductions in biomass, for instance by widespread deforestation, reduce rates of primary productivity and increase levels of CO_2. High levels of organic C stimulate microbial nitrogen fixation ($N_2 \rightarrow NH_3$, ◀ Section 3.12), and this in turn adds more fixed N to the pool for primary producers; low levels of organic C have just the opposite effect (Figure 21.6). High levels of ammonia (NH_3) stimulate primary production and nitrification (◀ Section 14.9), but inhibit nitrogen fixation. High levels of nitrate (NO_3^-), an excellent N source for plants and aquatic phototrophs, stimulate primary production but also increase the rate of denitrification (◀ Section 14.11); the latter removes fixed forms of N from the environment and feeds back in a negative way on primary production (Figure 21.6).

This simple example illustrates how nutrient cycles are anything but isolated entities. Instead, they are coupled systems that must maintain a delicate balance of inputs and outputs. Thus, one could expect the C and N cycles to respond to large inputs in specific components (for example, through inputs of CO_2 or nitrogen fertilizers) in ways that are not always beneficial to the biosphere and that can have unintended consequences for all life, including humans (Section 21.9).

UNIT 5

Mastering Microbiology
Art Activity: Figure 21.5 Coupled cycles

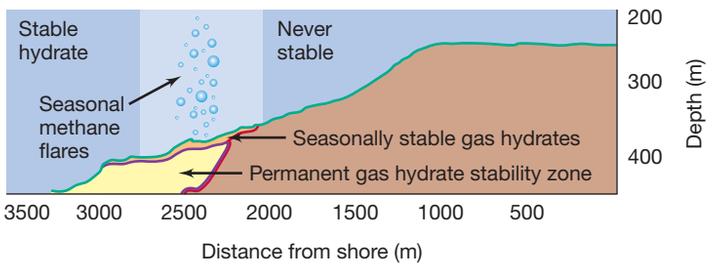


Figure 21.4 Seasonal flares of methane bubbling from methane hydrates. Methane hydrates in shallow coastal sediments are sensitive to seasonal changes in bottom water temperature. Flares of methane bubbles are observed when water temperatures warm by as little as 1–2°C.

Figure 21.5 Sulfur-oxidizing microbial mats overlying a deep-sea methane seep. This seep is located at 800 m depth in the Pacific Ocean, off Los Angeles, California. The oxidation of methane coupled to sulfate reduction in the subsurface produces abundant H_2S, which feeds a sediment surface community of filamentous sulfide-oxidizing bacteria. The bacteria are filled with inclusions of elemental sulfur (S^0), leading to the whitish color. The dark area near the bottom center is about 0.5 m long.

Check Your Understanding

- How is new organic matter made in nature?
- In what ways are oxygenic photosynthesis and respiration related?
- Why is microbially produced methane typically formed only in anoxic environments?

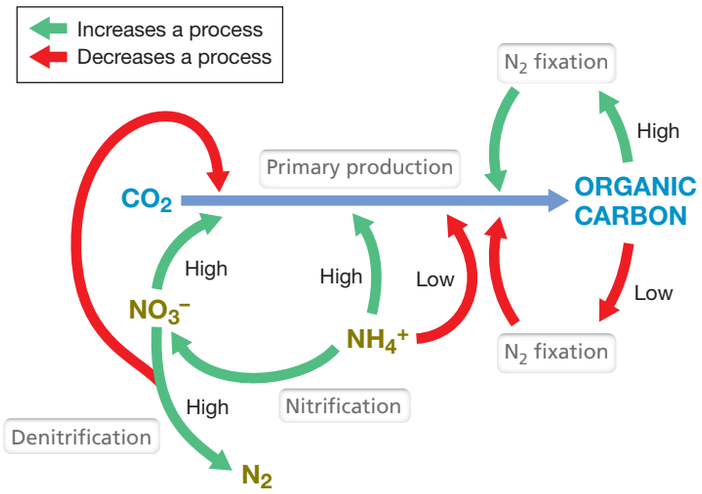


Figure 21.6 Coupled cycles. All nutrient cycles are interconnected, but the carbon and nitrogen cycles are intimately coupled. In the carbon cycle, CO_2 supplies the C for carbon compounds. The N cycle, shown in more detail in Figure 21.9, supplies N for many biological compounds.

21.2 Syntrophy and Methanogenesis

Most organic compounds are oxidized in nature by *aerobic* microbial processes. However, because oxygen (O_2) is a poorly soluble gas and is actively consumed when available, much organic carbon still ends up in anoxic environments. Methanogenesis, the biological production of CH_4, is a major process in anoxic habitats and is catalyzed by a large group of *Archaea*, the *methanogens*, which are strict anaerobes. We discussed the biochemistry of methanogenesis in Section 14.15 and other aspects of methanogens in Section 17.2.

Most methanogens can use CO_2 as a terminal electron acceptor in anaerobic respiration, reducing it to CH_4 with H_2 as electron donor. Only a very few other substrates, chiefly acetate, are directly converted to CH_4 by methanogens (◀ Section 14.15). To convert most organic compounds to CH_4, methanogens must team up with partner organisms called *syntrophs* that function to supply them with precursors for methanogenesis.

Anoxic Decomposition and Syntrophy

In Section 14.22 we discussed the biochemistry of **syntrophy**, a process in which two or more organisms cooperate in the anaerobic degradation of organic compounds. Here we consider the interactions of syntrophic bacteria with their partner organisms and their significance for the carbon cycle. Our focus will be anoxic freshwater sediments and anoxic wastewater treatment, both of which are major sources of CH_4.

Polysaccharides, proteins, lipids, and nucleic acids from dead organisms find their way into anoxic habitats, where they are catabolized. The monomers released by hydrolysis of these polymers become major electron donors for energy metabolism. For the breakdown of a typical polysaccharide such as cellulose, the process begins with *cellulolytic* bacteria (**Figure 21.7**). These organisms hydrolyze cellulose into glucose, which is catabolized by fermentative organisms to short-chain fatty acids (acetate, propionate, and butyrate), alcohols such as ethanol and butanol, and the gases H_2 and CO_2. Hydrogen (H_2) and acetate are consumed by methanogens directly, but the bulk of the carbon remains in the form of fatty acids and alcohols; these cannot be directly catabolized by methanogens and require the activities of syntrophic bacteria (◀ Section 14.22; Figure 21.7).

Syntrophic bacteria are called *secondary fermenters* because they ferment the products of the primary fermenters, yielding H_2, CO_2, and acetate as products. For example, *Syntrophomonas wolfei* oxidizes C_4 to C_8 fatty acids, yielding acetate, CO_2 (if the fatty acid was C_5 or C_7), and H_2 (**Table 21.1** and Figure 21.7). Other species of *Syntrophomonas* use fatty acids up to C_{18} in length, including some unsaturated fatty acids. *Syntrophobacter wolinii* specializes in propionate (C_3) fermentation, generating acetate, CO_2, and H_2, and *Syntrophus gentianae* degrades aromatic compounds such as benzoate to acetate, H_2, and CO_2 (Table 21.1).

Despite rather extensive metabolic diversity, syntrophs are unable to carry out any of these reactions in pure culture. Instead, they depend on a H_2-consuming partner organism because of the

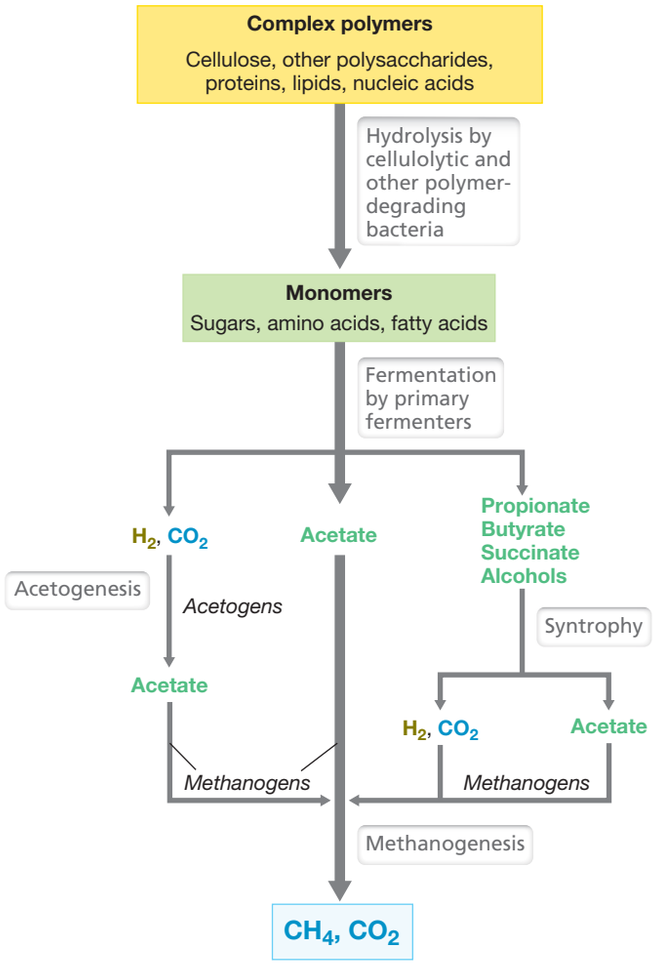


Figure 21.7 Anoxic decomposition. In anoxic decomposition, various groups of fermentative anaerobes cooperate in the conversion of complex organic materials to CH_4 and CO_2. This pattern holds for environments in which sulfate-reducing bacteria play only a minor role; for example, in freshwater lake sediments, sewage sludge, bioreactors, and the rumen.

Mastering Microbiology
Art Activity: Figure 21.6 Anoxic decomposition

UNIT 5

TABLE 21.1 Major reactions in the anoxic conversion of organic compounds to methane[a]

Reaction type	Reaction	Free energy change (kJ/reaction) $\Delta G^{0\prime}$[b]	Free energy change (kJ/reaction) ΔG[c]
Fermentation of glucose to acetate, H_2, and CO_2[d]	Glucose + 4 H_2O → 2 acetate$^-$ + 2 HCO_3^- + 4 H^+ + 4 H_2	−207	−319
Fermentation of glucose to butyrate, CO_2, and H_2	Glucose + 2 H_2O → butyrate$^-$ + 2 HCO_3^- + 2 H_2 + 3 H^+	−135	−284
Fermentation of butyrate to acetate and H_2	Butyrate$^-$ + 2 H_2O → 2 acetate$^-$ + H^+ + 2 H_2	+48.2	−17.6
Fermentation of propionate to acetate, CO_2, and H_2	Propionate$^-$ + 3 H_2O → acetate$^-$ + HCO_3^- + H^+ + H_2	+76.2	−5.5
Fermentation of ethanol to acetate and H_2	2 Ethanol + 2 H_2O → 2 acetate$^-$ + 4 H_2 + 2 H^+	+19.4	−37
Fermentation of benzoate to acetate, CO_2, and H_2	Benzoate$^-$ + 7 H_2O → 3 acetate$^-$ + 3 H^+ + HCO_3^- + 3 H_2	+70.1	−18
Methanogenesis from H_2 + CO_2	4 H_2 + HCO_3^- + H^+ → CH_4 + 3 H_2O	−136	−3.2
Methanogenesis from acetate	Acetate$^-$ + H_2O → CH_4 + HCO_3^-	−31	−24.7
Acetogenesis from H_2 + CO_2	4 H_2 + 2 HCO_3^- + H^+ → acetate$^-$ + 4 H_2O	−105	−7.1

[a]Data adapted from Zinder, S. 1984. Microbiology of anaerobic conversion of organic wastes to methane: recent developments. *Am. Soc. Microbiol. News 50*: 294.
[b]Standard conditions: solutes, 1 M; gases, 1 atm; 25°C.
[c]Concentrations of reactants in typical anoxic freshwater ecosystems: fatty acids, 1 mM; HCO_3^-, 20 mM; glucose, 10 μM; CH_4, 0.6 atm; H_2, 10^{-4} atm. Data from G_f^0 values in Table 3.3 and calculations described in Figure 3.7 and Section 3.3.
[d]Carbonic acid is formed by hydration of CO_2 dissolved in water. Since the deprotonated form of this acid is the major species at near neutral pH, HCO_3^- is commonly used in many microbial reactions involving CO_2 as a reactant or product.

unusual bioenergetics linked to the syntrophic process. As described in Section 14.22, H_2 consumption by a partner organism is absolutely essential for growth of syntrophic bacteria (in the absence of other electron acceptors), and the association of H_2 producer and H_2 consumer can be very intimate. In fact, H_2 transfer in some syntrophic associations may not be directly via H_2 transfer but through *direct conduction*, where electrons are transferred between species using electrically conductive wire-like structures (Section 21.5; ◀ Section 14.13 and Figure 14.31). But no matter the mechanisms, it is the transfer of H_2 (or electrons) that makes the syntrophic association work. How is this so?

When the reactions listed in Table 21.1 for the fermentation of butyrate, propionate, ethanol, or benzoate are written with all reactants at standard conditions (solutes, 1 M; gases, 1 atm; 25°C), the reactions yield free-energy changes ($\Delta G^{0\prime}$, ◀ Section 3.3) that are positive in arithmetic sign; that is, the reactions *require* rather than *release* energy. But the consumption of H_2 dramatically affects the energetics, making the reaction favorable and allowing energy to be conserved. This can be seen in Table 21.1, where the ΔG values (free-energy change measured under *actual conditions* in the habitat) are negative in arithmetic sign if H_2 concentrations are kept near zero by a H_2-consuming partner organism. This allows the syntrophic bacterium to conserve a small amount of energy that is used to produce ATP.

The final products of the syntrophic partnership are CO_2 and CH_4 (Figure 21.7), and any naturally occurring organic compound that enters a methanogenic habitat will eventually be converted to these products. This includes even complex aromatic and aliphatic hydrocarbons. Additional organisms other than those shown in Figure 21.7 may participate in such degradations, but eventually fatty acids and alcohols will be generated, and they will be converted into methanogenic substrates by syntrophs. Acetate produced by syntrophs (as well as by the activities of acetogenic bacteria, Figure 21.7 and ◀ Section 14.14) is a direct methanogenic substrate and is converted to CO_2 and CH_4 by various methanogens.

Methanogenic Symbionts and Acetogens in Termites

A variety of anaerobic protists that thrive under strictly anoxic conditions, including ciliates and flagellates (◀ Section 18.4), play a major role in the carbon cycle. Methanogenic *Archaea* live within some of these protist cells as H_2-consuming endosymbionts. For example, methanogens are present *within* cells of trichomonal protists inhabiting the termite hindgut (**Figure 21.8**) where methanogenesis and acetogenesis are major metabolic processes. Methanogenic symbionts of protists are species of the genera *Methanobacterium* or *Methanobrevibacter* (◀ Section 17.2 and Figure 17.5).

In the termite hindgut, endosymbiotic methanogens along with acetogenic bacteria are thought to benefit their protist hosts by consuming H_2 generated from glucose fermentation by cellulolytic protists. The acetogens are not endosymbionts but instead reside in the termite hindgut itself, consuming H_2 from primary fermenters and reducing CO_2 to make acetate. Unlike methanogens, acetogens can ferment glucose directly to acetate. Acetogens can also ferment methoxylated aromatic compounds to acetate. This is especially important in the termite hindgut because termites live on wood, which contains lignin, a complex polymer of methoxylated aromatic compounds (Figure 21.8*b*). The acetate produced by acetogens in the termite hindgut is consumed by the insect as its primary carbon and energy source.Microbial symbioses in the termite hindgut are discussed in more detail in Section 23.9.

Check Your Understanding

- Why does *Syntrophomonas* need a partner organism in order to ferment fatty acids or alcohols?
- To what extent does biogas formation depend on syntrophy?
- What substrates are used by acetogens to produce acetate?

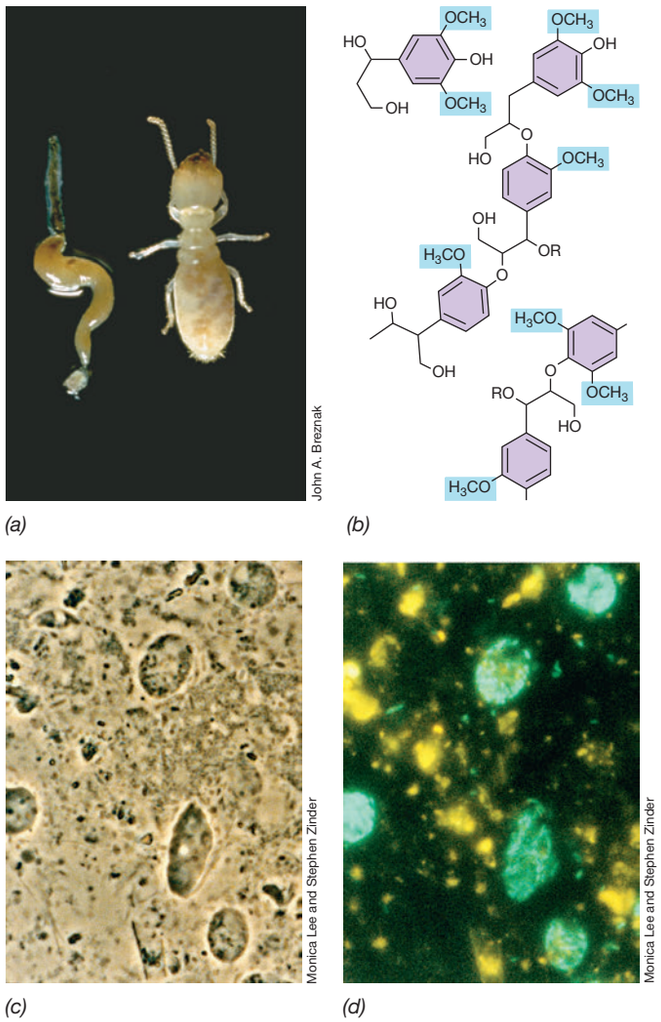


Figure 21.8 Termites and their carbon metabolism. *(a)* A subterranean termite worker larva shown to the right of a hindgut dissected from another worker. The animal is about 0.5 cm long. *(b)* Partial structure of lignin, a component of wood. The methyoxy groups (highlighted in blue) are substrates for acetogenesis. Two views of the same microscopic field show termite hindgut protists photographed by *(c)* phase-contrast and *(d)* epifluorescence. Endosymbiotic methanogens in the protist cells fluoresce blue-green because of the methanogenic coenzyme F_{420} (compare with ◀ Figure 14.37). The average diameter of the protist cells is 15–20 μm.

21.3 The Nitrogen Cycle

Nitrogen is an essential element for life (◀ Section 3.1) and exists in a number of oxidation states that are cycled by specific redox reactions. We have discussed four major microbial N transformations thus far: nitrification, denitrification, anammox, and nitrogen fixation (Chapter 14). These and other key N transformations are summarized in the redox cycle shown in **Figure 21.9**.

Nitrogen Fixation and Denitrification

Nitrogen gas (N_2) is the most stable form of N and is a major reservoir for N on Earth. However, only a relatively small number of *Bacteria* and *Archaea* are able to use N_2 as a cellular N source by the process of *nitrogen fixation* ($N_2 + 8\,H \rightarrow 2\,NH_3 + H_2$) (◀ Section 3.12). The N recycled on Earth is mostly already "fixed N"; that is, N in combination with other elements, such as in ammonia (NH_3) or nitrate (NO_3^-). In many environments, however, the short supply of fixed N puts a premium on biological nitrogen fixation, and in these habitats, nitrogen-fixing bacteria flourish.

We discussed the role of NO_3^- as an alternative electron acceptor in anaerobic respirationin Section 14.11. Under most conditions, the end product of NO_3^- reduction is N_2, nitric oxide (NO), or nitrous oxide (N_2O). The reduction of NO_3^- to these gaseous N compounds, called **denitrification** (Figure 21.9), is the main means by which N_2 and N_2O are formed biologically. Denitrification can be either beneficial or harmful. On the one hand, denitrification is a detrimental process. For example, if agricultural fields fertilized with NO_3^- fertilizer become waterlogged following heavy rains, anoxic conditions can develop and denitrification can be extensive; this removes fixed N from the soil. On the other hand, denitrification can aid in wastewater treatment (▶ Section 22.7). By converting NO_3^- in wastewater to volatile forms of N, denitrification minimizes the load of fixed N in discharge waters that triggers algal growth and loss of water quality (◀ Figure 20.19).

The production of N_2O and NO by denitrification can have other environmental consequences. Nitrous oxide can be photochemically oxidized to NO in the atmosphere. Nitric oxide reacts with ozone (O_3) in the upper atmosphere to form nitrogen dioxide (NO_2), which is further oxidized to form nitric acid (HNO_3) that returns to Earth as acid rain. In addition, N_2O is a very potent greenhouse gas. Although N_2O molecules persist on average only about 100 years because of their reactivity, on a per weight basis, the contribution of N_2O to global warming is about 300 times that of CO_2. Thus, denitrification contributes to global warming; to O_3 destruction, which increases passage of ultraviolet radiation to the surface of Earth; and to acid rain, which increases acidity of soils. Increases in soil acidity change microbial community structure and function and, ultimately, soil fertility, impacting both plant diversity and agricultural yields of crop plants.

Ammonification and Ammonia Fluxes

Ammonia is released during the decomposition of organic N compounds such as amino acids and nucleotides, a process called *ammonification* (Figure 21.9). Another process contributing to the generation of NH_3 is the respiratory reduction of NO_3^- to NH_3, called *dissimilative nitrate reduction to ammonia* (DNRA, Figure 21.9). DNRA dominates NO_3^- and nitrite (NO_2^-) reduction in electron donor–rich anoxic environments, such as highly organic marine sediments and the human gastrointestinal tract. Nitrate-reducing bacteria exploit this pathway primarily when NO_3^- is limiting because DNRA consumes eight electrons per NO_3^- reduced compared with the four and five electrons consumed when NO_3^- is reduced to N_2O or N_2, respectively.

At neutral pH, NH_3 exists as ammonium (NH_4^+). Much of the NH_4^+ released by aerobic decomposition in soils is rapidly recycled and converted to amino acids in plants and microorganisms. However, because NH_3 is volatile, some of it can be lost from alkaline soils by vaporization, and major losses of NH_3 to the atmosphere

Key Processes and Microbes in the Nitrogen Cycle

Processes	Example organisms
Nitrification ($NH_4^+ \rightarrow NO_3^-$)	
$NH_4^+ \rightarrow NO_3^-$	Comammox (*Nitrospira* species)
$NH_4^+ \rightarrow NO_2^-$	*Nitrosomonas, Nitrosopumilus* (*Archaea*)
$NO_2^- \rightarrow NO_3^-$	*Nitrobacter*
Denitrification ($NO_3^- \rightarrow N_2$)	*Bacillus, Paracoccus, Pseudomonas*
N_2 Fixation ($N_2 + 8\,H \rightarrow 2\,NH_3 + H_2$)	
Free-living	
Aerobic	*Azotobacter*
	Cyanobacteria
Anaerobic	*Clostridium*, purple and green phototrophic bacteria
	Methanobacterium (Archaea)
Symbiotic	*Rhizobium*
	Bradyrhizobium
	Frankia
Ammonification (organic-N $\rightarrow NH_4^+$)	
	Many organisms can do this
Anammox ($NO_2^- + NH_3 \rightarrow N_2$)	*Brocadia*

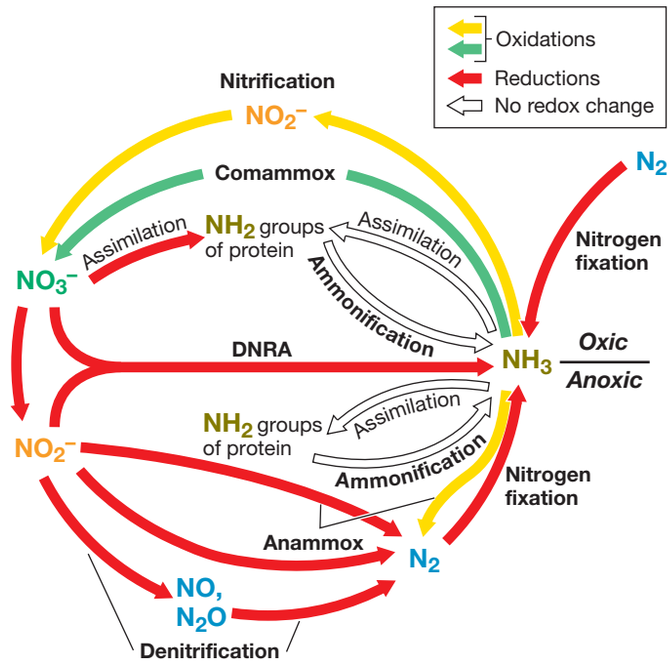


Figure 21.9 Redox cycle for nitrogen. The complete anammox reaction is $NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O$ (◀ Figure 14.26). DNRA, dissimilative nitrate reduction to ammonia.

occur in areas with dense animal populations (for example, cattle feedlots). On a global basis, however, NH_3 constitutes only about 15% of the N released to the atmosphere, the rest being primarily N_2 or N_2O from denitrification.

Nitrification

Nitrification, the oxidation of NH_3 to NO_3^-, is a major process in well-drained oxic soils at neutral pH and is carried out by the nitrifying *Bacteria* and *Archaea* (Figure 21.9). Whereas denitrification *consumes* NO_3^-, nitrification *produces* NO_3^-. If materials high in NH_3, such as manure or sewage, are added to soils, the rate of nitrification increases.

Nitrification was long considered an obligatory two-step process in which some species oxidize NH_3 to NO_2^- and then other species oxidize NO_2^- to NO_3^-. Many species of both *Bacteria* and *Archaea* can oxidize NH_3 (◀ Sections 14.9, 15.10, 17.5), whereas only species of *Bacteria* are known that oxidize NO_2^-. However, some *Bacteria*, such as certain *Nitrospira* species, can oxidize ammonia completely to nitrate; these have been called *comammox* (for *com*plete *amm*onia *ox*idizer) bacteria and seem to be especially important nitrifiers in freshwater lakes and rivers, drinking water systems, coastal sediments, and paddy soils.

Although NO_3^- is readily assimilated by plants, it is soluble and therefore rapidly leached (or denitrified) from waterlogged soils; consequently, nitrification is not beneficial for plant agriculture. Ammonium, on the other hand, is positively charged and strongly adsorbed to negatively charged soils. Anhydrous NH_3 is therefore used extensively as an agricultural fertilizer, but to prevent its conversion to NO_3^- by nitrification, chemicals are added to the NH_3 to inhibit the process. One common inhibitor is a pyridine compound called *nitrapyrin* (2-chloro-6-trichloromethylpyridine). Nitrapyrin specifically inhibits the *first* step in nitrification, the oxidation of NH_3 to NO_2^-. However, this effectively halts both steps in nitrification because the second step, $NO_2^- \rightarrow NO_3^-$, depends on the first (◀ Section 14.9). The addition of nitrapyrin to anhydrous NH_3 has greatly increased the efficiency of crop fertilization and has helped prevent pollution of waterways by NO_3^- leached from nitrified soils.

Anammox

In addition to oxygen-dependent nitrification, ammonia can be oxidized under anoxic conditions in a process called *anammox* (◀ Section 14.10). Anammox bacteria affiliate with five genera (*Brocadia, Kuenenia, Scalindua, Anammoxoglobus*, and *Jettenia*) in a single phylogenetically cohesive family (*Brocadiaceae*) within the *Planctomycetes* (◀ Section 16.16). Since anammox bacteria have yet to be isolated in pure culture, the genus and species names are prefaced by the term *Candidatus* to indicate their tentative taxonomic status (◀ Section 13.12).

In the anammox reaction, NH_3 is oxidized anaerobically with NO_2^- as the electron acceptor, forming N_2 as the final product (Figure 21.9; ◀ Figure 14.26*c*), which is released to the atmosphere. Although a major process in sewage and in anoxic marine basins and sediments, anammox is not a significant process in well-drained (oxic) soils. However, in wastewater processing plants (▶ Section 22.8 and Figure 22.22), anammox is highly beneficial because like denitrification, it removes fixed nitrogen from sewage and reduces the ability of wastewater effluents to trigger algal and other microbial blooms (◀ Figure 20.19*a*).

Check Your Understanding

- What is nitrogen fixation and why is it important to the nitrogen cycle?
- How do the processes of nitrification and denitrification differ? How do nitrification and anammox differ?
- How does denitrification impact arable land?

21.4 The Sulfur Cycle

Microbial transformations of sulfur (S) are more complex than those of N because of the large number of oxidation states of S and the fact that several transformations of S also occur spontaneously (abiotically). The major reactions of the sulfur cycle—sulfide oxidation and sulfate (SO_4^{2-}) reduction—were covered in Sections 14.7, 14.12, 15.11, and 15.12. The redox cycle for microbial sulfur transformations is shown in **Figure 21.10**.

Although a number of oxidation states of S are possible, only three are significant in nature: −2 (sulfhydryl, R–SH; and sulfide, HS^-), 0 (elemental sulfur, S^0), and +6 (sulfate, SO_4^{2-}). The bulk of S on Earth resides in sediments and rocks in the form of sulfate minerals, primarily gypsum ($CaSO_4$), and sulfide minerals (pyrite, FeS_2), but the oceans constitute the most significant reservoir of SO_4^{2-} in the biosphere. A significant amount of S, in particular sulfur dioxide (SO_2, a gas), enters the S cycle from human activities, primarily from the combustion of fossil fuels, especially coal.

Hydrogen Sulfide and Sulfate Reduction

Hydrogen sulfide (H_2S) is a major volatile sulfur compound. Hydrogen sulfide is produced from bacterial sulfate reduction ($SO_4^{2-} + 4\,H_2 \rightarrow H_2S + 2\,H_2O + 2\,OH^-$) (Figure 21.10) or emitted from sulfide springs and volcanoes. Although H_2S is volatile, different forms exist depending on pH: H_2S predominates below pH 7, and the nonvolatile HS^- and S^{2-} predominate above pH 7. Collectively, H_2S, HS^-, and S^{2-} are referred to as "sulfide."

Sulfate-reducing bacteria are a large and highly diverse group (◀ Sections 14.12 and 15.11) and are widespread in nature. However, in anoxic habitats such as freshwater sediments and many soils, sulfate reduction is limited by SO_4^{2-} availability. Moreover, because organic electron donors (or H_2, which is a product of the fermentation of organic compounds) are needed to support sulfate reduction, it only occurs where significant amounts of organic material are present.

In marine sediments, the rate of sulfate reduction is typically carbon-limited and can be greatly increased by an influx of organic matter. This is important because the disposal of sewage or garbage in the oceans or coastal regions can trigger sulfate reduction. Hydrogen sulfide is toxic to many plants and animals and therefore its formation is potentially detrimental; sulfide is toxic because it combines with the iron of cytochromes and blocks respiration. Sulfide is commonly detoxified in nature by combination with iron, forming the insoluble minerals FeS (pyrrhotite) and FeS_2 (pyrite). The black color of sulfidic sediments or sulfate-reducing bacterial cultures (◀ Figure 15.28*g*) is due to these metal sulfide minerals.

Sulfide and Elemental Sulfur Oxidation–Reduction

Under oxic conditions, sulfide rapidly oxidizes spontaneously at neutral pH. Sulfur-oxidizing chemolithotrophic bacteria, most of which are aerobes (◀ Sections 14.7 and 15.12), can catalyze the oxidation of sulfide. However, because of the rather rapid spontaneous reaction, microbial sulfide oxidation is significant only in areas where H_2S emerging from anoxic environments meets the atmosphere. Where light is available, there can be anoxic oxidation of sulfide, catalyzed by the phototrophic purple and green sulfur bacteria (◀ Sections 14.5, 15.4, and 15.6).

UNIT 5

Key Processes and Microbes in the Sulfur Cycle

Process	Example organisms
Sulfide/sulfur oxidation ($H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$)	
Aerobic	Sulfur chemolithotrophs (*Thiobacillus*, *Beggiatoa*, many others)
Anaerobic	Purple and green phototrophic bacteria, some chemolithotrophs
Sulfate reduction (anaerobic) ($SO_4^{2-} \rightarrow H_2S$)	*Desulfovibrio*, *Desulfobacter* *Archaeoglobus (Archaea)*
Sulfur reduction (anaerobic) ($S^0 \rightarrow H_2S$)	*Desulfuromonas*, many hyperthermophilic *Archaea*
Sulfur disproportionation ($S_2O_3^{2-} \rightarrow H_2S + SO_4^{2-}$)	*Desulfovibrio*, and others
Organic sulfur compound oxidation or reduction ($CH_3SH \rightarrow CO_2 + H_2S$) ($DMSO \rightarrow DMS$)	Many organisms can do this
Desulfurylation (organic–S $\rightarrow H_2S$)	Many organisms can do this

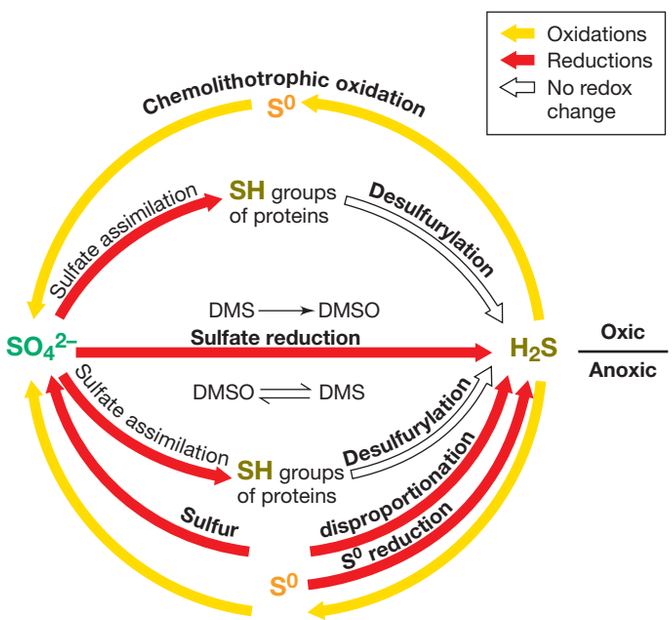


Figure 21.10 Redox cycle for sulfur. DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide.

S^0 is chemically stable but is readily oxidized by sulfur-oxidizing chemolithotrophic bacteria such as *Thiobacillus* and *Acidithiobacillus*. Because S^0 is insoluble, the bacteria that oxidize it must attach to the S^0 crystals to obtain their substrate (◀ Figure 14.19). The oxidation of S^0 forms sulfuric acid (H_2SO_4), and thus S^0 oxidation characteristically lowers the pH in the environment, sometimes drastically. For this reason, small amounts of S^0 can be added to alkaline agricultural soils as an inexpensive and natural way to lower the pH, relying on the ubiquitous sulfur chemolithotrophs to carry out the acidification process.

S^0 can be reduced as well as oxidized. The reduction of S^0 to sulfide (a form of anaerobic respiration) is a major ecological process of some *Bacteria* and hyperthermophilic *Archaea* (Chapter 17). Although sulfate-reducing bacteria can also reduce S^0, most S^0 reduction in nature occurs by the activities of the physiologically specialized sulfur reducers, organisms that are incapable of SO_4^{2-} reduction (◀ Section 15.11). However, the habitats of the sulfur reducers are generally those of the sulfate reducers, so from an ecological standpoint, the two groups form a metabolic guild unified by their formation of H_2S.

Organic Sulfur Compounds

In addition to *inorganic* forms of S, multiple forms of *organic* S compounds are also cycled in nature. Many of these foul-smelling compounds are highly volatile and can thus enter the atmosphere. Hundreds of different dissolved organic sulfur compounds have been identified in surface waters of the oceans. Many of these are rapidly cycled and are increasingly thought to play important roles in ecosystem dynamics. However, only a handful have been fully chemically characterized.

The most abundant organic S compound in nature is *dimethyl sulfide* [DMS, $(CH_3)_2S$)]; it is produced primarily in marine environments as a degradation product of dimethylsulfoniopropionate [DMSP], a major osmoregulatory solute in marine algae (◀ Section 4.15). As described previously (◀ Section 20.12), DMSP is catabolized by marine bacteria yielding DMS; in the atmosphere, DMS becomes photooxidized to sulfate, and this promotes the formation of clouds that help cool our planet (Section 21.9).

By contrast, DMS produced in anoxic habitats can be microbially transformed in at least three ways: (1) by methanogenesis (yielding CH_4 and H_2S), (2) as an electron donor for CO_2 fixation by certain phototrophic purple bacteria (yielding dimethyl sulfoxide, DMSO), and (3) as an electron donor in energy metabolism in certain chemoorganotrophs and chemolithotrophs (also yielding DMSO). DMSO can be an electron acceptor for anaerobic respiration (◀ Section 14.13), producing DMS. Many other organic S compounds affect the global sulfur cycle, including methanethiol (CH_3SH), dimethyl disulfide (CH_3SSCH_3), and carbon disulfide (CS_2), but on a global basis, DMS is the most significant.

Check Your Understanding

- Is H_2S a substrate or a product of the sulfate-reducing bacteria? Of the chemolithotrophic sulfur bacteria?
- What is the difference between chemotrophic and phototrophic sulfur oxidation?
- What organic sulfur compound is most abundant in nature, and how does it help keep Earth cool?

II • Other Nutrient Cycles

In addition to cycling major elements, microbes cycle many minor elements required for life—such as iron and phosphorus—as well as silicon and calcium required by certain marine phototrophs. Novel mechanisms of electron transfer are employed by microbes whose electron donors and acceptors are spatially distinct.

In Part II of this chapter we explore the interactions of microorganisms with metals—in particular the major processes of iron and manganese cycling—and with some nonmetals whose microbial transformations are also of major global significance.

21.5 The Iron and Manganese Cycles: Reductive Activities

Iron (Fe) is one of the most abundant elements in Earth's crust. On the surface of Earth, Fe exists naturally in two oxidation states, ferrous [Fe^{2+}, also Fe(II)] and ferric [Fe^{3+}, also Fe(III)]. A third oxidation state, Fe^0, is abundant in Earth's core and is also a major product of human activities from the smelting of iron ores to form cast iron.

In nature, iron cycles primarily between the Fe^{2+} and Fe^{3+} forms, and these redox transitions are one-electron oxidations and reductions. Ferric iron is reduced both chemically and as a form of anaerobic respiration, and Fe^{2+} is oxidized both chemically and as a form of chemolithotrophic metabolism (**Figure 21.11**). Manganese (Mn), although present at 5- to 10-fold lesser abundance than Fe in the near-surface environment, is another redox-active metal of microbiological significance, existing primarily in two oxidation states (Mn^{2+} and Mn^{4+}, see Figure 21.12).

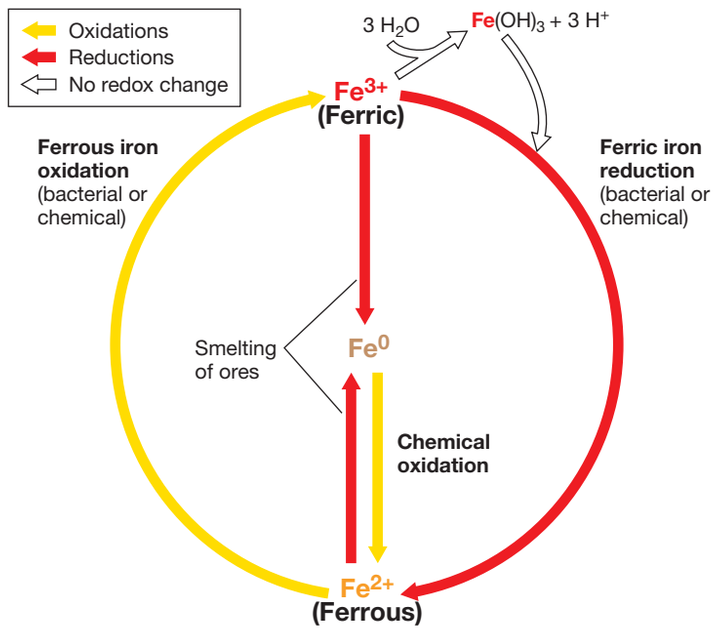


Figure 21.11 Redox cycle for iron. The major forms of iron in nature are Fe^{2+} and Fe^{3+}. Fe^0 is primarily a product of smelting of iron ores. Fe^{3+} forms various minerals such as ferric hydroxide, $Fe(OH)_3$.

A key feature of the iron and manganese cycles is the different solubilities of these metals in their oxidized versus reduced forms. Reduced iron (Fe^{2+}) and manganese (Mn^{2+}) are soluble. In contrast, oxidized minerals of iron such as iron oxide-hydroxides [e.g., $Fe(OH)_3$, FeOOH, and Fe_2O_3] and manganese oxide (MnO_2) are insoluble and tend to settle out in aquatic environments. As a consequence, these strong oxidants can comprise several percent by weight of marine and freshwater sediments, making them among the most abundant of potential electron acceptors in many anoxic systems (see Figure 21.12).

Bacterial Reduction of Iron and Manganese Oxides

Some *Bacteria* and *Archaea* can use Fe^{3+} as an electron acceptor in anaerobic respiration (◀ Section 15.13). These organisms also commonly have the capacity to use Mn^{4+} as an electron acceptor, and some have the capacity to reduce oxidized uranium (▶ Section 22.3). Ferric iron and manganese oxide reduction is common in waterlogged soils, bogs, and anoxic lake sediments (**Figure 21.12**). When soluble reduced iron and manganese reach oxic regions, for example, through diffusion from anoxic regions of sediments, they are oxidized chemically [e.g., $Fe^{2+} + \frac{1}{4}O_2 + 2\frac{1}{2}H_2O \rightarrow Fe(OH)_3 + 2H^+$] or microbiologically (Figure 21.12). The chemical oxidation of Fe^{2+} is very rapid at near-neutral pH. Although the spontaneous oxidation of Mn^{2+} is very slow at neutral pH, the rate of oxidation can be increased up to five orders of magnitude by a variety of manganese-oxidizing bacteria and even fungi. The oxidized metal oxides and hydroxides then precipitate, returning the oxidized metals to the sediments where they can again serve as electron acceptors, completing the cycle.

Oxidized iron (Fe^{3+}) and manganese (Mn^{4+}) are chemically very reactive and can alter the availability of both nutrients and toxic metals. For example, phosphate is less available when it is trapped as insoluble ferric phosphate precipitates. Essential and toxic metals [e.g., copper (Cu), cadmium (Cd), cobalt (Co), lead (Pb), and arsenic (As)] also form insoluble complexes with iron and manganese oxides. When these oxides are subsequently microbiologically reduced, the bound phosphate is liberated along with the soluble forms of these metals. In addition to sequestering phosphate and metals, abiotic oxidation of refractory organic compounds by manganese oxide may yield more available sources of carbon for microbial growth.

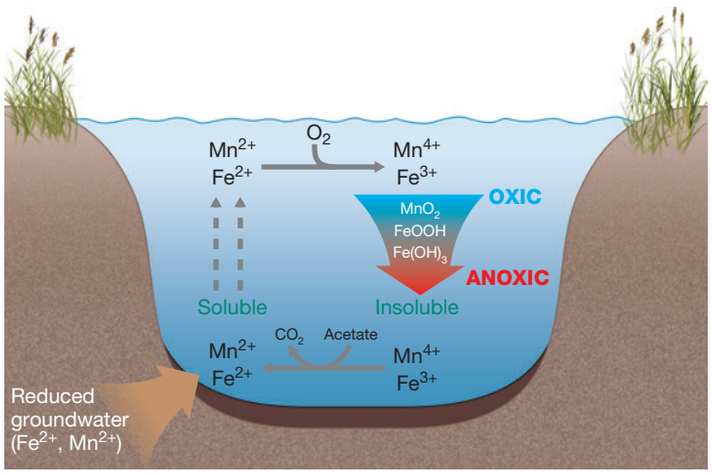


Figure 21.12 Iron and manganese redox cycling in a typical freshwater system. Iron and manganese oxides in sediments are used as electron acceptors by metal-reducing bacteria. The resulting reduced forms are soluble and diffuse into the oxic regions of the sediment or water column, where they are oxidized microbially or chemically. Precipitation of the insoluble oxidized metals then returns the metals to the sediments, completing the redox cycle.

Long-Range Electron Transfer to Insoluble Electron Acceptors

As we learned earlier (Chapters 3 and 14), in any form of respiration, electron disposal is necessary for energy conservation. When the electron acceptor is oxygen (O_2), nitrate (NO_3^-), or many other soluble substances used by bacteria as electron acceptors (◀ Sections 14.11–14.15), the final product diffuses away from the cell. Many bacteria reduce ferric iron (Fe^{3+}) as an electron acceptor under anoxic conditions, including the obligate anaerobe *Geobacter sulfurreducens* and the facultative aerobe *Shewanella oneidensis*. However, in contrast to soluble electron acceptors, Fe^{3+} is typically present in nature as an insoluble mineral, such as an iron oxide, and thus the reduction of Fe^{3+} must occur *outside* the cell. Under such conditions, the ferric iron functions as an electrical anode, and the bacterial cell facilitates transfer of electrons from the electron donor to the anode.

Regardless of the electron acceptor they use, when bacteria respire, they carry out oxidations and reductions that generate electricity. Thus, the surfaces and appendages of cells of bacteria that interact with iron and manganese oxides, such as *Geobacter* (**Figure 21.13*a***), are electrically conductive. *Geobacter* forms direct electrical connections with insoluble materials that can either accept or donate electrons. Electron transfer employs cytochromes localized along the length of pili that are generally 10–20 micrometers long (◀ Figure 14.31). These electrically conductive structures function as electrical nanowires, much as copper wires do in a household electrical circuit. Being conductive structures, nanowires can form direct electrical connections with insoluble materials that either accept or donate electrons; alternatively, nanowires can form connections between cells. In this way, electrons obtained by *Geobacter* from the oxidation of organic electron donors or from H_2 can be shuttled to a suitable electron acceptor.

Some electron transfers can occur over rather long distances, even longer than the cell itself. For example, hydrogen sulfide (H_2S) oxidation in some anoxic marine sediments is facilitated by filamentous bacteria composed of many interconnected cells that transfer electrons from sulfide (the donor) in the sediments to O_2 (the acceptor) in the overlying water, a distance of 2.5 cm (25000 μm, Figure 21.13*b*, *c*). The surface of the filamentous cells has ridges running along its entire length, and each microbial filament is surrounded by 15 to 17 such structures (Figure 21.13*b*). The structures function as electrical cables to transfer electrons from the sulfide oxidized at one end of the filament to the reduction of O_2 at the other end of the filament.

As for *Geobacter*, *Shewanella* is also capable of direct transfer of electrons to an insoluble exogenous electron acceptor. However, unlike *Geobacter* with its use of conductive pili, *Shewanella* uses cytochrome-rich extensions of its outer membrane to make physical

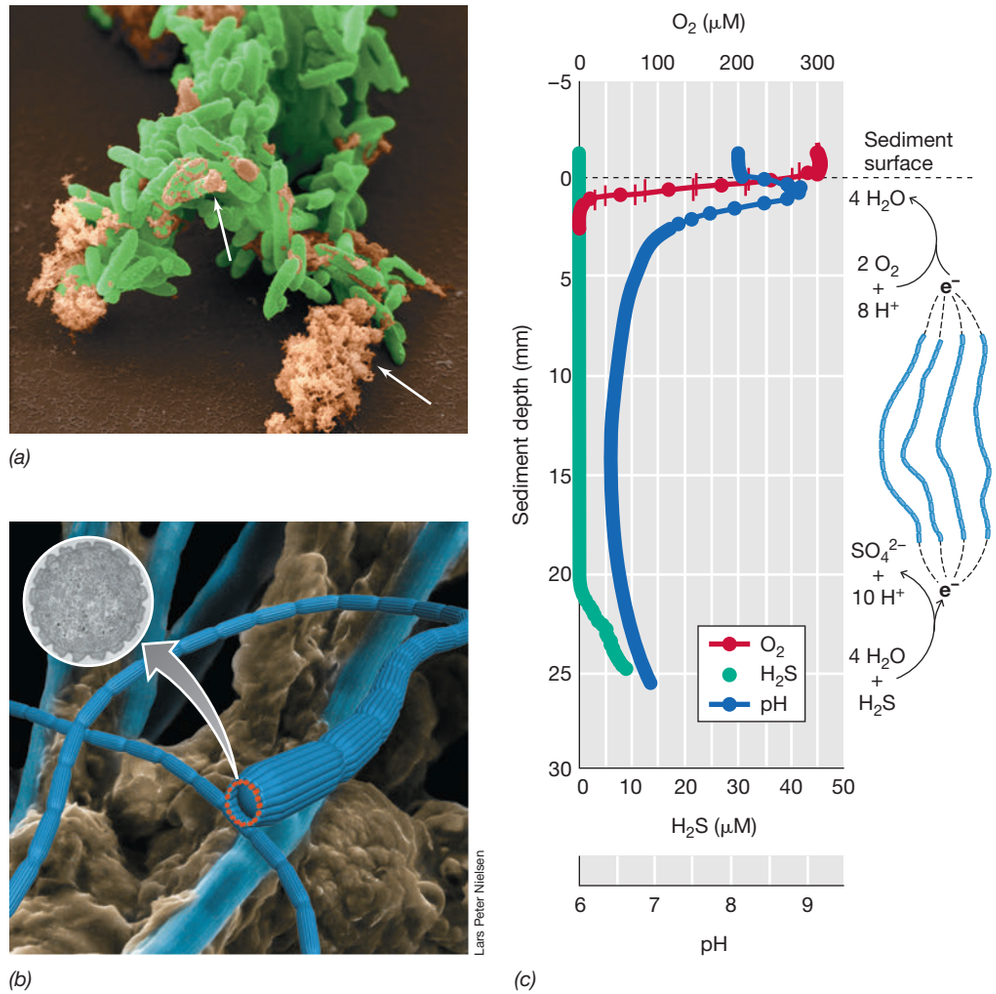


Figure 21.13 **Direct and long-range electron transfer.** *(a)* Cells of *Geobacter* attached to ferric iron precipitates (arrows) reduce Fe^{3+} to Fe^{2+}. *(b)* Three-dimensional rendering of cabled filamentous sulfide-oxidizers, with inset transmission electron microscopic cross-section displaying presumptive electrically conductive filaments surrounding the cell perimeter. *(c)* Microsensor profiles of sediments colonized by cabled filamentous bacteria, showing wide separation of depths where sulfide oxidation and oxygen reduction occur. The profiles are explained as follows: Oxygen (O_2) is quickly consumed by bacterial respiration in the upper regions of the sediment, whereas H_2S, produced in anoxic regions, only accumulates deeper in the sediments. The pH is more acidic lower in the sediments because sulfide oxidation yields protons. As O_2 is consumed near the sediment surface, protons are also consumed, and the pH rises.

contact with a mineral surface such as iron oxide. This system couples transfer of electrons, obtained from the oxidation of lactate or other organic compounds, to soluble cytochromes in the cell's periplasm and then to the cell surface using the Mtr complex of membrane-spanning multiheme proteins (**Figure 21.14**). From the Mtr complex, electrons can be shuttled (see next subsection) to the iron oxide by way of FMN (an electron carrier, ◀ Section 3.8) or directly transferred from MtrC, the outermost part of the Mtr protein complex, to iron oxide. An electrode poised at an electropositive potential can also function as an electron acceptor for this organism (Figure 21.14).

Extracellular Electron Shuttles

Another type of extracellular electron transfer is mediated by small, soluble, redox-active molecules called *extracellular electron shuttles* (EESs), typically aromatic organic compounds. The key functional attribute of an EES is the presence of conjugated double bonds that undergo redox transitions at a reduction potential (E_0') that facilitates transfer of electrons from the cell to an electron acceptor poised at a suitable potential difference. The acceptor can be an insoluble material such as oxidized metal or any other substance that can be reduced by the EES, including an actual electrode.

Naturally occurring humic substances (Section 21.1) often function as EESs. Since some constituents of humics can alternate between oxidized and reduced forms, they can function to shuttle electrons from the bacterium to reduce iron or manganese oxides (**Figure 21.15**). However, in addition to using shuttles that are available in the environment, a number of microorganisms secrete EESs to facilitate transfer of electrons to exogenous electron acceptors. These may be common cellular cofactors, such as flavin mononucleotide (FMN) secreted by *Shewanella oneidensis* (Figure 21.14), or more specialized shuttles such as the virulence factor pyocyanin (**Figure 21.16*a***), a derivative of phenazine, made by *Pseudomonas aeruginosa*. Pyocyanin has many effects, including enhancing

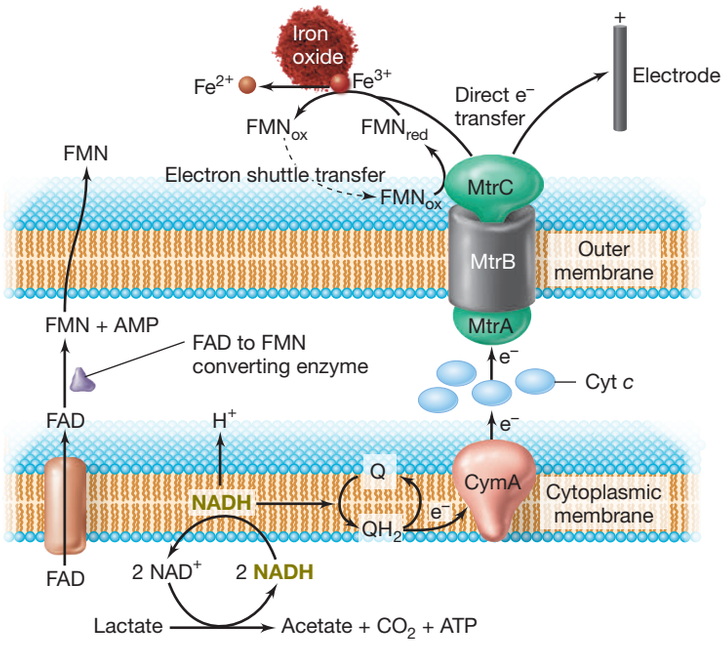


Figure 21.14 ***Shewanella*** **electron transfer to insoluble electron acceptors.** Electrons from lactate oxidation enter the quinone pool (Q, QH_2) through a membrane-bound NADH dehydrogenase. Reduced quinone (QH_2) is oxidized by a membrane-associated multiheme cytochrome (CymA), which transfers electrons to the outer membrane–spanning Mtr complex via periplasmic *c*-type cytochromes. The outer part of the Mtr complex (MtrC) then delivers electrons to an insoluble electron acceptor via direct interaction, such as to an iron oxide or an electrode, or via flavin (FMN) electron shuttles. FMNs are actively secreted by *Shewanella*. Although some proton efflux may be associated with electron transfer to CymA, ATP generation is primarily by substrate-level phosphorylation.

substrate-level ATP synthesis by oxidizing excess intracellular redox cofactors such as NADH, promoting biofilm formation, generating peroxide by reduction of molecular oxygen, and a capacity to liberate ferrous iron from an infected host's iron reserves. Pyocyanin is present in the lungs of patients suffering from the genetic disease

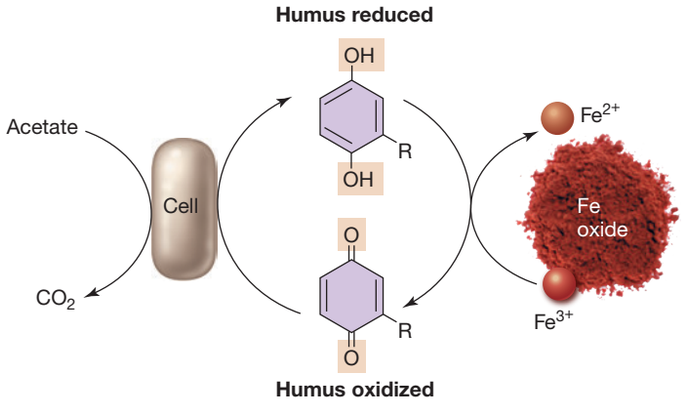


Figure 21.15 **Role of humic substances in humus as an electron shuttle in microbial metal reduction.** Quinone-like functional groups in humus are reduced by acetate-oxidizing bacteria. The reduced humus then donates electrons to metal oxides, releasing reduced iron (Fe^{2+}) and oxidized humus. The cycle continues as oxidized humus is again reduced by the bacteria.

cystic fibrosis, where vigorous biofilms of this bacterium develop (◀ Section 8.10); maintenance of the bacterial infection is promoted in part by the organism's ability to reduce ferric iron to the soluble ferrous form and sequester it through activity of the Feo iron transport membrane protein (Figure 21.16*b*).

Both direct electron transfer (as seen in ▶ Section 23.3) and the use of EESs are also important for transfer of electrons between symbiotically associated microorganisms. In nature, electrical communication between bacterial cells may be a major way by which electrons generated from microbial metabolism in anoxic habitats are shuttled to oxic regions. Moreover, research on the microbiology of the process indicates that this microbial electricity could be harnessed in the form of microbial "fuel cells" that could oxidize toxic and waste carbon compounds in anoxic environments, with the resulting electrons coupled to power generation. In such a scheme, bacteria would be exploited to function as catalysts for diverting electrons from electron donors directly to artificial anodes (Figure 21.14), with the resulting electrical current being siphoned off to supply a portion of human power needs. Such a system would have to be developed on a very large scale, of course. However, large-scale systems for microbial growth are already well established in wastewater treatment facilities (▶ Sections 22.6–22.8).

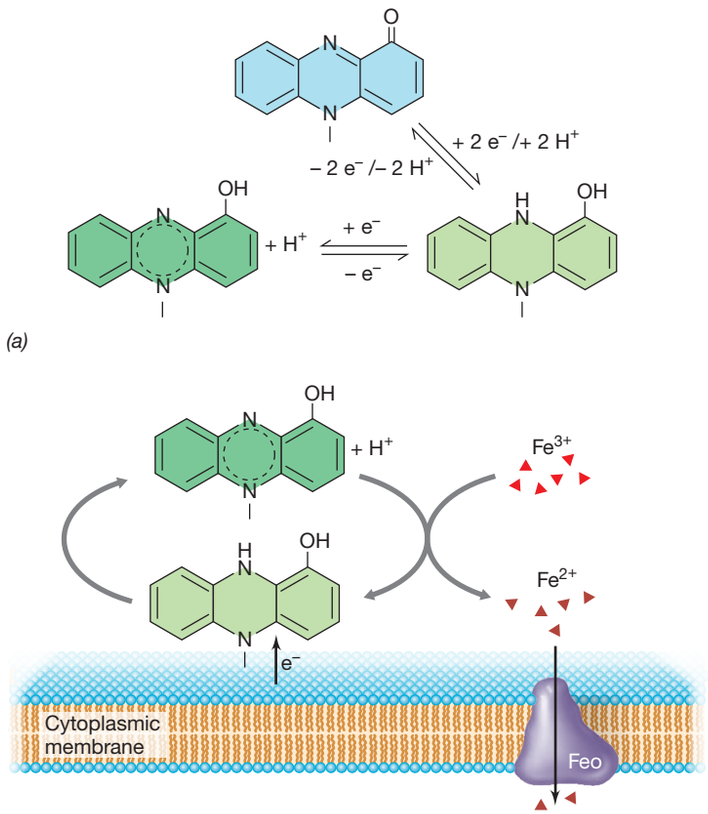


Figure 21.16 **Pyocyanin electron shuttling.** *(a)* Pyocyanin undergoes single and double electron transfer reactions between cells and environmentally available electron acceptors. *(b)* In *Pseudomonas* species, the pyocyanin electron shuttle is a virulence factor, the reduced pyocyanin functioning to reduce ferric iron to acquire iron from the infected host and incorporate it through the Feo uptake system.

Check Your Understanding

- In what oxidation states are Fe in $Fe(OH)_3$ and Mn in MnO_2?
- What alternative mechanisms are used by iron-respiring microorganism to deliver electrons to insoluble electron acceptors?

21.6 The Iron and Manganese Cycles: Oxidative Activities

Besides functioning as electron *acceptors* in their oxidized forms, in their reduced forms, iron and manganese can also function as electron *donors* (◀ Section 14.8). In this section, we explore this option as important links in the iron and manganese cycles.

Microbial Oxidation of Reduced Iron

At neutral pH, ferrous iron (Fe^{2+}) is oxidized abiotically in oxic environments. By contrast, at *acidic* pH (pH <4), Fe^{2+} is not oxidized spontaneously. Thus, much of the research on microbial oxidation of iron has been focused on acidic, ferrous-iron-rich habitats, where acidophilic chemolithotrophs such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* oxidize Fe^{2+} to Fe^{3+} (**Figure 21.17**). As we will see in Chapter 22, this activity can be both beneficial and harmful. On the one hand, because the oxidation of ferrous iron generates acidity, this can be devastating if large amounts of iron and other metal sulfides are released into freshwaters or soils, such as from mining activities. The acidity produced from iron oxidation can make the environments unsuitable for plant and animal life (▶ Figure 22.5). On the other hand, the generation of acidity and the ability of acidophilic chemolithotrophs to oxidize ferrous iron is exploited in the mining industry to extract metals such as copper from low-grade ores (▶ Section 22.1 and Figure 22.2).

Figure 21.17 Oxidation of ferrous iron (Fe^{2+}). A microbial mat growing in the Rio Tinto, Spain. The mat consists of acidophilic green algae (eukaryotes) and various iron-oxidizing chemolithotrophic bacteria. The Rio Tinto has a pH of about 2 and contains high levels of dissolved metals, in particular Fe^{2+}. The red-brown precipitates consist of $Fe(OH)_3$ and other ferric minerals.

In addition to oxidation by acidophilic microorganisms, ferrous iron can be oxidized microbially at neutral pH. However, since the abiotic oxidation of reduced iron also occurs under these conditions, iron-oxidizing bacteria that inhabit nonacidic environments are restricted to a very narrow zone in which anoxic ferrous-iron-rich water mixes with oxygenated water. These microoxic habitats include freshwater and coastal sediments (Figure 21.12; ◀ Figure 20.22), seeps of iron-rich groundwater, slow-moving streams, ferrous-iron-rich waters from springs, and hydrothermal vents (**Figure 21.18**). When ferrous-iron-rich groundwaters are exposed to air, Fe^{2+} is oxidized at the interface of these two zones by iron-oxidizing bacteria such as *Leptothrix* and *Gallionella* (Figure 21.18*b*, *c*, *d*; ◀ Sections 14.8 and 15.14). Thus, their physiology dictates that they maintain a position within a narrow environment of low levels of O_2 and high levels of reduced metals. Although O_2 is the most environmentally significant electron acceptor, Fe^{2+} oxidation can also be coupled to NO_3^- reduction by some anaerobic microbes (◀ Sections 14.8 and 15.14), and Fe^{2+} functions as an electron donor in photosynthesis for some anoxygenic phototrophs (◀ Sections 14.5, 15.2, and 15.5). Neutrophilic aerobic iron-oxidizing bacteria are members of two proteobacterial classes. The freshwater species are members of several closely related genera in the *Betaproteobacteria* whereas all known marine species are *Zetaproteobacteria* (*Proteobacteria* are discussed in Chapter 16). All characterized *Zetaproteobacteria* are autotrophic and aerobic iron-oxidizers, and all but the bacterium *Ghiorsea bivora*—which can oxidize either Fe^{2+} or H_2 as electron donors—are obligate iron-oxidizers. Although primarily associated with marine environments, the *Zetaproteobacteria* have also been found in saline terrestrial aquifers and springs, and in biofilms associated with steel corrosion (▶ Section 22.12).

As we saw in Section 21.5, organisms that reduce insoluble metal oxides can use extracellular conductors for electron transfer, such as electrically conductive pili or cell-surface-associated cytochromes. Cytochromes participate in both iron reduction and iron oxidation, and genomes of metal-oxidizing *Gallionella* and *Sideroxydans* species contain genes encoding periplasmic *c*-type cytochromes that resemble those encoding proteins known to reduce metal oxides in *Shewanella* (Figure 21.14), indicating that mechanistically related electron transfer pathways are used for both the reduction and oxidation of extracellular metals.

Although possibly sharing similar electron transfer mechanisms, metal-oxidizing bacteria are confronted with the problem that the precipitates they form can encase the cell in an iron oxide shell. To prevent this, metal oxidizers secrete organic substances that capture metal oxides and deposit them some distance away from the cell. For example, the iron oxidizer *Gallionella* produces extended organic stalks that become encrusted with metal oxides away from the cell (Figure 21.18*d*, *f*, and *g*; see also Figure 15.36). Similar structures are made by marine *Zetaproteobacteria* of the genus *Mariprofundus*; the species *Mariprofundus ferrooxydans* deposits iron oxides as a twisted stalk while *Mariprofundus ferrinatatus* makes shorter ropelike filaments of iron oxides (**Figure 21.19**). Laboratory experiments employing opposing gradients of Fe^{2+} and O_2 showed that *M. ferrooxydans* uses its iron oxide stalk as both a holdfast and a dynamic structure to position cells at an optimum point within the gradient of oxygen and Fe^{2+}. An alternative strategy is used by *Leptothrix* and *Sphaerotilus*

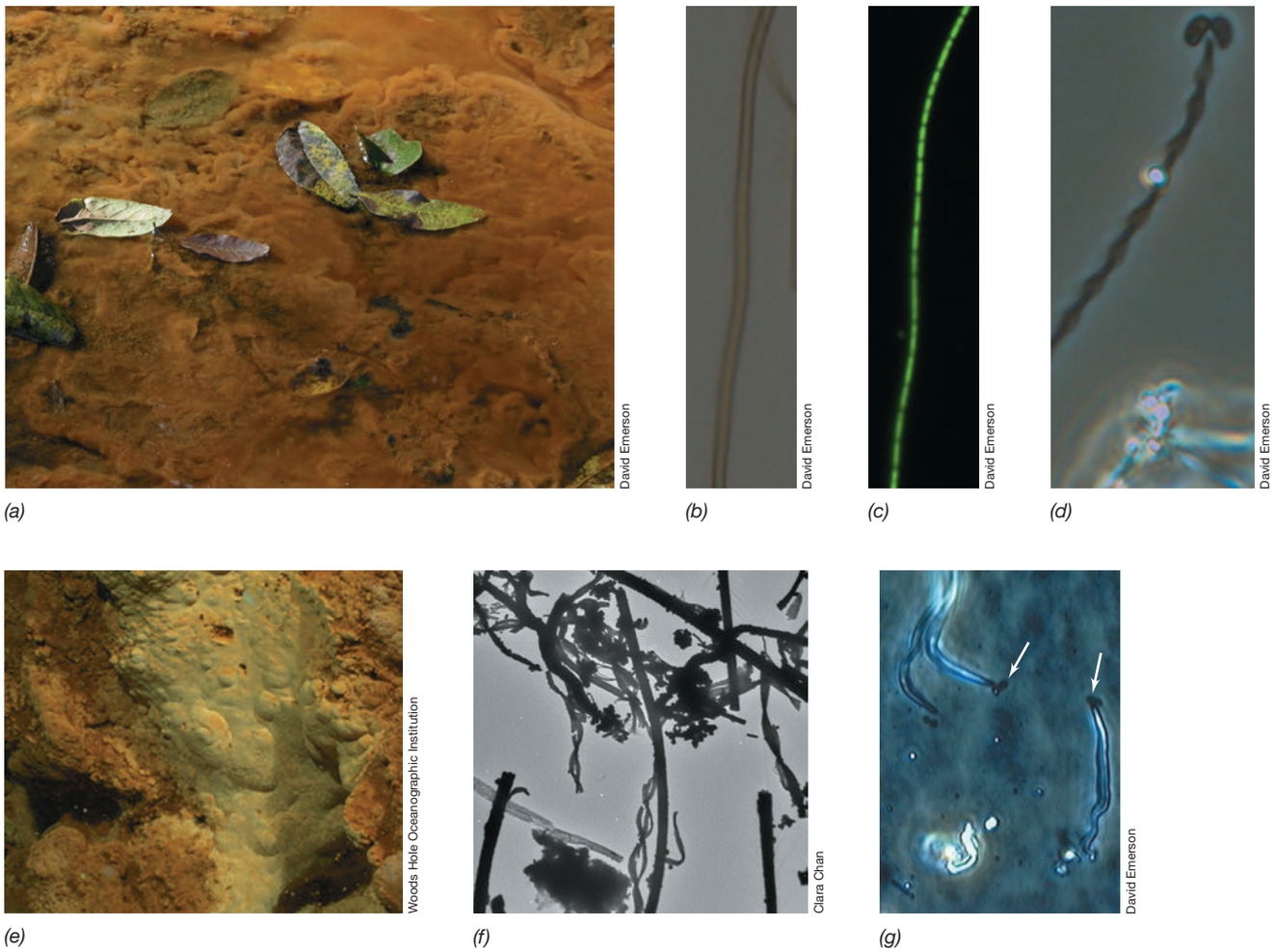


Figure 21.18 Fe-oxidizing microbial mats. *(a)* Freshwater microbial mat in a slow-moving stream where Fe^{2+}-enriched groundwater is mixing with oxygenated surface water, triggering growth of Fe^{2+}-oxidizing bacteria and precipitation of iron oxides. *(b–d)* Fe-oxidizing *Bacteria*. *(b, c)* Phase-contrast and epifluoresence photomicrographs of the sheath-forming Fe-oxidizer *Leptothrix ochracea* (the sheath is approximately 2 μm wide). *(d)* The stalk-forming Fe^{2+}-oxidizer *Gallionella ferruginea* showing bean-shaped cells in the process of cell division at the end of the iron oxide–encrusted stalk (each bean-shaped cell is about 2 μm long). *(e)* An iron-oxidizing mat at a deep-sea hydrothermal vent (1000-meter depth) at Lō'ihi Seamount. *(f)* TEM image of biogenic oxides produced at Lō'ihi; note the variety of helical stalks and tubular sheathlike filaments (the filaments vary from 2 to 4 μm wide). *(g)* Phase-contrast photomicrograph of marine Fe^{2+}-oxidizers growing at the ends of iron oxide filaments (cells denoted by arrows) from an experimental incubation at Lō'ihi (filaments are approximately 2 μm wide).

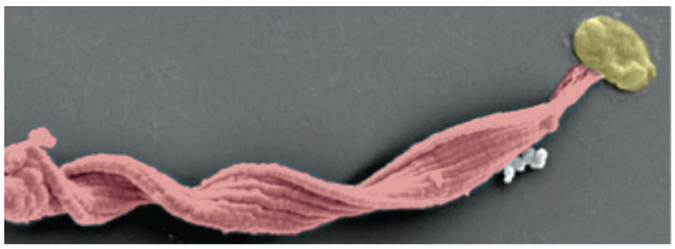
UNIT 5

species. These bacteria produce an organic sheath surrounding the cells that becomes encrusted with metal oxides (Figure 21.18*b* and ◀ Figure 15.55). Later, the cells move out of the sheath, leaving the metal oxide crust behind. Although not all metal oxidizers produce such morphologically conspicuous structures, it is thought that most, if not all, metal oxidizers are forced to produce some form of extracellular organic material in order to sequester the insoluble product of their energy metabolism.

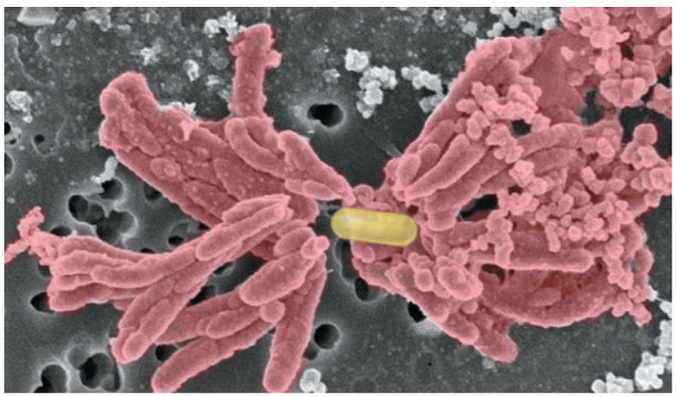
Microbial Oxidation of Reduced Manganese

Many bacteria catalyze Mn^{2+} oxidation, and the different species known are ubiquitous in nature, inhabiting fresh and marine waters, soils, and virtually anywhere significant levels of Mn^{2+} are present. Reduced manganese is present along with Fe^{2+} in anoxic waters that reach oxic zones, the Mn^{2+} resulting from the anaerobic respiration of Mn^{4+} (Figure 21.12; ◀ Figure 20.22); these anoxic/oxic interfaces establish a niche for the obligately aerobic Mn^{2+}-oxidizing bacteria. Because the reduction potential of the Mn^{2+}/Mn^{4+} couple is so high, very near to that of the O_2/H_2O couple (◀ Figure 14.1), O_2 is the only electron acceptor that can couple with Mn^{2+} oxidation; anaerobic respirations of Mn^{2+} are thus not possible.

Several genera of *Bacteria* can oxidize Mn^{2+} including *Sphaerotilus* and *Leptothrix*—both *Betaproteobacteria*—and *Hyphomicrobium* and *Pedomicrobium*—both *Alphaproteobacteria* (◀ Figures 15.51, 15.55, and 15.56). Some *Pseudomonas* species can also oxidize Mn^{2+}. Most of these organisms can be cultured and therefore Mn^{2+} oxidation easily measured. However, a major unanswered question surrounding this entire physiological group is whether they actually benefit from Mn^{2+} oxidation. Chemolithotrophic (and autotrophic) growth on Mn^{2+} has been achieved with one *Pseudomonas* species whose cells were shown to contain carboxysomes (◀ Figure 15.30*a*). Carboxysomes are storage granules of the key enzymes of the Calvin



(a)



(b)

Figure 21.19 Scanning electron micrograph of iron oxide minerals formed by species of marine *Zetaproteobacteria*. *(a)* Twisted mineral stalk (colorized red) formed by a cell of *Mariprofundus ferrooxydans* (yellow). The cell is about 1.2 μm long. *(b) Mariprofundus ferrinatatus* forms short iron oxide structures (colorized red), which are shed from the single cell (outlined in yellow) that is covered by the mineral deposits. The cell is about 1 μm long. Images reproduced from McAllister, S.M., Moore, R.M., Gartman, A., Luther, G.W., III, Emerson, D., and C.S. Chan. 2019 *FEMS Microbiol. Ecol.* doi:10.1093/femsec/fiz015.

cycle (◀ Section 3.12), the main autotrophic pathway of aerobic chemolithotrophs. Convincing chemolithotrophic growth of other species of Mn^{2+} oxidizers has not been achieved. A possible alternative reason for Mn^{2+} oxidation is to convert soluble Mn^{2+}—which can be a toxic metal when present in high enough concentrations—into nontoxic manganese oxides. Whatever the reason(s) for bacterial Mn^{2+} oxidation, the process can be a major one in nature and is typically linked to neutral pH habitats where extensive iron oxidation is occurring simultaneously.

Check Your Understanding

- Both *Acidithiobacillus* and *Mariprofundus* are iron oxidizers, but their habitats differ dramatically. Explain.
- Although some iron-oxidizing bacteria can grow on iron anaerobically, manganese-oxidizing bacteria cannot grow on manganese anaerobically. Explain.

21.7 The Phosphorus, Calcium, and Silicon Cycles

Mastering Microbiology
Art Activity: Figure 21.15 The marine calcium cycle

Many other chemical elements undergo microbial cycling and we focus on three key ones here: phosphorus (P), calcium (Ca), and silicon (Si). The cycling of these elements is important in aquatic environments, particularly in the oceans, which are major reservoirs of Ca and Si and where large amounts of Ca and Si are incorporated into the exoskeletons of certain microbes. However, unlike the C, N, and S cycles, in the Ca and Si cycles there are no redox changes or gaseous forms that can escape and alter Earth's atmospheric chemistry. However, different redox states of P are biogeochemically significant. From a global perspective, keeping these cycles in balance—especially that of Ca—is important for maintaining sustainable life on Earth.

Phosphorus and Phosphonates

Phosphorus exists in nature primarily as organic and inorganic phosphates. Phosphorus reservoirs include phosphate-containing minerals in rocks, dissolved phosphates in freshwaters and marine waters, and the nucleic acids and phospholipids of living organisms. Although P has multiple oxidation states, most environmental phosphates are at the +5 oxidation state (for example, hydrogen phosphate, HPO_4^{2-}). In nature, P cycles through living organisms (as cellular P), waters and soils (as inorganic and organic P), and Earth's crust (as inorganic P). P is typically the limiting nutrient for photosynthesis in freshwaters, which receive it from the weathering of rocks.

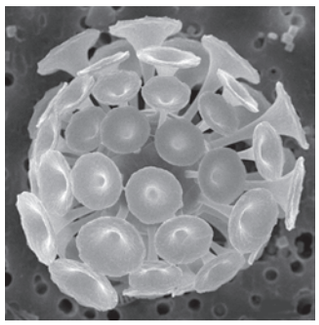
In the oceans, a fraction of dissolved P is organic, in the form of phosphate esters and *phosphonates*. Phosphonates, also referred to as *phosphonic acids* when in the protonated forms, are organophosphate compounds that contain a direct bond between the P and C atoms. In this form the P atom is more reduced (+3 oxidation state) than in phosphate (+5 oxidation state). Phosphonates are produced by certain microorganisms and comprise about a quarter of the organic P pool in nature. For many organisms, phosphonates are a less available source of P than is HPO_4^{2-} because the organisms lack the enzymes required to degrade phosphonates. Such organisms can be phosphorus-limited even when sufficient P is present as phosphonates.

Phosphonates and reduced inorganic forms of P—phosphite ($H_2PO_3^-$, +3 oxidation state) and hypophosphite ($H_2PO_2^-$, +1 oxidation state)—are rapidly cycled in the marine environment by producers and consumers. This cycling of P is important for organisms that live in P-depleted environments and have the capacity to make or consume these alternative forms of phosphorus. Why marine organisms produce so much of these reduced phosphorus species is unknown. However, the fact that some marine microorganisms produce methylphosphonic acid may have solved another marine metabolic conundrum. The degradation of methylphosphonic acid (CH_5O_3P) by some marine microorganisms—a process that liberates methane (CH_4)—likely explains the previously puzzling observation that relatively high levels of CH_4 are present in the oxygenated surface waters of the ocean (see Explore the Microbial World, "Solving the Marine Methane Paradox").

Calcium

The major global reservoirs of calcium (Ca) are calcareous rocks and the oceans. In the oceans, where dissolved Ca exists as Ca^{2+}, calcium cycling is a highly dynamic process although the concentration of Ca^{2+} in seawater remains constant at about 10 mM. Several marine eukaryotic phototrophic microorganisms take up Ca^{2+} to form their calcareous exoskeletons; these include in particular species of haptophytes called *coccolithophores* (◀ Section 18.7 and Figure 18.15) and *Rhizaria* called *foraminifera* (◀ Section 18.6) (**Figure 21.20**). The calcium-cycling activities of these planktonic phototrophs are also tightly coupled with inorganic components of the carbon cycle.

(a)



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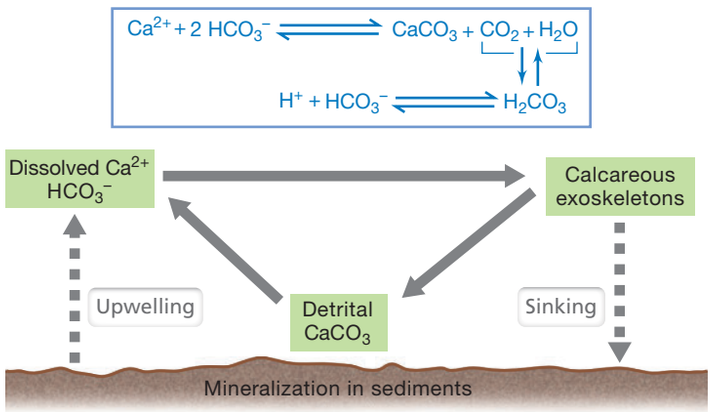


(c)

Figure 21.20 The marine calcium cycle. Scanning electron micrographs of cells of the calcareous phytoplankton *(a) Emiliania huxleyi* and *(b) Discosphaera tubifera*. The exoskeletons of these coccolithophores are made of calcium carbonate ($CaCO_3$). A cell of *E. huxleyi* is about 8 μm wide and a cell of *D. tubifera is* about 12 μm wide. *(c)* The marine calcium cycle; dynamic pools of Ca are shaded in green. Detrital $CaCO_3$ is that in fecal pellets and other organic matter from dead organisms. Note how H_2CO_3 formation lowers ocean pH when it dissociates to form H^+ and HCO_3^-.

The precipitation of calcium carbonate ($CaCO_3$) to form the shells of calcareous phytoplankton controls both CO_2 flux into ocean surface water and inorganic C transport into deep ocean waters and the sediments. Moreover, the formation of $CaCO_3$ both depletes surface dissolved bicarbonate (HCO_3^-) and increases the level of dissolved CO_2 (Figure 21.20*c*). The latter reduces the influx of atmospheric CO_2 into surface ocean waters, and this helps maintain the slightly alkaline pH of the oceans. When these calcareous organisms die and sink toward the sediments, Ca^{2+} and inorganic and organic C are transported to the deep ocean from which they are only slowly released over long periods (see also page 621).

The formation of $CaCO_3$ exoskeletons brings into play a delicate balance between Ca^{2+} and C and is a process sensitive to changes in atmospheric CO_2 levels. This is because increased levels of atmospheric CO_2 increase the formation of carbonic acid (H_2CO_3), and as this dissociates to form HCO_3^- and H^+, $CaCO_3$ dissolves and seawater pH decreases (Figure 21.20*c*). Greater ocean acidity resulting from rising atmospheric CO_2 is predicted to reduce the rate of formation of calcareous shells, which will likely have effects on other microbial nutrient cycles and plant and animal communities (Section 21.9).

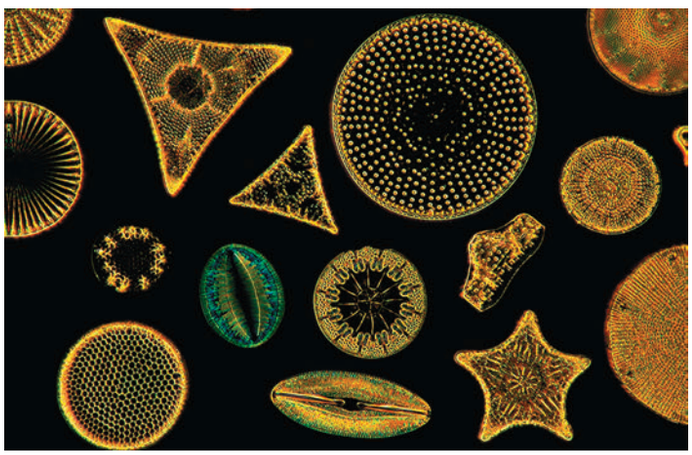
Silicon

The marine Si cycle is controlled primarily by unicellular eukaryotes (diatoms and other *Stramenopiles* referred to as silicoflagellates, and radiolarians) that build ornate external cell skeletons called *frustules* (diatoms, **Figure 21.21*a***; ◀ Section 18.5 and Figure 18.12) or tests (radiolarians, ◀ Section 18.6). These structures are not constructed of $CaCO_3$ as in the coccolithophores, but of silica (SiO_2, also called opal), whose formation begins with the uptake by the cell of dissolved silicic acid (Figure 21.21*b*).

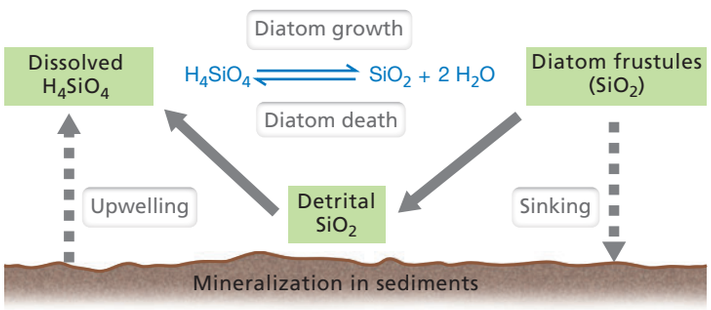
Mastering Microbiology
Art Activity: Figure 21.16 The marine silica cycle

Diatoms are rapidly growing phototrophic eukaryotes and often dominate blooms of phytoplankton in coastal and open ocean waters. However, unlike other major phytoplankton groups, diatoms require Si and can become silicon-limited when blooms develop. Also, because of their large size, diatom cells tend to sink faster than other organic particles, and in this way, they contribute significantly to the return of Si and C to deeper ocean waters. The transport of organic material produced through primary production in near-surface waters to deeper ocean waters, primarily by sinking particles, is called the *biological pump* and is an important aspect of the carbon cycle in terms of carbon burial and mineralization in marine environments (Figure 21.1).

In addition to the major nutrient requirements of any phototrophic organism (CO_2, N, P, Fe), diatoms require sufficient dissolved Si, and in nature this originates primarily from Si released from the skeletons of dead diatoms (Figure 21.21*b*). Although Si is released fairly rapidly



(a)



(b)

Figure 21.21 The marine silicon cycle. *(a)* Dark-field photomicrograph of a collection of diatom shells (frustules). The frustules are made of silica (SiO_2). *(b)* The marine silicon cycle; dynamic pools of Si are shaded in green.

UNIT 5

Solving the Marine Methane Paradox

An anomaly in the concentration of dissolved methane in oxygenated ocean surface waters was first observed in the latter half of the last century. A region from the surface to approximately 300 meters depth is supersaturated in methane relative to the concentration expected if the ocean was in equilibrium with atmospheric methane (**Figure 1*a***). About 75% of the oceanic flux of methane to the atmosphere derives from this source (other sources being melting methane hydrates and methane generated in marine sediments; see Figures 21.3, 21.4, and 21.5). Because methane is a major greenhouse gas (Section 21.9), identifying its marine origins has been considered essential for predicting how changes in ocean temperatures might exacerbate climate change.

An initial clue came from the light isotopic composition of carbon in methane from marine surface waters, a result consistent with a microbial source (◀ Section 19.10). However, this presented a conundrum—only obligately *anaerobic* methanogens are known to produce methane (◀ Section 17.2), and marine surface waters are highly oxic. Thus, at first glance, a microbial source for this methane did not make sense. However, a solution to this paradox ultimately came from studies of the marine phosphorus cycle.

Dissolved phosphorus, often a limiting nutrient controlling primary production in the ocean, is present in both inorganic (typically as phosphate) and organic forms. About 10% of the dissolved organic phosphorus is in the form of phosphonates, compounds characterized by a C–P bond (Figure 1*b*, *c*). About 10% of marine microbes are capable of phosphonate biosynthesis; however, not all marine organisms are capable of using phosphonate. Cleavage of the C–P bond, required for release of inorganic phosphate, requires an enzyme called *C–P lyase*. Since phosphonates are abundant in upper ocean waters and the C–P bond can be cleaved by many marine microbes, it was hypothesized that methylphosphonate, or the protonated form methylphosphonic acid (Figure 1*b*), could be the source of the surface water methane observed.

Subsequent experiments showed that several aerobic marine bacteria could cleave methylphosphonate and use the released phosphate to support growth. However, this brought up another question: What was the source of the methylphosphonic acid? This riddle was solved when a pathway for its biosynthesis was discovered in the marine ammonia-oxidizing archaeon *Nitrosopumilus maritimus*[1] (Figure 1*b*). Identification of the biochemical pathway and supporting genes from this organism facilitated surveys of other marine microbes and marine metagenomes for similar genes. These studies revealed that the pathway for methylphosphonic acid biosynthesis is widely distributed in marine bacteria, including the abundant chemotrophic bacterium *Pelagibacter* and phototroph *Prochlorococcus* (◀ Sections 20.11 and 20.12).

Quantitative studies have confirmed that levels of methylphosphonate in the marine pool of dissolved organic phosphorus can account for the marine methane anomaly, solving the methane paradox and identifying an important new process in the cycling of carbon and phosphorus in the ocean.[2] But in addition to marine sources of phosphonates, a few phosphonates are of medical or agricultural significance, including the antibiotic fosfomycin—a potent clinical weapon for treating urinary tract infections—and the widely used herbicide glyphosate (marketed as Roundup®) (Figure 1*c*). Glyphosate can be catabolized as a sole source of carbon, nitrogen, and phosphate by several different bacteria. However, because of the large amounts of this herbicide used agriculturally, microbial ecological studies of glyphosate biodegradation is an area in need of much further research if we are to understand the fate of terrestrial phosphonates as well as we now do marine phosphonates.

[1]Metcalf et al. 2012. Synthesis of methylphosphonic acid by marine microbes: a source for methane in the aerobic ocean. *Science 337*: 1104.
[2]Repeta et al. 2016. Marine methane paradox explained by bacterial degradation of dissolved organic matter. *Nat. Geosci. 9*: 884.

following cell death, during periods of high diatom production in relatively shallow waters, a significant fraction of dissolved Si can become buried in sediments and remain there for millions of years. This has consequences for continued diatom growth and their phototrophic consumption of dissolved CO_2 from ocean waters. The flux of CO_2 into and out of ocean water affects its pH (Figure 21.20*c*), and through this link, the Si and C cycles are coupled in a manner similar to what we have seen with the Ca and C cycles (Figure 21.20).

Although the nutrient cycles we have seen thus far are mainly driven by microbial activities (plus plants in the case of the C cycle), humans can have major impacts on nutrient cycling through major inputs of C, N and toxic elements, and we consider these next.

Check Your Understanding

- How does the formation of $CaCO_3$ skeletons by calcareous phytoplankton retard movement of CO_2 from the atmosphere to oceans and help maintain ocean water pH?
- How might Si depletion in the photic zone influence the biological pump?

III • Humans and Nutrient Cycling

In addition to steadily increasing levels of CO_2, intensive agriculture and fossil fuel burning have increased levels of nitrous oxide and methane and have accelerated the mobilization of mercury. Human inputs into major nutrient cycles will likely affect all life forms, be they microbes, flora, or fauna.

Humans have a profound impact on microbial nutrient cycles by adding and removing specific components of the cycles in large amounts. Here we consider human inputs of three major chemical groups: mercury (Hg), CO_2 and other atmospheric gases, and various fixed N compounds. These compounds originate primarily from the burning of fossil fuels or from agricultural activities, and either cause toxicity problems (Hg) or affect Earth in globally significant ways (gases and N compounds). We begin with the very toxic metal Hg, which is transformed by bacteria in many different ways.

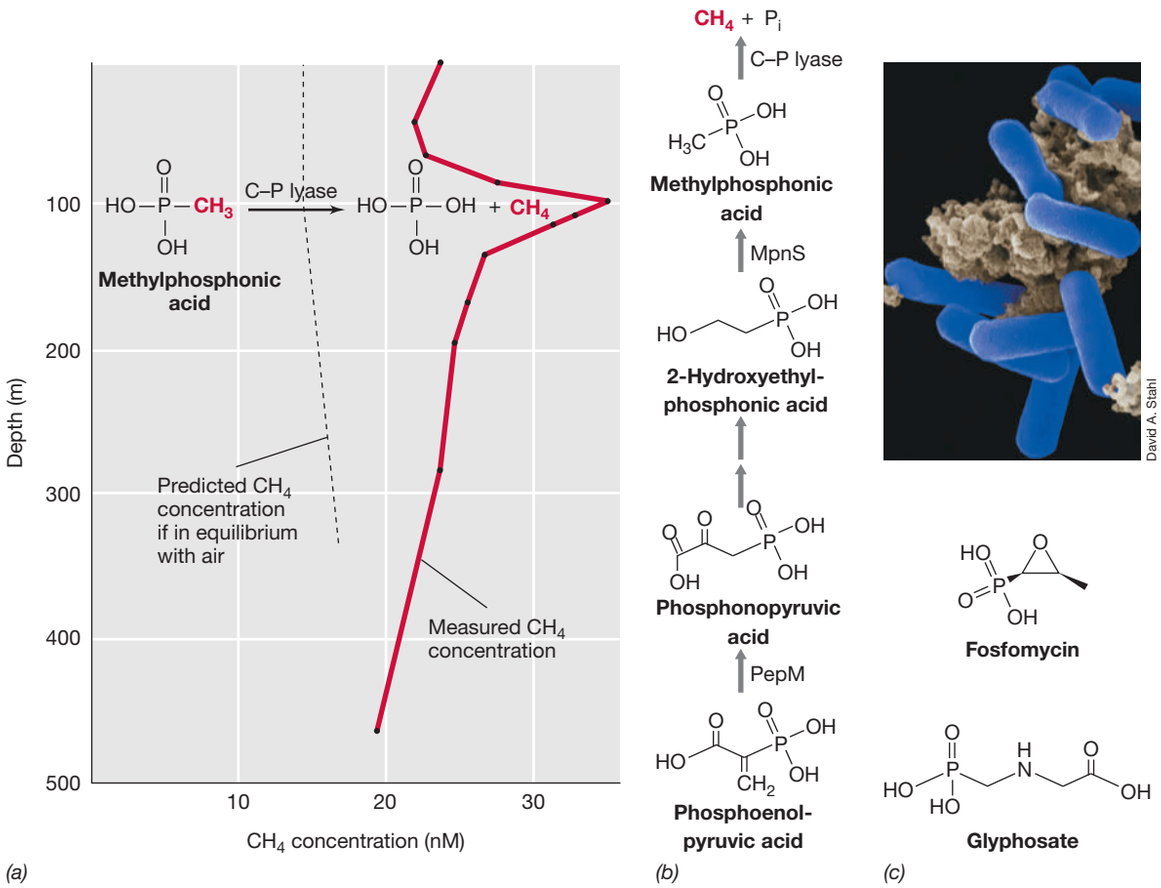


Figure 1 The marine methane paradox. *(a)* Representative profile of dissolved methane in surface waters of the Atlantic Ocean showing supersaturation of methane in the upper 300 meters. *(b)* The pathway for methylphosphonic acid biosynthesis, first identified in the marine ammonia-oxidizing archaeon *Nitrosopumilus maritimus* (scanning electron micrograph). Phosphoenolpyruvic acid (PEP) is an intermediate in glycolysis (◀ Section 3.6 and Figure 3.11). Methylphosphonic acid synthase (MpnS) generates the methylphosphonic acid that is subsequently cleaved to methane and inorganic phosphate by the enzyme C–P lyase present in many marine bacteria. The enzyme phosphoenolpyruvic acid mutase (PepM) catalyzes the first step of all known phosphonic acid biosynthesis pathways, and the presence of the *pepM* gene is diagnostic for organisms having the capacity for phosphonic acid biosynthesis. *(c)* Structures of the phosphonate antibiotic fosfomycin and the phosphonate herbicide glyphosate.

21.8 Mercury Transformations

Mercury is not a microbial nutrient (◀ Figure 4.1), but microbial transformations of various mercuric compounds are natural processes and help to detoxify some of the most toxic forms. Mercury is a widely used industrial product, especially in the electronics industry. Mercury is also an active ingredient in many pesticides, a pollutant from the chemical and mining industries and from the combustion of coal and municipal wastes, and a common contaminant of aquatic ecosystems and wetlands. Because of its propensity to concentrate in living tissues, Hg is of considerable environmental importance. The major form of Hg in the atmosphere is elemental mercury (Hg^0), which is volatile and is oxidized to mercuric ion (Hg^{2+}) photochemically. Most Hg thus enters aquatic environments as Hg^{2+} (**Figure 21.22**).

Microbial Redox Cycle for Mercury

Mercuric ion readily adsorbs to particulate matter, and when deposited in anoxic environments, such as lake or marine sediments, Hg^{2+} can be metabolized from there by anaerobic microbes. Microbial activity methylates Hg, yielding *methylmercury*, CH_3Hg^+ (Figure 21.22), and this can be further methylated to form *dimethylmercury*, $Hg(CH_3)_2$. Methylmercury formation is associated with the activities of sulfate-reducing and iron-reducing *Bacteria* and methanogenic *Archaea*; very few aerobes have been shown to methylate mercury. In contrast to methylmercury, the formation of dimethylmercury is likely not due to microbes but instead to abiotic processes.

Methylmercury and dimethylmercury are readily absorbed through the skin of animals and are potent neurotoxins. Dimethylmercury is an extremely toxic neurotoxin and only a few microliters of liquid is sufficient to kill a human. Symptoms are slow to develop but unstoppable as this uncharged form crosses the blood–brain barrier. In addition, CH_3Hg^+ is soluble and thus easily concentrated in the food chain, primarily in the muscle tissues of fish, and its concentration increases with each trophic level (a process called *biomagnification*), causing a threat to humans whose diets rely heavily on fish. Methylmercury and Hg^{2+} are about 100 times more toxic than Hg^0, and their accumulation in the aquatic food chain is particularly acute in freshwater lakes and marine coastal waters where

Mastering Microbiology
Art Activity: Figure 21.17 Biogeochemical cycling of mercury

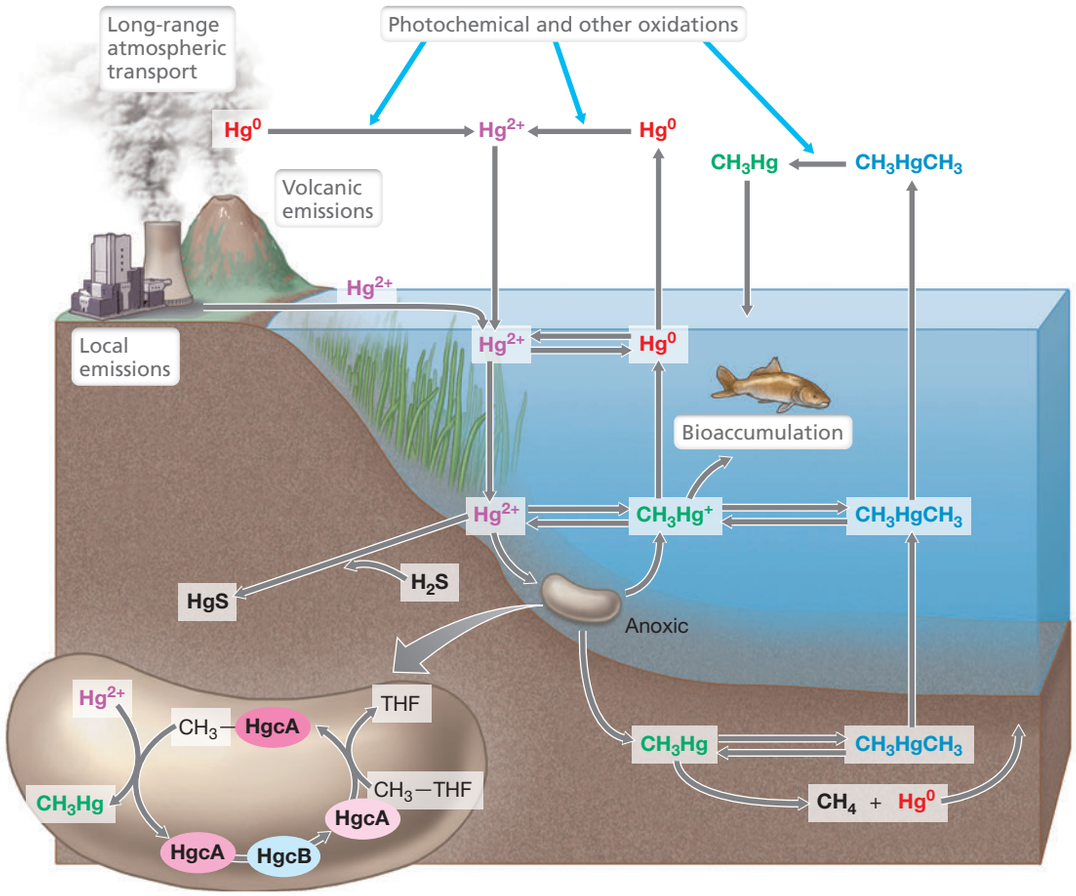


Figure 21.22 Biogeochemical cycling of mercury. The major reservoirs of mercury are water and sediments. Mercury in water can be concentrated in animal tissues; it can be precipitated as HgS from sediments. The volatile forms of mercury are Hg^0 and CH_3HgCH_3. The enlarged bacterial cell shows the enzyme system responsible for mercury methylation by sulfate-reducing bacteria. Common forms of mercury are shown in different colors. THF, tetrahydrofolate. HgcA and HgcB are proteins that function to methylate mercury.

enhanced levels of CH_3Hg^+ have been detected in fish caught for human consumption. In addition to their neurotoxicity, mercuric compounds also cause liver and kidney damage in humans and other animals, and methylmercury is a suspected human carcinogen.

Biochemistry of Mercury Methylation

Methylation of Hg^{2+} by sulfate-reducing bacteria requires two genes, *hgcA* (encoding a methyltransferase corrinoid protein) and *hgcB*, encoding a [4Fe-4S] ferredoxin-containing protein (Figure 21.22). In the reaction sequence (Figure 21.22), a methyl group is transferred from methylated HgcA to inorganic Hg^{2+}. HgcB then regenerates the reduced form of HgcA that accepts a methyl group from the coenzyme methyltetrahydrofolate (Figure 21.22). Identification of *hgcA* and *hgcB* has fueled surveys of their distribution in genomic and metagenomic sequences and has revealed that genes for mercury methylation are widely distributed in species of *Bacteria* and methanogenic *Archaea*. The discovery of mercury methylation genes paves the way for developing genetic tools for surveying environments for their potential to methylate mercury and should contribute to our understanding of the physiological significance of methylation. It also allows for the identification of environmental variables that control the expression of mercury methylation genes and insight into the observation that mercury methylation also occurs in oxic marine surface waters.

Several other microbial transformations of mercury occur in anoxic sediments, including reactions catalyzed by sulfate-reducing bacteria ($H_2S + Hg^{2+} \rightarrow HgS$) and methanogens ($CH_3Hg^+ \rightarrow CH_4 + Hg^0$) (Figure 21.22). The solubility of mercuric sulfide (HgS) is very low, so in sulfidic sediments, most Hg exists as HgS. But upon aeration, HgS can be oxidized to Hg^{2+} and SO_4^{2-} by sulfide-oxidizing bacteria (◀ Sections 14.7 and 15.12), and the Hg^{2+} is eventually converted to CH_3Hg^+. However, it is not the Hg in HgS that is oxidized here, but instead the *sulfide*, probably by organisms related to *Acidithiobacillus* or other sulfur-oxidizing bacteria (◀ Section 15.12).

Mercury Resistance

At sufficiently high concentrations, Hg^{2+} and CH_3Hg^+ can be toxic to microorganisms as well as to macroorganisms. However, several gram-positive and gram-negative bacteria convert toxic forms of Hg to nontoxic or less toxic forms. These mercury-resistant bacteria employ the enzyme *organomercury lyase* to degrade the highly toxic CH_3Hg^+ to Hg^{2+} and methane (CH_4), and the NADPH (or NADH)-linked enzyme *mercuric reductase* to reduce Hg^{2+} to Hg^0, which is volatile and thus mobile (**Figure 21.23**).

In many mercury-resistant bacteria, which are predominantly aerobes, genes encoding Hg resistance (*mer* genes) reside on plasmids or transposons (◀ Sections 6.2 and 9.11). Mer genes are arranged in an operon under control of the regulatory protein MerR, which can function as either a repressor or an activator of transcription (◀ Section 7.3), depending on Hg availability. In the absence of Hg^{2+}, MerR functions as a *repressor* and binds to the operator region of the *mer* operon, thus preventing transcription of the structural genes, *merTPABD*. However, when Hg^{2+} is present, it forms a complex with MerR, which then binds to the *mer* operon and functions as an *activator* of transcription of *mer* structural genes (Figure 21.23).

MerP is a periplasmic mercuric ion–binding protein; it binds Hg^{2+} and transfers it to the membrane transport protein MerT, which interacts with mercuric reductase (MerA) to reduce Hg^{2+} to Hg^0 (Figure 21.23*b*). Thus, Hg^{2+} is not released into the cytoplasm and the final result is the release of Hg^0 from the cell. Mercuric ion

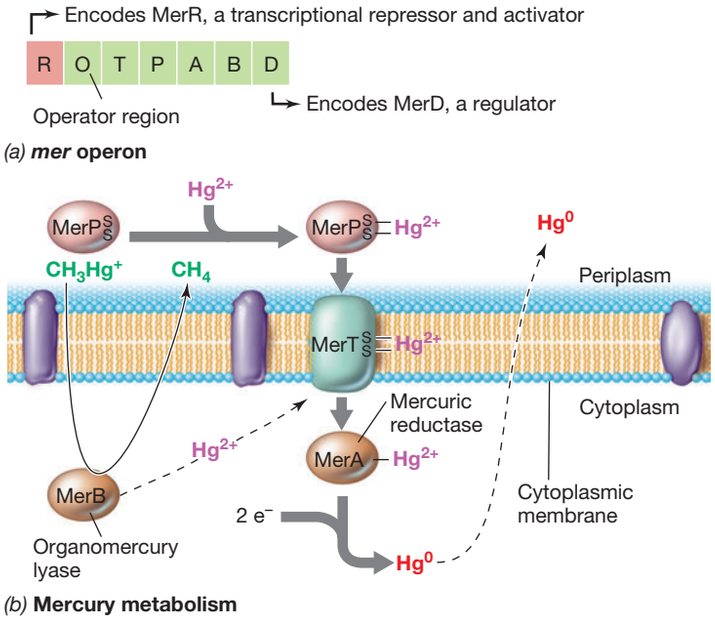


Figure 21.23 Mechanism of mercury transformations and resistance. *(a)* The *mer* operon. MerR can function as either a repressor (in the absence of Hg^{2+}) or a transcriptional activator (in the presence of Hg^{2+}). *(b)* Transport and reduction of Hg^{2+} and CH_3Hg^+; the Hg^{2+} is bound by cysteine residues in the MerP and MerT proteins. MerA is the enzyme mercuric reductase and MerB is organomercury lyase.

produced from the activity of MerB is trapped by MerT and reduced by MerA, again releasing Hg^0 (Figure 21.23*b*). In this way, Hg^{2+} and CH_3Hg^+ are converted to the relatively nontoxic Hg^0, which is nonpolar and exits the cell through the cytoplasmic membrane.

Check Your Understanding

- What forms of mercury are most toxic to organisms?
- How is mercury methylated by microbes?
- How is mercury detoxified by bacteria?

21.9 Human Impacts on the Carbon and Nitrogen Cycles

Human activities have major effects on the carbon and nitrogen cycles, and these effects have significance for the health of our planet in general. The period of marked human influence on these nutrient cycles began with the Industrial Revolution in the 1800s and is informally termed the *Anthropocene*, a new geological epoch. Although the greatest human impacts have been on the release of CO_2 through the burning of fossil fuels (oil, gas, and coal) and from extensive and ongoing deforestation, human activity has also profoundly affected the nitrogen cycle. We discussed earlier the close coupling of the carbon and nitrogen cycles (Section 21.1), and here we consider some of the projected biogeochemical consequences of human alteration of these two critical nutrient cycles.

CO_2, Other Trace Gases, and Global Warming

Atmospheric CO_2 levels have increased approximately 40% since the beginning of the Industrial Revolution and are now higher than at any time in the last 800,000 years. In mid-2019, atmospheric levels of CO_2 reached 415 parts per million—its highest level in human history (**Figure 21.24**). Carbon dioxide is one of several *trace gases* (primarily water vapor, CO_2, CH_4, and N_2O) that comprise less than 0.5% of the atmosphere but contribute significantly to terrestrial and atmospheric warming due to the *greenhouse effect*, the ability of these gases to trap the infrared radiation emitted by Earth. Atmospheric concentrations of all of these trace gases are rapidly increasing as a consequence of human activities (Figure 21.24). The change in global warming potential resulting from the addition of these gases to the atmosphere is expressed as **radiative forcing**, defined as the difference between sunlight energy absorbed by Earth and energy radiated back to space, measured in watts per square meter of Earth's surface ($W\ m^{-2}$; Figure 21.24*d*). Radiative forcing is used to calculate an Annual Greenhouse Gas Index (AGGI), defined as the ratio of the total direct radiative forcing due to long-lived greenhouse gases for a given year to that which was present in 1990 (the baseline year for the Kyoto Protocol for controlling greenhouse gas emissions).

In 2018, the AGGI was 1.43, an increase in radiative forcing of 43% since 1990. Climate models incorporate both the radiative forcing values and the atmospheric lifetimes of the major greenhouse gases. Although methane and N_2O have greater radiative forcing per unit molecule than CO_2 (58- and 206-fold, respectively), they are at much lower concentrations and therefore contribute less to warming (Figure 21.24*d*). CO_2 is the major contributor to the AGGI in terms of both amount and rate of increase. The atmospheric lifetime of a molecule of N_2O is around 150 years. In contrast, there is more uncertainty about the atmospheric lifetimes of methane and CO_2, now estimated at about 10 and 120 years, respectively. The long lifetimes of N_2O and CO_2 mean that once these gases are added to the atmosphere, they will impact Earth's climate for centuries. In contrast, the much shorter lifetime of methane indicates that any reductions in emissions would have a much more immediate impact on warming.

Also shown in Figure 21.24*d* is radiative forcing attributed to chlorofluorocarbons (CFCs). There is no natural source of CFCs. These compounds were chemically synthesized for use as refrigerants, aerosol propellants, and cleaning solvents. Following the discovery that they were destroying stratospheric ozone, their manufacture was effectively suspended through an international agreement, the Montreal Protocol on substances that deplete the ozone layer, which entered into force on January 1, 1989. Although the levels of major CFCs now appear to be declining, their long atmospheric lifetimes mean they will remain in the atmosphere for over 100 years. Unfortunately, analyses have identified a 25% increase in CFC release originating in eastern Asia since 2012, pointing to the difficulties in enforcing international Earth-benefiting agreements.

CO_2 and Its Effects on Aquatic Microbial Ecosystems

The increase in atmospheric CO_2 concentration, measured across a global network of sampling stations (Figure 21.24*a*), is currently about 2 parts per million per year. This increase would be much more rapid were it not for the high solubility of CO_2 in water, which produces carbonic acid (H_2CO_3); much anthropogenic CO_2 thus dissolves in the oceans (Figures 21.1 and 21.20*c*). The surface waters

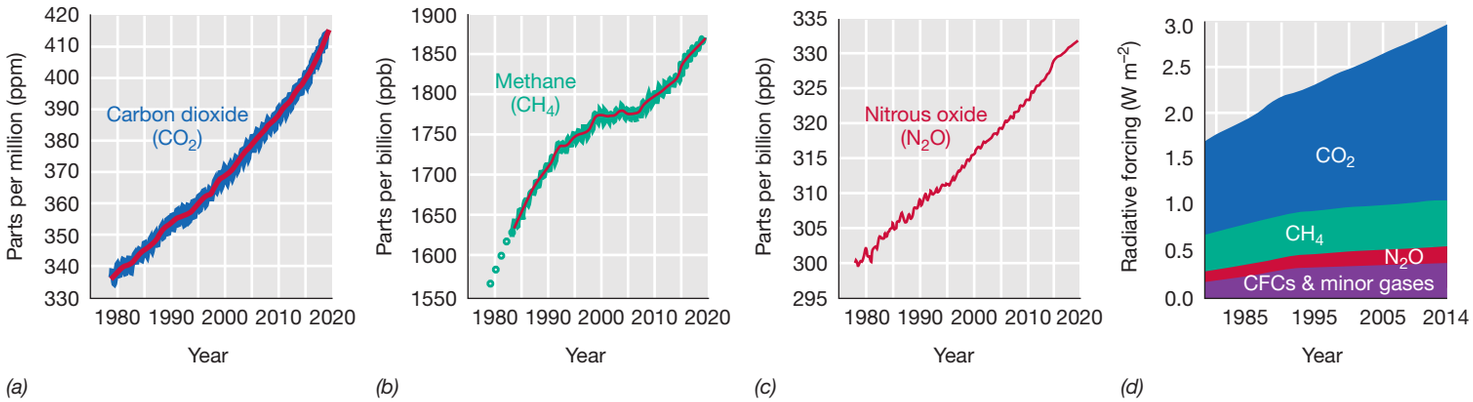


Figure 21.24 Past 40-year increases in greenhouse gases and associated radiative forcing. Global average values for *(a)* CO_2, *(b)* CH_4, *(c)* N_2O. Increases in CO_2 have averaged 1.4 ppm per year before 1995 and 2 ppm per year thereafter. *(d)* Radiative forcing from chlorofluorocarbons (CFCs) is now stable or declining as a result of the Montreal Protocol banning production of substances that deplete the ozone layer. Greenhouse gas measurements are continuously collected by the National Oceanic and Atmospheric Association on Mauna Loa, Hawaii.

of the oceans have taken up an estimated 500 billion tons of CO_2 from the atmosphere out of a total of 1300 billion tons of total anthropogenic emissions, thus modulating the greenhouse effect somewhat. The increase in average Earth air temperature, estimated to have increased 0.75°C in the twentieth century and projected to increase by anywhere from 1.1 to 6.4°C in the twenty-first century, would also have been more rapid without the heat absorption of the oceans. Since three orders of magnitude more energy is required to raise the temperature of a cubic meter of water than a cubic meter of air, it can be calculated that over 80% of the heat retained on Earth as a result of the greenhouse effect thus far has actually entered the ocean.

Although there is considerable uncertainty about the consequences of ocean warming and CO_2 consumption on Earth's biological systems, there is agreement on how these changes will affect biogeochemistry. Warmer ocean surface waters are more buoyant because of their lower density than are deeper waters. Thus, as occurs seasonally in lakes (◀ Section 20.9), the oceans will become more stratified with future global warming. Stratification tends to slow the transfer of nutrients from deeper waters that are needed to nourish phototrophic microbes at the base of the food web in surface waters. This in turn reduces ocean productivity and export of a portion of that production to the deeper ocean through sedimentation (the biological pump, Figure 21.1).

Ocean warming is also contributing to the expansion of oxygen minimum zones (OMZs), regions of naturally occurring low O_2 concentration in subsurface waters between 100 and 1000 m in depth (◀ Section 20.10). OMZs are a consequence of both the reduced solubility of O_2 in warmer water and the increasing stratification associated with surface warming, which reduces mixing of surface and subsurface waters. Animals will be excluded from the expanding OMZs in contrast to the enhancement of anaerobic microbial processes, such as denitrification and anammox, that directly influence the nitrogen cycle and production of the greenhouse gas N_2O.

Acidification of the ocean resulting from the ongoing dissolution of anthropogenic CO_2 has reduced ocean pH by 0.1 pH units since the beginning of the Industrial Revolution and may further reduce the pH by 0.3–0.4 units by the year 2100. The ongoing reduction in carbonate (CO_3^{2-}) concentration, a consequence of increasing acidification, is expected to be detrimental to marine calcifiers (organisms synthesizing $CaCO_3$ shells or skeletons, Figure 21.20). Since the concentration of Ca in seawater is relatively constant, continued reduction in CO_3^{2-} will ultimately reach a point where the dissolution of existing $CaCO_3$ is chemically favored, ultimately releasing more dissolved CO_2 (Figure 21.20), which reduces the capacity of the oceans to absorb more atmospheric CO_2.

Although the biological response to ocean acidification is unknown, it is likely that coral reef ecosystems, a major component of the marine biosphere (▶ Section 23.13), will cease to occur naturally on Earth if CO_2 emissions continue at their present rate (Figure 21.24*a* and see the chapter opener on page 729). Calcification in foraminifera (◀ Section 18.6) will likely be impaired significantly by ocean acidification, as will calcification in coccolithophores (Figure 21.20; ◀ Section 18.7). Over periods of a century or so, the invasion of anthropogenic CO_2 into the deep ocean will ultimately result in a significant reduction in the levels of $CaCO_3$ sequestered there, and this will likely affect the carbon cycle in major but as yet unpredictable ways.

Methane and Global Warming: Wetlands, Permafrost, and Sea Ice

Increases in atmospheric methane have contributed to about one-fifth of the increase in radiative forcing since 1750. There are many natural sources of methane as well as several sources linked to human activities (**Figure 21.25**). Major natural sources of methane are wetlands (including melting permafrost), ruminants, and rice cultivation. Analysis of the stable isotopic composition of new atmospheric methane (◀ Section 19.10) suggests that the uptick since 2007 is primarily fueled by emissions from tropical wetlands, the world's largest natural source of atmospheric methane (Figure 21.25). On the human side, fossil fuel and biomass burning make up nearly a quarter of all methane emissions, and the steadily increasing numbers of domesticated ruminants used as a food source constitute a major source of methane as well.

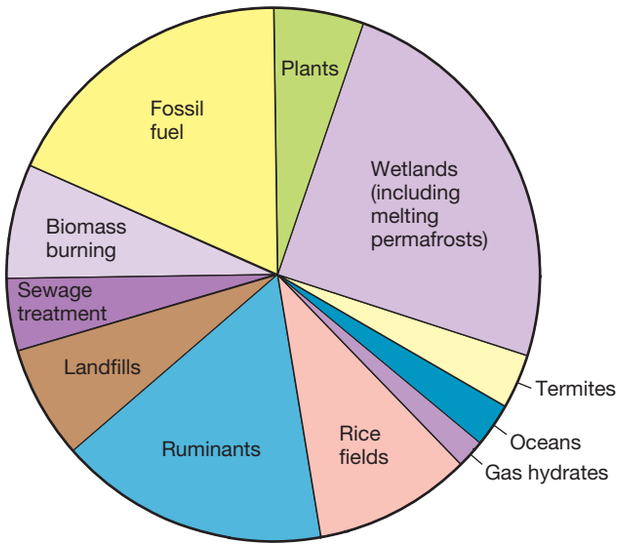


Figure 21.25 Major sources of global methane emissions. Total methane emissions are 500–600 million metric tons per year.

Ongoing planetary warming will also increase atmospheric inputs of methane by destabilizing coastal methane hydrates (Figure 21.4) and by the melting of permafrost. That is, there is *positive climate feedback* linked to methane release. As Earth warms in response to the accumulation of greenhouse gases, attention has focused on high-latitude regions where temperatures are rising twice as fast as the global average. As temperatures rise, normally frozen ground (the permafrost subsurface layer in tundra) thaws, making vast stores of organic carbon available for microbial decomposition. Accumulated over thousands of years from past plant and animal life, the pool of carbon in the permafrost zone is estimated to be 1300–1600 billion tons. This is about 50% of global soil carbon and twice as much carbon as is currently in the atmosphere.

Predicting the rate and extent of microbial conversion of permafrost organic carbon to greenhouse gases (primarily CO_2 and CH_4) is essential in developing future climate scenarios. It is estimated that 5–15% of permafrost carbon will decompose in this century, releasing primarily CO_2 at a rate unlikely to cause abrupt climate change in the near future (a portion of the methane will be oxidized to CO_2 by methanotrophs). However, thawing permafrost will impact global climate for centuries, with current models estimating that about 60% of total permafrost carbon emissions will occur past the year 2100. Predicting the end result of such releases on the habitability of Earth is impossible at this point except to say that microbial responses to continued warming will unquestionably occur and will have consequences for all life on Earth.

An additional positive climate feedback is associated with the rapid loss of summer sea ice in the Arctic Ocean, reduced by more than 11% since 1979. Instead of reflecting back most sunlight as ice does, the sea ice melt has opened a new expanse of dark open water to absorb the sun's energy. This extra energy input, and associated flux of moisture and heat to the Arctic atmosphere, is contributing to a strong local positive feedback called the *Arctic amplification*. As a result, surface temperatures in the Arctic are rising twice as fast as at lower latitudes and should increase releases of methane from hydrates and of methane and CO_2 from permafrost accordingly. The enormous loads of organic carbon in the Arctic place a special focus on this region as an area where microbial activities will continue to surge significantly and accelerate global warming.

Anthropogenic Effects on the Nitrogen Cycle

Anthropogenic impacts on the microbial ecology of the nitrogen cycle are nearly as profound as those on the carbon cycle. The yearly industrial production of nitrogenous fertilizers through the Haber–Bosch process, which combines $N_2 + H_2$ to form NH_3 under high temperature and pressure, is now comparable to the amount of fixed nitrogen entering the biosphere through biological nitrogen fixation, a key link in the nitrogen cycle (Section 21.3). Most of the industrially produced N is used in agriculture, and a significant fraction runs off to the oceans and contributes to coastal eutrophication (◀ Section 20.10). Large amounts are also lost as gaseous nitrogen compounds (N_2, N_2O, and NO), primarily from nitrification of NH_3 and denitrification of NO_3^- (Section 21.3). N_2O emission is now increasing at a rate of 0.2–0.3% per year (Figure 21.24*c*). Agriculture—including the microbial breakdown of manure and urine—contributes about 80% of N_2O emissions in the United States. Lesser amounts come from motor vehicle emissions and industrial production of fertilizers and nitrogen-based polymers.

Transport of N from industrial and agricultural centers through the atmosphere fertilizes both terrestrial and marine systems and could cause one of at least two effects. On the one hand, if deposition suppresses microbial nitrogen fixation, this would to some degree mitigate the fertilization effect. On the other hand, a greater supply of both CO_2 and iron (caused by greater deposition of dust from areas of increasing desertification, ◀ Sections 20.6 and 20.7) along with increased N depositions could enhance primary production, since iron is also often a limiting nutrient. Either way, major effects on the carbon cycle should be expected from human inputs in the nitrogen cycle.

Although changes in Earth's biosphere from human intervention in microbial nutrient cycles are a certainty, precisely what these changes will be is less clear. However, because major nutrient cycles are closely coupled (Section 21.1 and Figure 21.6), it is likely that the significant changes already in play in the carbon and nitrogen cycles will have feedback effects on other nutrient cycles as well. Collectively, these events could upset the balance and interrelationships of Earth's nutrient cycles in general—cycles driven by the activities of microbial communities keenly attuned to their environments—and have significant (and likely negative) consequences for plants, animals, and humans.

Check Your Understanding

- What is the greenhouse effect, and what causes it?
- How does nitrogen fixed by human processes contribute to an imbalance of the global nitrogen cycle?
- Why are the OMZs expanding, and what are the likely impacts on nutrient cycles?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Carbon, Nitrogen, and Sulfur Cycles

21.1 The oxygen and carbon cycles are interconnected through the complementary activities of autotrophic and heterotrophic organisms. Microbial decomposition is the single largest source of CO_2 released to the atmosphere.

Q Which metabolic class of microorganisms is considered primary producers, and where are they typically found in nature?

21.2 Under anoxic conditions, organic matter is degraded to CH_4 and CO_2. Methane is formed primarily from acetate and the reduction of CO_2 by H_2. The H_2 and acetate are supplied by syntrophic bacteria that depend on H_2 consumption by a partner as the basis of their energetics.

Q How can organisms such as *Syntrophobacter* and *Syntrophomonas* grow when their metabolism is based on thermodynamically unfavorable reactions? How does coculture of these syntrophs with certain other bacteria allow them to grow?

21.3 The principal form of nitrogen on Earth is N_2, which can be used as a N source only by nitrogen-fixing bacteria. Ammonia produced by nitrogen fixation or by ammonification can be assimilated into organic matter or oxidized to NO_3^-. Denitrification and anammox cause major losses of fixed nitrogen from the biosphere.

Q Compare the two major pathways of anaerobic microbial N_2 formation in terms of the organisms and intermediates involved as well as their relevance in the context of greenhouse gas formation.

21.4 Bacteria play major roles in both the oxidative and reductive sides of the sulfur cycle. Sulfur- and sulfide-oxidizing bacteria produce SO_4^{2-}, whereas sulfate-reducing bacteria consume SO_4^{2-}, producing H_2S. Because sulfide is toxic and reacts with various metals, SO_4^{2-} reduction is an important biogeochemical process. Dimethyl sulfide is the major organic sulfur compound of ecological significance in nature.

Q If sulfur chemolithotrophs had never evolved, would there be a problem in the microbial cycling of sulfur compounds? What impact would coastal marine eutrophication have on microbial sulfur transformations?

II • Other Nutrient Cycles

21.5 Iron and manganese exist naturally in two oxidation states: Fe^{2+}/Fe^{3+} and Mn^{2+}/Mn^{4+}, and typically as insoluble minerals. Bacteria reduce the oxidized metals in anoxic environments, having evolved mechanisms for exporting electrons to the insoluble electron acceptors outside the cell to carry out the anaerobic respiration.

Q Compare the systems employed by *Geobacter* and *Shewanella* for reducing metals outside the cell.

21.6 The reduced forms of iron and manganese can be oxidized by a wide variety of bacteria. Ferrous iron is soluble at acidic pH, but at neutral pH, Fe^{2+} and Mn^{2+} are only soluble under anoxic conditions, and thus organisms that oxidize these metals must live at anoxic–oxic interfaces.

Q Contrast the habitats of the iron-oxidizing bacteria *Acidithiobacillus* and *Gallionella*.

21.7 P, Ca, and Si are elements cycled by microbial activities, primarily in aquatic environments. Calcium and silicon play important roles in the biogeochemistry of the oceans as components of the exoskeletons of coccolithophores and diatoms, respectively.

Q In what ways are Ca and Si cycling in ocean waters similar, and in what ways do they differ? How do the calcium and silicon cycles couple to the carbon cycle?

III • Humans and Nutrient Cycling

21.8 A major toxic form of Hg in nature is CH_3Hg^+, which can yield Hg^{2+} that is reduced by bacteria to Hg^0. Genes conferring resistance to the toxicity of Hg, such as those that encode enzymes that can detoxify or pump out the metal, often reside on plasmids or transposons.

Q Where do Hg-resistant genes reside? How are they controlled?

21.9 Anthropogenic inputs of CO_2 and reactive nitrogen are impacting major nutrient cycles. Although some consequences are reasonably well understood, including expansion of oxygen minimum zones (OMZs) and impaired growth of calcareous organisms, the long-term changes to the nutrient cycles that sustain Earth's biosphere remain unclear.

Q Describe the Haber–Bosch process used to make fertilizers and the relative amounts of fixed nitrogen it produces.

APPLICATION QUESTIONS

1. Compare and contrast the carbon, sulfur, and nitrogen cycles in terms of the physiologies of the organisms that participate in each cycle. Which physiologies are part of one cycle but not another?
2. ^{14}C-labeled cellulose is added to a vial containing some anoxic freshwater lake sediments and sealed under anoxic conditions. A few hours later, $^{14}CH_4$ appears in the vial. Discuss what has happened to yield such a result.
3. Methane and nitrous oxide are important greenhouse gases that contribute to global warming and climate change. Discuss how bacteria can help in reducing methane and nitrous oxide emissions.

CHAPTER GLOSSARY

Denitrification anaerobic respiration in which NO_3^- or NO_2^- is reduced to nitrogen gases, primarily N_2

Global warming the warming of the atmosphere and oceans attributed to anthropogenic release of greenhouse gases, primarily CO_2, that trap infrared radiation emitted by Earth, ultimately leading to major changes in climate

Humus dead organic matter, some of which functions as electron shuttles for the microbial reduction of metal oxides

Radiative forcing the difference between sunlight energy absorbed by Earth and energy radiated back to space

Syntrophy a process whereby two or more microorganisms cooperate to degrade a substance neither can degrade alone

22 Microbiology of the Built Environment

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MICROBIOLOGYNOW

Sending Microbes to Clean Up after Polluters

The chlorinated solvents tetrachloroethene and trichloroethene have long been used as degreasing agents, dry cleaning fluids, and chemical feedstocks. Unfortunately, their improper disposal has contributed to extensive contamination of groundwater at thousands of sites throughout the United States. Since these solvents can be widely dispersed in the subsurface, the only feasible approach to their cleanup is to deploy microbes that can degrade them in place, a process called *in situ bioremediation*.

Bioremediation of chlorinated contaminants exploits the ability of some bacteria to use these chemicals as electron acceptors through reductive dechlorination, a form of anaerobic respiration. Although dehalogenating bacteria are common, most fail to remove all of the chlorines, leaving the even more toxic and carcinogenic products dichloroethylene and vinyl chloride behind. Thus, the discovery of the bacteria *Dehalococcoides* and *Dehalogenimonas* that can completely dechlorinate these solvents and produce the benign gas ethene was critical to effective cleanup.

If these dehalogenating bacteria are naturally present, then *biostimulation* by injecting organic carbon is used to trigger bioremediation. However, if these microbes are absent, *bioaugmentation* (the addition of microorganisms expressing a desired activity) is needed to jump-start cleanup. At a remediation site, large numbers of *Dehalococcoides* are injected along with an inexpensive carbon source such as molasses (shown here in the photo of a contaminated manufacturing site). Fermentation of the molasses by various bacteria produces the acetate and H_2 needed by *Dehalococcoides* to dehalogenate the solvents. It was initially unclear how long *Dehalococcoides* would continue to degrade solvents remaining in the groundwater after injection stopped. However, environmental microbiology research showed that these physiologically specialized microbial cleanup agents remain active for at least four years after injection is stopped, all the time continuing to protect humans from our past poor environmental stewardship.

Source: Schaefer, C.E., Lavorgna, G.M., Haluska, A.A., and Annable, M.D. 2018. Long-term impacts on groundwater and reductive dechlorination following bioremediation in a highly characterized trichloroethene DNAPL source area. *Groundwater Monit. Rem. 38*: 65.

In this chapter we address the microbiology of "built" systems. These include the infrastructure for drinking water and wastewater distribution and treatment, gas and oil transmission, building materials, private and public spaces, and environments modified for mineral extraction or for the cleanup of pollutants. By their very nature, built systems create new microbial habitats, and these promote both desired and undesired microbial activities. From the standpoint of microbial ecology, these activities are simply the natural result of microbes exploiting resources provided to them.

Microbial activity in the built environment is the proverbial double-edged sword. Examples of built systems that promote *desirable* microbial activities include biological reactors for the treatment of wastewater and the stimulation of microbial activity in aquifers to clean up environmental pollutants. By contrast, an *undesirable* activity is microbially influenced corrosion of the pipelines used for the transmission of wastewater, drinking water, and oil, activities that destroy billions of dollars' worth of essential infrastructure yearly in the United States alone.

I • Mineral Recovery and Acid Mine Drainage

It is often remarked that microorganisms are "Earth's greatest chemists," and the activities of these tiny biochemical factories have been exploited in many ways. Here we consider how microbial activities help extract valuable metals from low-grade ores, and the environmental consequences thereof.

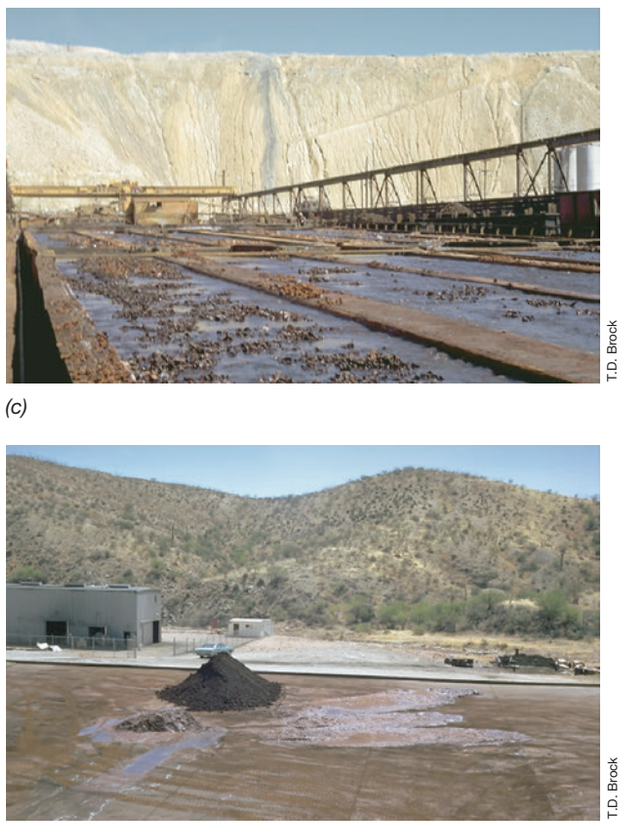
22.1 Mining with Microorganisms

One of the most common forms of iron in nature is **pyrite** (FeS_2), which is often present in coal and in metal ores. Sulfide (HS^-) also forms insoluble minerals with many metals, and many ores mined as sources of these metals are sulfide ores. If the concentration of metal in the ore is low, it may be economically feasible to mine the ore only if the desired metals are first concentrated by **microbial leaching** (Figure 22.1). The promotion of acid production and dissolution of FeS_2 by acidophilic bacteria such as *Acidithiobacillus ferrooxidans* is used to leach the metal ores in large-scale mining operations. Leaching is especially useful for copper ores because copper sulfate ($CuSO_4$), formed during the oxidation of copper sulfide ores, is very water-soluble. Indeed, approximately a quarter of all copper mined worldwide is obtained by microbial leaching.

The Leaching Process

The susceptibility to oxidation varies among minerals, and those minerals that are most readily oxidized are most amenable to microbial leaching. Thus, iron and copper sulfide ores such as pyrrhotite (FeS) and covellite (CuS) are readily leached, whereas lead and molybdenum ores are much less so. In microbial leaching, low-grade ore is dumped in a large pile called the *leach dump* and a dilute sulfuric acid solution at pH 2 is percolated down through the pile (Figure 22.1*a*). The liquid emerging from the bottom of the pile (Figure 22.1*b*) is rich in dissolved metals and is transported to a precipitation plant (Figure 22.1*c*) where the desired metal is precipitated and purified (Figure 22.1*d*). The liquid is then pumped back to the top of the pile and the cycle repeated. As needed, acid is added to maintain an acidic pH.

(a) (b)



(c) (d)

Figure 22.1 The leaching of low-grade copper ores using iron-oxidizing bacteria. *(a)* A typical leaching dump. The low-grade ore has been crushed and dumped in such a way that the surface area exposed is as high as possible. Pipes distribute the acidic leach water over the surface of the pile. The acidic water slowly percolates through the pile and exits at the bottom. *(b)* Effluent from a copper leaching dump. The acidic water is very rich in Cu^{2+}. *(c)* Recovery of copper as metallic copper (Cu^0) by passage of the Cu^{2+}-rich water over metallic iron in a long flume. *(d)* A small pile of metallic copper removed from the flume, ready for further purification.

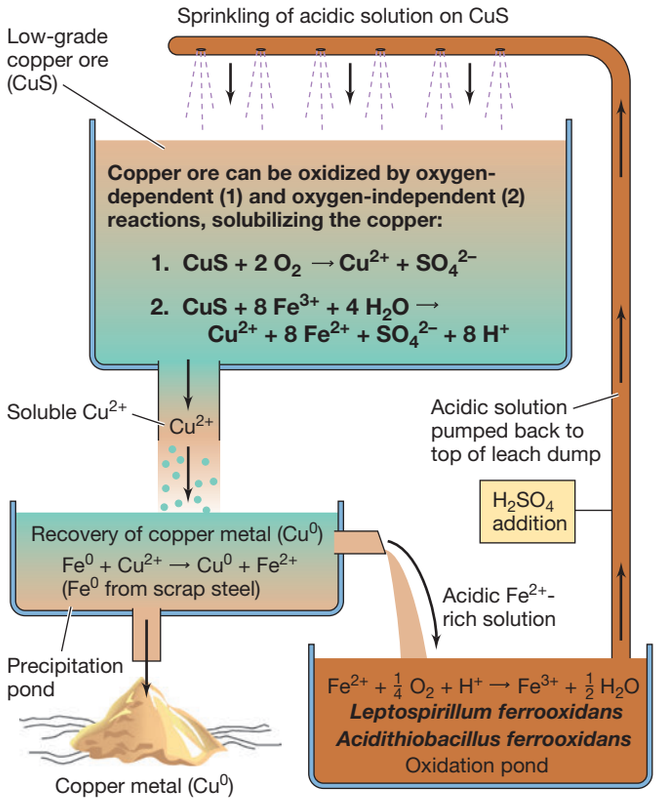


Figure 22.2 Arrangement of a leaching pile and reactions in the microbial leaching of copper sulfide minerals to yield metallic copper. Reaction 1 occurs both biologically and chemically. Reaction 2 is strictly chemical and is the most important reaction in copper-leaching processes. For reaction 2 to proceed, it is essential that the Fe^{2+} produced from the oxidation of sulfide in CuS to sulfate be oxidized back to Fe^{3+} by iron chemolithotrophs (see chemistry in the oxidation pond).

We illustrate microbial leaching of copper with the common copper ore CuS, in which copper exists as Cu^{2+}. *A. ferrooxidans* oxidizes the sulfide in CuS to SO_4^{2-}, releasing Cu^{2+} as shown in **Figure 22.2**. However, this reaction can also occur spontaneously. Indeed, the key reaction in copper leaching is actually not the bacterial oxidation of sulfide in CuS but the spontaneous oxidation of sulfide by ferric iron (Fe^{3+}) generated from the bacterial oxidation of ferrous iron (Fe^{2+}) (Figure 22.2). In any copper ore, FeS_2 is also present, and its oxidation by bacteria leads to the formation of Fe^{3+}. The spontaneous reaction of CuS with Fe^{3+} proceeds in the absence of O_2 and forms Cu^{2+} plus Fe^{2+} (Figure 22.2).

Mastering Microbiology
Art Activity: Figure 22.2 Arrangement of a leaching pile and reactions in the microbial leaching of copper sulfide minerals to yield metallic copper

Metal Recovery

The precipitation plant is where the Cu^{2+} from the leaching solution is recovered (Figure 22.1*c*, *d*). Shredded scrap iron (a source of elemental iron, Fe^0) is added to the precipitation pond to reduce Cu^{2+} (abiotically) and recover metallic copper (Cu^0) from the leach liquid by the chemistry shown in the lower part of Figure 22.2. This produces a Fe^{2+}-rich liquid that is pumped to a shallow oxidation pond where iron-oxidizing chemolithotrophs oxidize the Fe^{2+} to Fe^{3+}. This now ferric-iron-rich acidic liquid is pumped to the top of the pile and the Fe^{3+} is used to oxidize more CuS (Figures 22.1 and 22.2). The entire CuS leaching operation is thus driven by the oxidation of Fe^{2+} to Fe^{3+} by iron-oxidizing bacteria.

Temperatures rise in a leaching dump and this leads to shifts in the iron-oxidizing microbial community. *A. ferrooxidans* is a mesophile, and when heat generated by microbial activities raises temperatures above about 30°C inside a leach dump, this bacterium is outcompeted by mildly thermophilic, iron-oxidizing chemolithotrophic *Bacteria* such as *Leptospirillum ferrooxidans* and *Sulfobacillus*. At even higher temperatures (60–80°C), hyperthermophilic *Archaea* such as *Sulfolobus* (◀ Section 17.10) predominate in the leach dump.

Other Microbial Leaching Processes: Uranium and Gold

Bacteria are also used in the leaching of uranium (U) and gold (Au) ores. In uranium leaching, *A. ferrooxidans* oxidizes U^{4+} to U^{6+} with O_2 as an electron acceptor. However, U leaching depends more on the abiotic oxidation of U^{4+} by Fe^{3+} with *A. ferrooxidans* contributing to the process mainly through the reoxidation of Fe^{2+} to Fe^{3+}, as in copper leaching (Figure 22.2). The reaction observed is as follows:

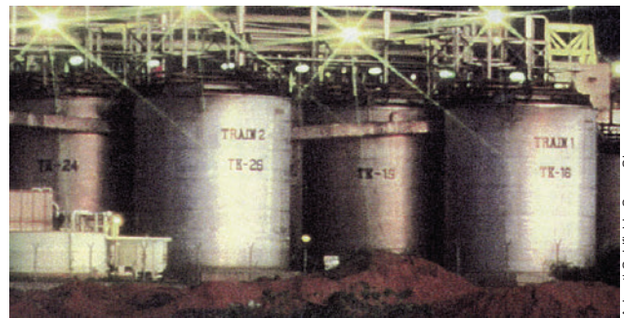
$$\underset{(U^{4+})}{UO_2} + \underset{(Fe^{3+})}{Fe_2(SO_4)_3} \rightarrow \underset{(U^{6+})}{UO_2SO_4} + \underset{(Fe^{2+})}{2\,FeSO_4}$$

Unlike UO_2, the uranyl sulfate (UO_2SO_4) formed is highly soluble and is concentrated by other processes.

Gold is typically present in nature in deposits associated with minerals containing arsenic (As) and FeS_2. *A. ferrooxidans* and related bacteria can leach the arsenopyrite minerals, releasing the trapped Au:

$$2\,FeAsS[\,Au\,] + 7\,O_2 + 2\,H_2O + H_2SO_4 \rightarrow Fe_2(SO_4)_3 + 2\,H_3AsO_4 + [\,Au\,]$$

The Au is then complexed with cyanide (CN^-) by traditional gold-mining methods. Unlike copper leaching, which is done in a huge dump (Figure 22.1*a*), gold leaching is done in small bioreactor tanks (**Figure 22.3**), where more than 95% of the trapped Au can be released. Moreover, the potentially toxic As and CN^- residues from the mining process are removed in the gold-leaching bioreactor. Arsenic is removed as a ferric precipitate, and CN^- is removed by its bacterial oxidation to CO_2 plus urea in later stages of the Au recovery process. Small-scale microbial-bioreactor leaching has thus become popular as an alternative to the environmentally devastating gold-mining techniques that leave a toxic trail of As and CN^- at the extraction site. Pilot processes are also being developed for bioreactor leaching of zinc, lead, and nickel ores.



Ashanti Goldfields Corp., Ghana

Figure 22.3 Gold bioleaching. Gold leaching tanks in Ghana (Africa). Within the tanks, a mixture of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* solubilizes the pyrite/arsenic mineral containing trapped gold, which releases the gold.

We now examine the other side of the coin—environmental damage from microbial mining.

Check Your Understanding

- What is required to oxidize CuS under anaerobic conditions?
- What key role does *Acidithiobacillus ferrooxidans* play in the copper leaching process?

22.2 Acid Mine Drainage

Although microbial leaching has tremendous value in mining operations, the same process has contributed to extensive environmental destruction where mining operations improperly handle or dispose of pyrite-containing coal and mineral deposits. Bacterial and spontaneous oxidation of sulfide minerals is the major cause of **acid mine drainage**, an environmental problem worldwide caused by surface mining operations. As described for the oxidation of copper sulfides promoted in microbial mining (Section 22.1), the oxidation of FeS_2 is a combination of chemically and bacterially catalyzed reactions, and two electron acceptors participate in the process: O_2 and Fe^{3+}. When FeS_2 is first exposed in a mining operation (**Figure 22.4*a, b***), a slow chemical reaction with O_2 begins (Figure 22.4*c*). This reaction, called the *initiator reaction*, leads to the oxidation of HS^- to SO_4^{2-} and the development of acidic conditions as Fe^{2+} is released. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* then oxidize Fe^{2+} to Fe^{3+}, and the Fe^{3+} formed under these acidic conditions, being soluble, reacts spontaneously with more FeS_2 and oxidizes the HS^- to sulfuric acid (H_2SO_4), which immediately dissociates into SO_4^{2-} and H^+:

$$FeS_2 + 14\,Fe^{3+} + 8\,H_2O \rightarrow 15\,Fe^{2+} + 2\,SO_4^{2-} + 16\,H^+$$

Again, the bacteria oxidize Fe^{2+} to Fe^{3+}, and this Fe^{3+} reacts with more FeS_2. Thus, there is a progressive, rapidly increasing rate at which FeS_2 is oxidized, called the *propagation cycle* (Figure 22.4*c*). Under natural conditions some of the Fe^{2+} generated by the bacteria leaches away and is subsequently carried by anoxic groundwater into surrounding streams. However, bacterial or spontaneous oxidation of Fe^{2+} then takes place in the aerated streams, and because O_2 is present, the insoluble $Fe(OH)_3$ is formed.

As we have seen (Figure 22.4*c*), the breakdown of FeS_2 ultimately leads to the formation of H_2SO_4 and Fe^{2+}; in waters in which these products have formed, pH values can be lower than 1. Mixing of acidic mine waters into rivers (**Figure 22.5**) and lakes seriously degrades water quality because both the acid and the dissolved metals (iron, aluminum, and heavy metals such as cadmium and lead) are toxic to aquatic organisms.

The O_2 requirement for the oxidation of Fe^{2+} to Fe^{3+} explains how acid mine drainage develops. As long as the pyritic material is not mined, FeS_2 cannot be oxidized because O_2, water, and the bacteria cannot reach it. However, when a mineral or coal seam is exposed (Figure 22.4*b*), O_2 and water are introduced, making both spontaneous and bacterial oxidation of FeS_2 possible. The acid formed can then leach into surrounding aquatic systems (Figure 22.5).

Where acid mine drainage is extensive and Fe^{2+} levels high, a strongly acidophilic species of *Archaea*, *Ferroplasma*, is often present. This aerobic iron-oxidizing organism is capable of growth at pH 0 and at temperatures up to 50°C. Cells of *Ferroplasma* lack a cell wall and are phylogenetically related to *Thermoplasma*, also a cell-wall-lacking

Figure 22.5 Acid mine drainage from a surface coal-mining operation. The yellowish-red color is due to the precipitated iron oxides in the drainage (see Figure 22.4*c* for the reactions in acid mine drainage).

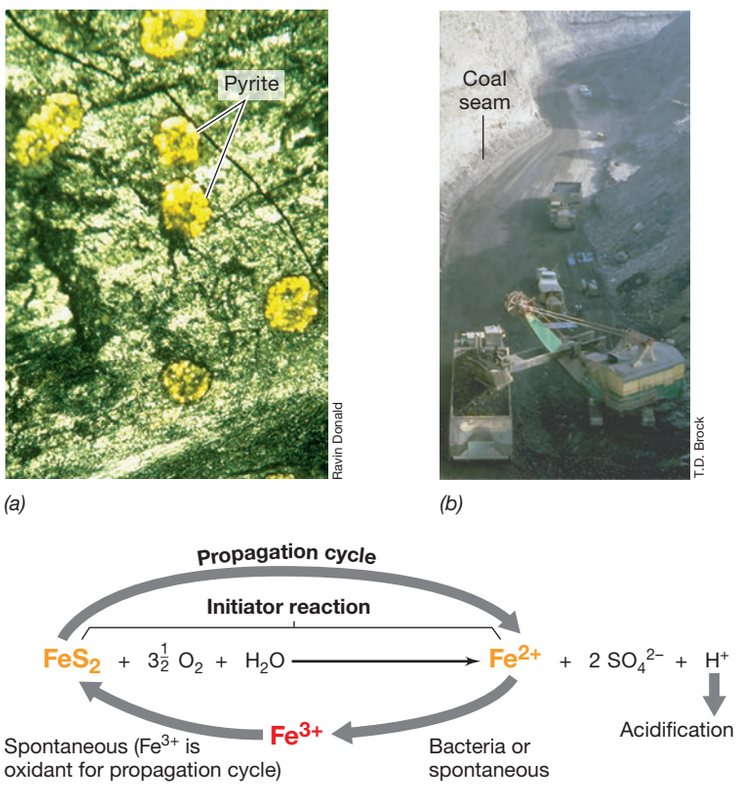


Figure 22.4 Coal and pyrite. *(a)* Coal from the Black Mesa formation in northern Arizona (USA); the gold-colored spherical discs (about 1 mm in diameter) are particles of pyrite (FeS_2). *(b)* A coal seam in a surface coal-mining operation. Exposing the coal to oxygen and moisture stimulates the activities of iron-oxidizing bacteria growing on the pyrite in the coal. *(c)* Reactions in pyrite degradation. The primarily abiotic initiator reaction sets the stage for the primarily bacterial oxidation of Fe^{2+} to Fe^{3+}. The Fe^{3+} attacks and oxidizes FeS_2 abiotically in the propagation cycle.

and strongly acidophilic (but chemoorganotrophic) member of the *Archaea* (◀ Section 17.3).

In Part II, we explore the use of microbes in cleaning up several undesirable agents that, one way or the other, end up in the environment.

Check Your Understanding

- In what oxidation state is iron in the mineral $Fe(OH)_3$? In FeS? How is $Fe(OH)_3$ formed?
- Natural pyritic deposits, such as underground coal seams, do not contribute to acid mine drainage; why not?

II • Bioremediation

Bioremediation is the microbial cleanup of oil, toxic chemicals, or other environmental pollutants by stimulating the degradative activities of indigenous microbes or by the addition of microbes having the needed degradative capacity. These pollutants include both natural materials, such as petroleum products, and synthetic chemicals new to nature.

Bioremediation is the process by which toxic or otherwise dangerous materials are cleaned up using microbes as the cleaning agents. Major successes thus far have been registered with spills of crude oil or the leakage of hydrocarbons from bulk storage tanks because the microbiology of hydrocarbon cleanup is well founded. But many other materials can be bioremediated, including chlorinated pollutants such as solvents and pesticides, and even radioactive waste in uranium-contaminated environments, a legacy of past mining of uranium for nuclear fuel and weapons. We begin with a consideration of this very toxic pollutant.

22.3 Bioremediation of Uranium-Contaminated Environments

Major classes of inorganic pollutants are metals and radionuclides that cannot be destroyed but only altered in chemical form. Often the extent of environmental pollution is so great that physical removal of the contaminated material is impossible. Thus, *containment* is the only real option, and a common goal in the bioremediation of inorganic pollutants is to change their mobility, making them less likely to move in groundwater and contaminate surrounding environments. The containment of uranium is a prime example.

Bioremediation of Uranium

Uranium contamination of groundwater has occurred at sites in the United States and elsewhere where uranium ores have been processed or stored (**Figure 22.6**), and the movement of radioactive materials offsite via groundwater is a threat to environmental and human health. Because the contamination is often widespread, making mechanical methods of recovery very expensive, microbiologists have joined forces with engineers to develop biological treatments that exploit the ability of some bacteria to reduce U^{6+} to U^{4+}. Uranium as U^{6+} is soluble, whereas U^{4+} forms an immobile uranium mineral called *uraninite* (UO_2), thus limiting the movement of U into groundwater and potential contact with humans and other animals.

Bacterial Transformations of Uranium

In order to change the oxidation state of U in major uranium contaminants to a form that will stabilize the element, *Bacteria*, including metal-reducing *Shewanella* and *Geobacter* species (◀ Section 15.13) and sulfate-reducing *Desulfovibrio* species (◀ Section 15.11), couple the oxidation of organic matter and H_2 to the reduction of U^{6+} to U^{4+}.

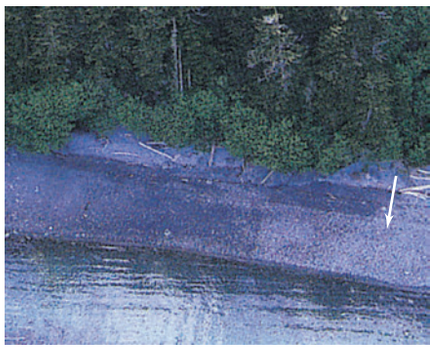
Field studies in which organic electron donors such as acetate have been injected into uranium-contaminated aquifers to stimulate U^{6+} reduction have shown that this can lower U levels to below the U.S. Environmental Protection Agency's drinking water standard of 0.126 μM. Natural levels of organic matter are typically very low in the subsurface (◀ Section 20.8), but can be sufficient to support microbial activities including anaerobic respiration using U^{6+} as electron acceptor. However, even though uraninite is stable under reducing conditions, if conditions become oxic, it reoxidizes (primarily abiotically by manganese oxide minerals) and once again becomes mobile.

Ken Williams

Figure 22.6 Uranium bioremediation. An experimental plot at a United States Department of Energy uranium-contaminated site. Organic carbon (acetate) is being infused into the site (see inset photo) and travels in groundwater in the direction of the arrow shown in the main photo. Acetate is an electron donor for reduction of U^{6+} to U^{4+}, which immobilizes the uranium.

US Environmental Protection Agency

(a)


US Environmental Protection Agency

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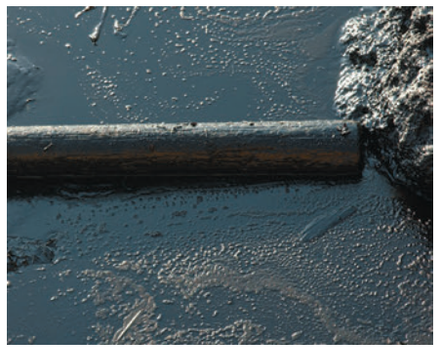

Bassam Lahoud, Lebanese American University

(c)

Figure 22.7 Environmental consequences of large oil spills and the effect of bioremediation. *(a)* A contaminated beach along the coast of Alaska containing oil from the *Exxon Valdez* spill of 1989. *(b)* The rectangular plot (arrow) was treated with inorganic nutrients to stimulate bioremediation of spilled oil by microorganisms, whereas areas above and to the left were untreated. *(c)* Oil spilled into the Mediterranean Sea from the Jiyeh (Lebanon) power plant that flowed to the port of Byblos during the 2006 war in Lebanon.

Much ongoing uranium bioremediation research is focused on questions of whether microbially reduced uranium is stable if the composition of the microbial community changes or if oxidants, such as O_2, NO_3^-, and Fe^{3+}, are introduced via groundwater. This is obviously an important question because uraninite stability must be targeted for the long term in order to account for the long half-life of nuclear decay of uranium; U^{238}, the most common isotope of uranium, has a half-life approximately equal to the age of Earth (4.5 billion years)!

Check Your Understanding

- Which reaction, oxidation or reduction, is key to uranium bioremediation?
- Why is immobilization a good strategy for dealing with uranium pollution?

22.4 Bioremediation of Organic Pollutants: Hydrocarbons

Organic pollutants, unlike inorganic pollutants, can generally be completely degraded by microorganisms, eventually to CO_2. This is true of petroleum released in oil spills (**Figure 22.7**), which can be attacked by many different microorganisms. These organisms have been exposed to complex mixtures of hydrocarbons through natural or accidental oil and gas seeps (◀ Figures 20.23 and 20.24) for millennia and thus have evolved the catabolic machinery necessary to degrade this naturally occurring pollutant. In contrast, artificial pollutants (xenobiotics, see Section 22.5) tend to be more persistent and are degraded by more specialized groups of microorganisms. In this section we focus on hydrocarbons and in the next section on xenobiotics.

Petroleum and Hydrocarbon Bioremediation

Petroleum is a rich source of organic matter, and because of this, microorganisms readily attack hydrocarbons when petroleum is pumped to Earth's surface and comes into contact with air and moisture. Under some circumstances, such as in bulk petroleum storage tanks, microbial growth is undesirable. However, in oil spills, biodegradation is desirable and can be promoted by the addition of inorganic nutrients to balance the huge influx of organic carbon from the oil (Figure 22.7*b*).

The biochemistry of hydrocarbon catabolism was covered in Sections 14.23 and 14.24. Both aerobic and anaerobic biodegradation is possible. We emphasized that under oxic conditions, oxygenase enzymes play an important role in introducing oxygen atoms into the hydrocarbon. Our discussion here will focus on *aerobic* processes because it is only when O_2 is present that oxygenase enzymes can function and hydrocarbon bioremediation can be effective in a relatively short time.

Diverse bacteria, fungi, and a few green algae can oxidize petroleum products aerobically. Small-scale oil pollution of aquatic and terrestrial ecosystems from human as well as natural activities is common. Oil-oxidizing microorganisms develop rapidly on oil films and slicks, and hydrocarbon oxidation is most extensive if the temperature is warm enough and supplies of inorganic nutrients (primarily N and P) are sufficient. Moreover, because oil is insoluble in water and is less dense, it floats to the surface and forms slicks. There, hydrocarbon-degrading bacteria attach to the oil droplets (**Figure 22.8**) and eventually decompose the oil and disperse the slick. Certain oil-degrading bacteria are specialist species. For example, the bacterium *Alcanivorax borkumensis* grows only on hydrocarbons, fatty acids, or pyruvate. This organism produces surfactant chemicals that help break up the oil and solubilize it. Once solubilized, the oil can

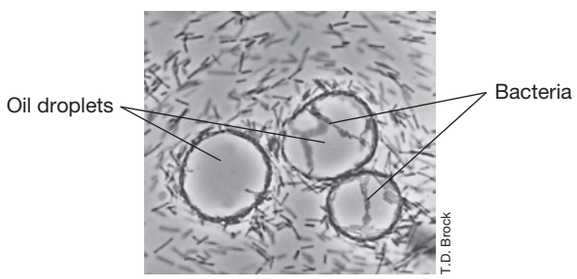


Figure 22.8 Hydrocarbon-oxidizing bacteria in association with oil droplets. The bacteria are concentrated in large numbers at the oil–water interface but are actually not within the droplet itself.

be incorporated more readily and catabolized as an electron donor and carbon source.

In large surface oil spills such as those shown in Figure 22.7, volatile hydrocarbons, both aliphatic and aromatic, evaporate quickly without bioremediation, leaving nonvolatile components for cleanup crews and microorganisms to tackle. Microorganisms consume oil by oxidizing it to CO_2. When bioremediation activities are promoted by inorganic nutrient application, oil-oxidizing bacteria typically develop quickly after an oil spill (Figure 22.7*b*), and under ideal conditions, 80% or more of the nonvolatile oil components can be oxidized within one year. However, certain oil fractions, such as those containing branched-chain and polycyclic hydrocarbons, are not preferred microbial substrates and remain in the environment much longer. Spilled oil that finds its way into sediments is even more slowly degraded and can have a significant long-term impact on fisheries that depend on unpolluted waters for productive yields.

A notable exception to the more common surface spill of oil was the 2010 sinking of the Deepwater Horizon offshore drilling platform in the Gulf of Mexico, resulting in the rupture of the wellhead at a depth of 1.5 km and the release of over 4 million barrels (635 million liters) of oil and hydrocarbon gases into the deep ocean (◀ Section 20.10 and Figure 20.23). About 35% of the resulting hydrocarbon plume was comprised of low-molecular-weight components and natural gas (methane, ethane, propane). The availability of these more easily degraded oil components likely accelerated the natural degradation process by stimulating the development of a bloom of bacteria having the capacity to oxidize both the easily degraded and more recalcitrant hydrocarbon components. Chemical dispersants were also added directly to the oil plume to accelerate microbial degradation, and much of the oil eventually disappeared through a combination of volatilization and microbial activities.

Degradation of Stored Hydrocarbons

Interfaces where oil and water meet often form on a large scale. Besides water that separates from crude petroleum during storage and transport, moisture can condense inside bulk fuel storage tanks (**Figure 22.9**) where there are leaks. This water eventually accumulates in a layer beneath the petroleum. Gasoline and crude oil storage tanks are thus potential habitats for hydrocarbon-oxidizing microorganisms. If sufficient sulfate (SO_4^{2-}) is present in the oil, as is often the case with crude oils, sulfate-reducing bacteria can grow in the tanks, consuming hydrocarbons under anoxic conditions (◀ Sections 14.12, 14.24, and 15.11). The sulfide (H_2S) produced is highly corrosive and causes pitting and subsequent leakage of the tanks along with souring of the fuel. Aerobic degradation of stored fuel components is less of a problem because the storage tanks are sealed and the fuel itself contains little dissolved O_2.

Figure 22.9 Bulk petroleum storage tanks. Fuel tanks often support microbial growth at oil–water interfaces.

Check Your Understanding

- Why do petroleum-degrading bacteria need to attach to the *surface* of oil droplets?
- What is unusual about the physiology of the bacterium *Alcanivorax*?

22.5 Bioremediation and Microbial Degradation of Major Chemical Pollutants: Chlorinated Organics and Plastics

Unlike hydrocarbons, many chemicals that humans put into the environment have never been there before; these are **xenobiotics**, substances not previously present on Earth and new to microbial communities.

Catabolism of Organohalogens: The Chlorinated Organics

Major chemical pollutants are primarily xenobiotics. These include pesticides, polychlorinated biphenyls (PCBs), munitions, dyes, and chlorinated solvents, among many other chemicals. Some xenobiotics differ chemically in such major ways from anything organisms have experienced in nature that they are slow to biodegrade. In these cases, human intervention is often required to promote and accelerate degradation. We focus here on microbial degradation of major chlorinated compounds that are among the most common of environmental pollutants, either through widespread targeted application, as for pesticides, or through improper disposal or manufacture of chemicals used primarily in industrial applications.

Microorganisms that degrade chlorinated compounds are widespread in nature and evolved a natural halogen cycle long before the introduction of manufactured chemicals. Some natural chlorinated compounds originate from forest fires and volcanic eruptions. Others are the products of microbial synthesis of specific bioactive compounds, including haloalkanes and secondary metabolites produced by algae, fungi, bacteria, plants, and some animals. These likely provide the producing organisms a competitive advantage over predators or competitors, but few have been fully characterized. Today, well over 5000 different natural organohalogens have been identified and some are being studied for use in clinical medicine. A notable example is a bioactive secondary metabolite produced by the marine actinobacterium *Salinispora tropica*; the metabolite, salinosporamide A (**Figure 22.10**), is in

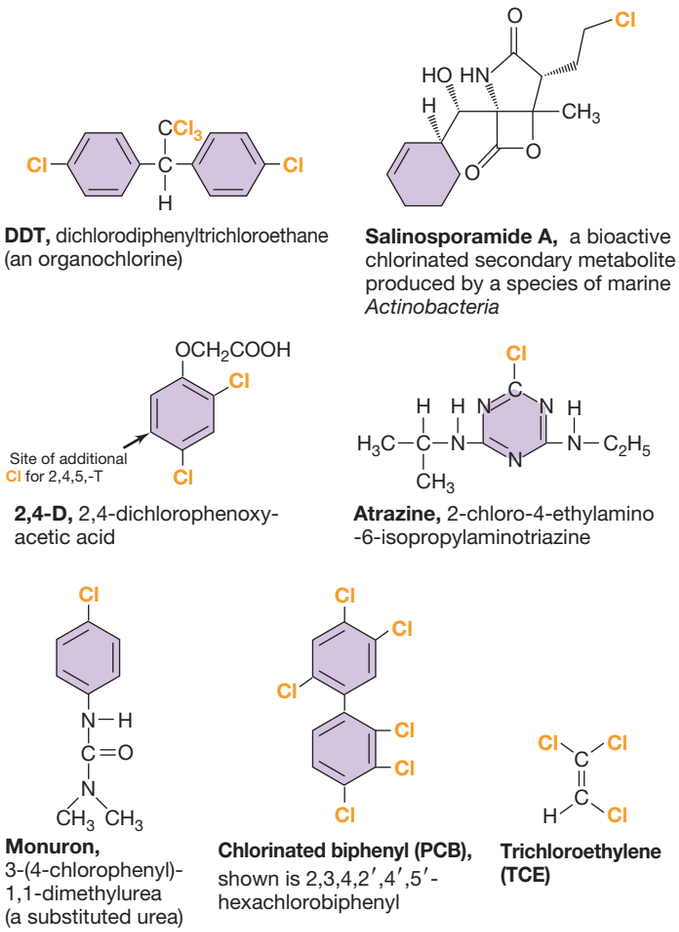


Figure 22.10 Examples of xenobiotic compounds. Although none of these compounds is a natural compound (except for salinosporamide A), microorganisms exist that can break them down.

clinical development for treatment of myeloma, a cancer of white blood cells.

The microbial synthesis and degradation of naturally occurring organohalogens provides a rich natural source of enzymes for the degradation of the manufactured organohalogens that now pollute the environment. Although some organohalogens are degraded only very slowly, most function as either electron donors or terminal electron acceptors for microbial growth, and virtually all are metabolized by microorganisms.

Over 1000 pesticides have been marketed worldwide and include *herbicides, insecticides*, and *fungicides*. Pesticides display a wide variety of chemistries and include chlorinated, aromatic, and nitrogen- and phosphorus-containing compounds (Figure 22.10 and see Explore the Microbial World, "Solving the Marine Methane Paradox," in Chapter 21). Highly chlorinated compounds are typically the pesticides most resistant to microbial attack. For example, chlorinated compounds such as DDT persist relatively unaltered for years in soils, whereas compounds such as 2,4-D (Figure 22.10) are significantly degraded in just a few weeks. Environmental factors, such as temperature, pH, aeration, and organic content of the soil, influence the rate of pesticide decomposition, and some pesticides can disappear from soils nonbiologically by volatilization, leaching, or spontaneous chemical breakdown. In addition, some pesticides are degraded only when other organic material is present that can be used as the primary energy source, a phenomenon called *cometabolism*. In many cases, pesticides that are cometabolized are only partially degraded, generating new xenobiotic compounds that may be even more toxic or difficult to degrade than the original compound. Thus, from an environmental standpoint, cometabolism of a pesticide is not always a good thing.

Aerobic and Anaerobic Dechlorination

The microbial degradation of chlorinated xenobiotics (Figure 22.10) is generally initiated by *dechlorination*. In aerobes, dechlorination is most often catalyzed by halogenase enzymes having specific substrate requirements. For example, a specific monooxygenase catalyzes removal of the first chlorine from the aromatic ring of the insecticide pentachlorophenol (**Figure 22.11**). This is followed by reductive elimination of two additional chlorines, dioxygenase ring cleavage, and elimination of the final chlorine to generate products that enter the citric acid cycle (◀ Section 3.6). In contrast to this very specific pathway for catabolism of pentachlorophenol, oxidative degradation of trichloroethylene (TCE) and vinyl chloride is initiated by cometabolism in which a relatively nonspecific monooxygenase generates an unstable epoxide that decomposes abiotically to form harmless products (see Figure 22.12).

Although the aerobic breakdown of chlorinated xenobiotics is undoubtedly ecologically important, **reductive dechlorination** may be even more so because of the rapidity with which anoxic conditions develop in polluted microbial habitats. We previously described reductive dechlorination as a form of anaerobic respiration in which chlorinated organic compounds such as chlorobenzoate ($C_7H_4O_2Cl^-$) are terminal electron acceptors that when reduced release chloride (Cl^-), a nontoxic substance (◀ Section 14.13).

Many compounds can be reductively dechlorinated, including dichloro-, trichloro-, and tetrachloro- (perchloro-) ethylene,

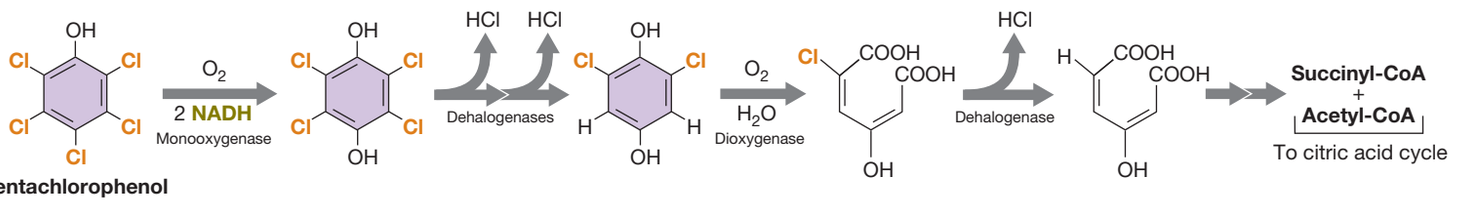


Figure 22.11 Pentachlorophenol metabolism by *Sphingobium chlorophenolicum*. A monooxygenase catalyzes the first chlorine removal from pentachlorophenol, followed by two reductive dehalogenase steps before the aromatic ring is cleaved by a dioxygenase. A subsequent dehalogenation reaction yields a product that can enter central metabolism.

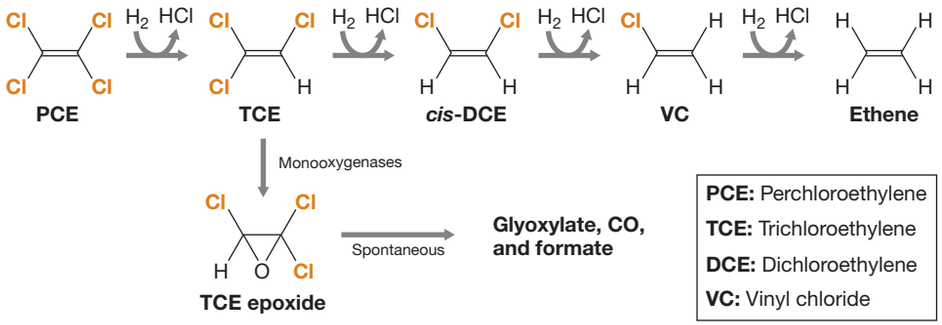


Figure 22.12 Anaerobic and aerobic pathways for PCE and TCE degradation. Top panel: Anaerobic pathway for reductive dechlorination by *Dehalococcoides* species. Lower panel: cometabolic epoxide formation and abiotic product decay catalyzed aerobically by a monooxygenase enzyme. Monooxygenases generate unstable epoxides of both TCE and PCE.

chloroform, dichloromethane, and polychlorinated biphenyls (some of these are shown in Figure 22.10). In addition, several brominated and fluorinated organic compounds can be dehalogenated in analogous fashion. Many of these halogenated compounds are highly toxic and some have been linked to cancer (particularly trichloroethylene and vinyl chloride). Some of these compounds, such as PCBs, have been widely used as insulators in electrical transformers and enter anoxic environments from slow leakage of the transformer or from leaking storage containers. Moreover, perchloroethylene (PCE) is commonly used as a dry-cleaning solvent. Eventually these compounds end up in groundwater or sediment, where they are among the most common contaminants detected in the United States. There is therefore great interest in reductive dechlorination as a bioremediation strategy for their removal from anoxic environments, as we now consider.

Bioremediation of PCE and TCE

The organohalogens TCE and PCE are poorly soluble, but both can be degraded aerobically by monooxygenase enzymes, such as methane monooxygenase of methanotrophic bacteria (◀ Section 14.16), to form nontoxic organic compounds (**Figure 22.12**). Early remediation efforts employed this activity to eliminate TCE from polluted sites. However, this is a cometabolic process that yields no energy for growth of methanotrophs, and maintaining activity required the continuous injection of both oxygen and methane—an explosive gas mixture—into the subsurface.

More successful remediation is now based on stimulating the growth of organisms capable of using organohalogens as electron acceptors (anaerobic respiration), thus avoiding the need to inject gases into the subsurface. Many facultative or obligate anaerobes capable of organohalogen respiration are known (**Figure 22.13**). *Obligate* organohalide-respiring bacteria grow only by organohalide respiration, using H_2 as an electron donor, and with the exception of *Dehalobacter* are members of the *Chloroflexi* (Figure 22.13). In contrast, *facultative* organohalide-respiring bacteria are more physiologically and phylogenetically diverse (Figure 22.13), being able to use organic electron donors and common electron acceptors such as Fe^{3+}, SO_4^{2-}, and NO_3^-.

Since the biologically available energy from organohalide respiration decreases with decreasing number of chlorines on the molecule, the much less favorable electron acceptor vinyl chloride (VC, Figure 22.12) often accumulates at polluted sites. This chemical is more toxic than its parent compounds, and remediation is successful only if conversion to the fully dechlorinated product, ethene, is achieved. Most organohalide-respiring bacteria are unable to fully dechlorinate PCE or TCE, but the bacterium *Dehalococcoides* can do this and supports a full bioremediation technology.

Successful bioremediation of PCE or TCE requires hydrogen (H_2) to stimulate the growth of *Dehalococcoides*. This is achieved by the injection of an inexpensive fermentable carbon source to the contaminated site, most commonly molasses or emulsified vegetable oil (EVO). Fermentation of these generates H_2 and acetate used as the electron donor for dehalogenation and as a carbon source, respectively, by *Dehalococcoides*. Nutrient (nitrogen and phosphate) injection coupled with groundwater extraction and recirculation accelerates solvent degradation. *Bioaugmentation*—the injection of an active *Dehalococcoides* culture into the subsurface—also accelerates the process or provides an inoculum if a resident *Dehalococcoides* population is absent (see MicrobiologyNow on page 754).

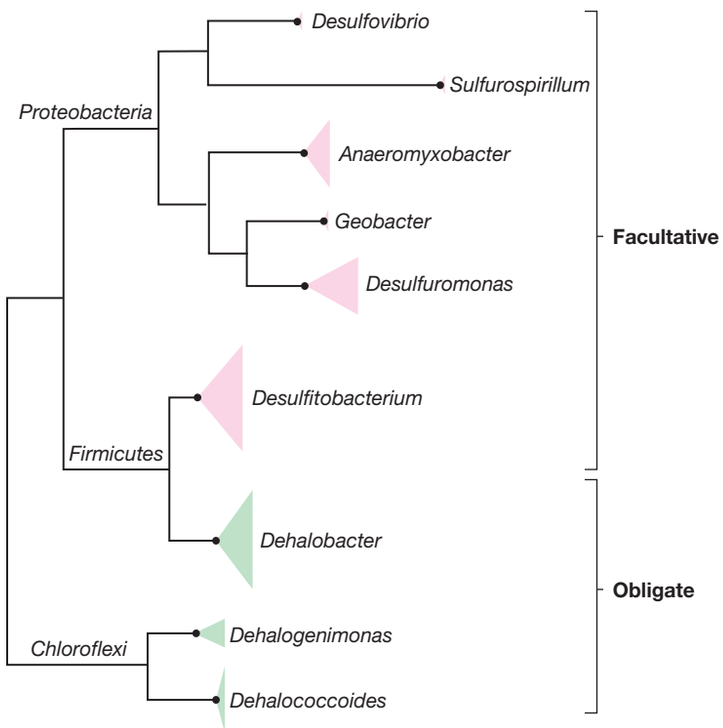


Figure 22.13 Phylogeny of organohalide-respiring bacteria. Facultative organohalide respirers use other electron acceptors, such as nitrate, in addition to organohalides, and a greater variety of electron donors, including pyruvate, lactate, ethanol, and butyrate. Obligate organohalorespirers are restricted to H_2 as electron donor and organohalides as electron acceptors.

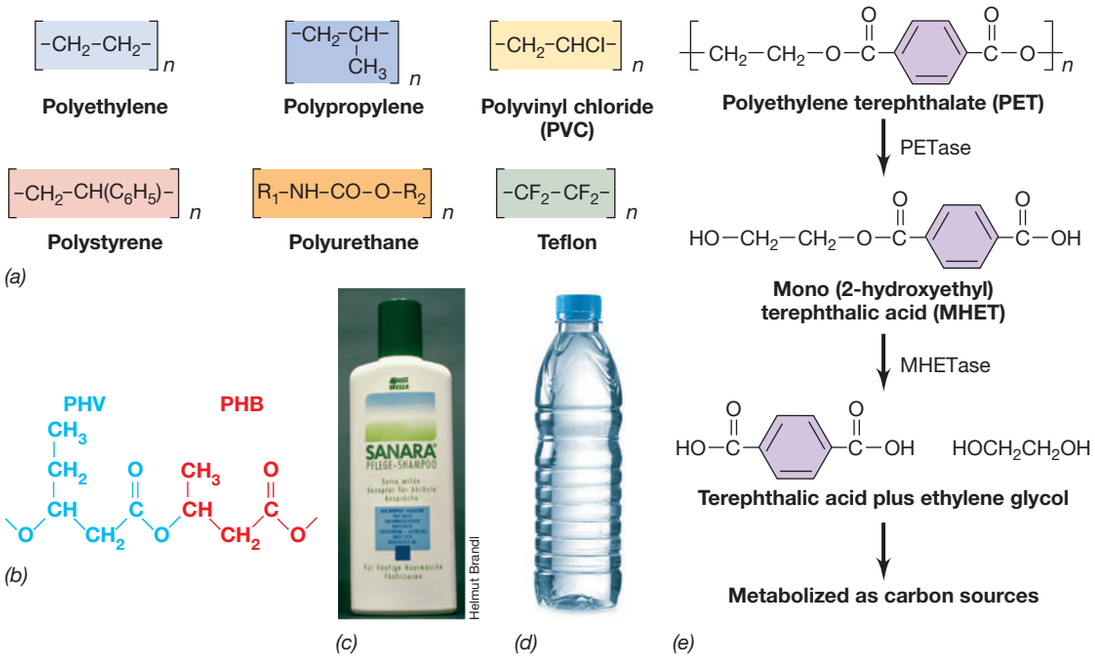


Figure 22.14 Oil-based and biodegradable plastics. *(a)* The monomeric structure of several synthetic plastics. *(b)* Structure of the copolymer of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV). *(c)* A brand of shampoo previously marketed in Germany and packaged in a bottle made of the PHB/PHV copolymer. *(d)* Drinking bottle made from PET, the only oil-based plastic now known to be significantly biodegraded by microorganisms. *(e)* Pathway of PET degradation by the bacterium *Ideonella sakaiensis* to generate monomers as a carbon and energy source.

Plastics

Plastics are classic examples of xenobiotics, and the plastics industry worldwide produces over 300 million tons of plastic per year, almost half of which are discarded rather than recycled. Plastics are polymers of various chemistries (**Figure 22.14**). Many plastics remain essentially unaltered for long periods in landfills, refuse dumps, and as litter in the environment. A significant amount of synthesized plastic enters the marine environment, and this is of particular environmental concern. Weathering of plastic debris in the ocean causes fragmentation into small particles called *microplastics* (◀ Section 20.4 and Figure 20.7) that marine invertebrates can ingest, possibly disrupting important marine food webs. Microplastics also clog the baleen filter used by baleen whales to harvest small marine invertebrates and fish.

Among commonly used plastics, only polyethylene terephthalate (PET) has hydrolyzable ester bonds that are susceptible to biodegradation (Figure 22.14*d, e*). Over 50 million tons of PET is produced yearly for plastic products such as soft drink and water bottles and polyester fabrics. Although the fungus *Fusarium* can slowly cometabolize PET to release terephthalic acid, the bacterium *Ideonella sakaiensis* (*Betaproteobacteria*, ◀ Section 16.2) can attach to the surface of PET, express enzymes that depolymerize the plastic (Figure 22.14*e*), and use PET as a carbon and energy source. Two hydrolase enzymes of *I. sakaiensis* depolymerize PET: the exoenzyme PETase releases mono(2-hydroxyethyl) terephthalic acid (MHET), which is converted into terephthalic acid and glycol by the cell surface enzyme MHETase (Figure 22.14*e*). These substances then support growth of *I. sakaiensis.* Since PET is the most common plastic used for bottled drinking water, discovery of the metabolic capacities of *I. sakaiensis* offers some hope that the tons of PET water bottles that have been casually disposed of into the environment in recent decades may eventually disappear through microbial action.

The persistence of plastic products in the environment has also fueled research into improved biodegradable alternatives to synthetic plastics including natural microbial polymers. Polyhydroxyalkanoates (PHAs) are a common bacterial storage polymer (◀ Section 2.7), are readily biodegraded, and have many of the desirable properties of synthetic plastics. PHAs can be biosynthesized in various chemical forms, each with its own unique physical properties (stiffness, shear and impact strength, and the like). A PHA *copolymer* containing equal amounts of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate was marketed in the past as a container for shampoo (Figure 22.14*b, c*). However, because synthetic plastics are currently less expensive than microbially synthesized plastics, plastics such as PET make up virtually the entire plastics market today.

Check Your Understanding

- Why might the addition of inorganic nutrients stimulate oil degradation whereas the addition of glucose would not?
- What is reductive dechlorination and how does it differ from the reactions shown in Figure 22.11?
- How does the bacterium *Ideonella sakaiensis* degrade an insoluble substance such as PET that it cannot transport into the cell?

UNIT 5

III • Wastewater and Drinking Water Treatment

Appropriate treatment of wastewater is essential for maintaining environmental quality and for reducing the spread of waterborne diseases. Methods to purify wastewaters rely on the activities of microbial communities that reduce levels of organic carbon, fixed nitrogen, and phosphorus from the waste stream.

Water is the most important potential common source of infectious diseases because a single water source often serves large numbers of people, as, for example, in major cities. Thus, the microbiology of water, water transport systems, and treatments of both

wastewater and drinking water are of the utmost importance to public health.

Here we examine systems built for the chemical and biological treatment of water and the transmission systems used for delivering treated water to consumers. Existing water transmission and treatment systems consume about 4% of all electricity in the USA, and we consider more efficient technologies now being brought into application. We also examine the human health consequences of the microbial communities that develop within the pipes of municipal water distribution systems and premise plumbing.

22.6 Primary and Secondary Wastewater Treatment

Wastewater is domestic sewage or liquid industrial waste that cannot be discarded in untreated form into lakes or streams because of public health, economic, environmental, and aesthetic considerations. Wastewater treatment employs physical and chemical methods as well as industrial-scale use of microorganisms. Wastewater enters a treatment plant and, following treatment, the **effluent water**—treated wastewater discharged from the wastewater treatment facility—is suitable for release into surface waters such as lakes and streams or to drinking water purification facilities (**Figure 22.15**).

Mastering Microbiology
Art Activity: Figure 22.13 Wastewater treatment processes

Wastewater and Sewage

Wastewater from sewage or industrial sources cannot be discarded in untreated form into lakes or streams. **Sewage** is liquid effluent contaminated with human or animal fecal materials and may contain potentially harmful chemicals as well as pathogenic microorganisms. Domestic wastewater is made up of sewage, "gray water" (the water resulting from washing, bathing, cooking, and drinking), and wastewater from small-scale food processing in homes and restaurants. Industrial wastewater includes liquid discharged from the petrochemical, pesticide, food and dairy, plastics, pulp and paper, pharmaceutical, metallurgical, and manufacturing industries. All of these wastewaters must be treated—typically using physical, chemical, and microbiological processes—to remove or neutralize contaminants.

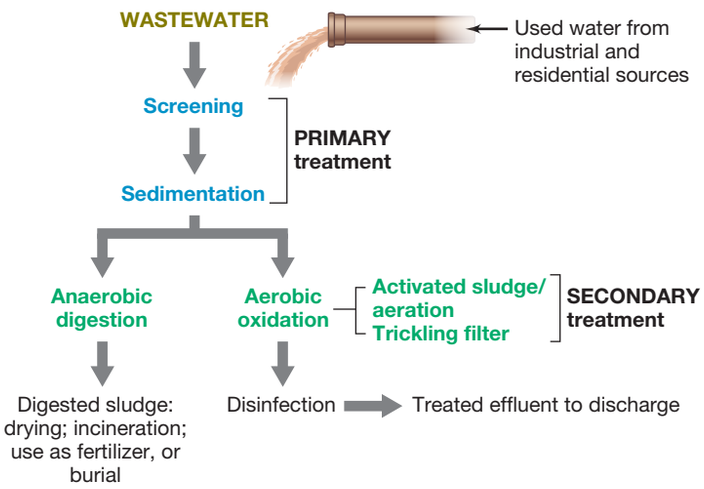


Figure 22.15 Wastewater treatment processes. Effective water treatment plants use the primary and secondary treatment methods shown here. Water from primary treatment undergoes aerobic oxidation, while the sludge collected from screening and sedimentation, along with flocs and activated sludge from aerobic oxidation of wastewater, undergoes anaerobic digestion (Section 22.8 and Figure 22.22). Tertiary treatment, not shown, may also be used to reduce nutrient levels in waters released to the environment, reducing biochemical oxygen demand (BOD), nitrogen, and phosphorus to very low to undetectable levels.

Industrial wastewater may contain highly toxic substances and are "pretreated" biologically or chemically to remove substances such as cyanide, heavy metals such as arsenic, lead, and mercury, and organic materials such as acrylamide and benzene. These substances are converted to less toxic forms by treatment with chemicals or microorganisms capable of neutralizing, oxidizing, precipitating, or volatilizing these wastes. The pretreated wastewater can then be released to the municipal wastewater treatment facility.

The major objective of wastewater treatment is to reduce organic and inorganic materials in wastewater to a level that no longer supports microbial growth and to eliminate other potentially toxic materials. The efficiency of treatment is expressed in terms of a reduction in the **biochemical oxygen demand (BOD)**, the relative amount of dissolved oxygen consumed by microorganisms to completely oxidize all organic and inorganic matter in a water sample (◀ Section 20.9). High levels of organic and inorganic materials in the wastewater result in a high BOD. Typical BOD values for domestic wastewater, including sewage, are approximately 200 mg of O_2 per liter. For industrial wastewater from sources such as dairy plants, the values can be as high as 1500 mg/liter. An efficient wastewater treatment facility reduces BOD levels to less than 5 mg/liter in the final treated water.

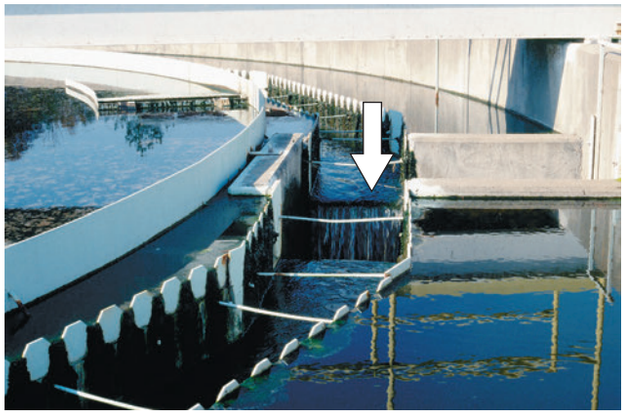
Primary Treatment: Physical Removal Processes

Wastewater treatment is a multistep operation that relies on several different physical and biological processes (Figure 22.15). *Primary*, *secondary*, and sometimes additional (*tertiary*) treatments are employed to reduce biological and chemical contamination in the wastewater, and each higher level of treatment employs more complex technologies.

Primary wastewater treatment uses a series of grates and screens to physically remove large particulate materials (bottles and other heavy solids) from the wastewater. The effluent is allowed to settle for a few hours and the solids that settle to the bottom of the separation reservoir are typically treated in an anaerobic digester that consumes these solids and generates methane for energy recovery (see Figure 22.22*b*). The effluent (**Figure 22.16**) is drawn off for secondary treatment.

Secondary Treatment: Activated Sludge and Trickling Filters

Municipalities that provide only primary treatment discharge extremely polluted water with high BOD into adjacent waterways, and this triggers undesirable microbial growth and a reduction in water quality. Thus, secondary treatment is a necessity. **Secondary wastewater treatment** uses oxidative degradation reactions



John M. Martinko and Deborah O. Jung

Figure 22.16 Primary treatment of wastewater. Wastewater is pumped into the reservoir (left) where solids settle. As the water level rises, the water spills through the grates to successively lower levels. Water at the lowest level, now virtually free of solids, enters the spillway (arrow) and is pumped to a secondary treatment facility.

catalyzed by microorganisms under *oxic* conditions (**Figure 22.17*a*, *b***) to treat wastewater containing relatively low levels of organic material. In general, wastewaters that originate from residential sources can be treated efficiently using only aerobic treatment. Secondary treatment exploits the tendency of bacteria to attach to particulate material and form biofilms (◀ Sections 4.9, 8.10, and 20.4), producing slimy, extracellular polymeric substances to form *activated sludge* flocs.

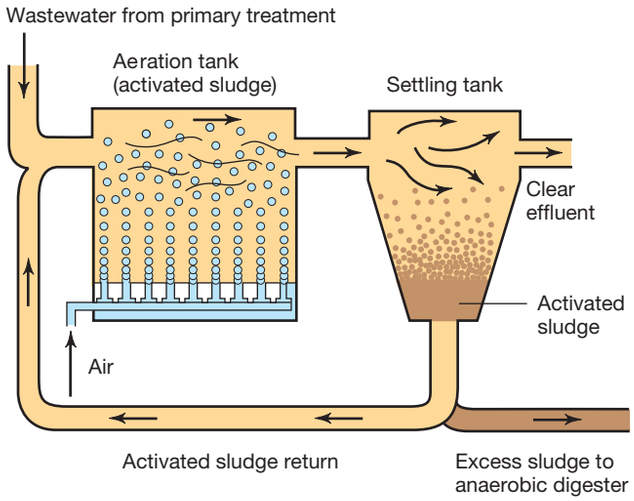
Activated sludge consists of a community of slime-forming aerobic bacteria, including in particular *Zoogloea ramigera* (*Betaproteobacteria*, ◀ Section 16.2) (**Figure 22.18**). Protists, small animals, filamentous bacteria, and fungi also attach to the flocs. The activated sludge is mixed, aerated, and circulated in the treatment tanks to promote oxidation of organic carbon by the bacteria (Figure 22.17*a*, *b*). The aerated effluent containing the flocs is then pumped into a holding tank or clarifier where the flocs settle. Some of the activated sludge is then returned to the aerator as inoculum for new wastewater, and the rest is commonly pumped to an anaerobic sludge digester (see Figure 22.22*c*). Because of the volumes of wastewater needing treatment, wastewaters normally stay in an activated sludge tank for 5–10 hours, insufficient for complete oxidation of all organic matter. However, during this time much soluble organic matter is adsorbed to the flocs and incorporated by microbial cells, reducing the BOD by up to 95% (most of the material with high BOD is now in the settled flocs). The flocs are then transferred to the anoxic sludge digester for anaerobic degradation to CO_2 and CH_4 (Figure 22.17*b*).

The *trickling filter* is an alternative method of aerobic secondary treatment (Figure 22.17*c*). A trickling filter is a bed of crushed rocks, about 2 m thick. Wastewater is sprayed on top of the rocks and slowly passes through the bed. The organic material in the wastewater adsorbs to the rocks, and microorganisms grow and form biofilms on the large, exposed rock surfaces. The complete mineralization of organic matter to CO_2, ammonia, nitrate, sulfate, and phosphate takes place in the extensive microbial biofilm that develops on the rocks.

Following secondary treatment, whether by activated sludge or trickling filter, and assuming tertiary treatment (Section 22.7) is

John M. Martinko and Deborah O. Jung

(a)



(b)

John M. Martinko and Deborah O. Jung

(c)

Figure 22.17 Secondary aerobic wastewater treatment processes. Parts *a* and *b* show the activated sludge method. *(a)* Aeration tank of an activated sludge installation in a metropolitan wastewater treatment plant. The tank is 30 m long, 10 m wide, and 5 m deep. *(b)* Wastewater flow through an activated sludge installation. Recirculation of activated sludge to the aeration tank introduces microorganisms responsible for oxidative degradation of the organic components of the wastewater. *(c)* Trickling filter method. The rotating arms distribute wastewater slowly and evenly on the rock bed. The rocks are 10–15 cm in diameter and the bed is 2 m deep.

Mastering Microbiology
Art Activity: Figure 22.15 Secondary aerobic wastewater treatment process

UNIT 5

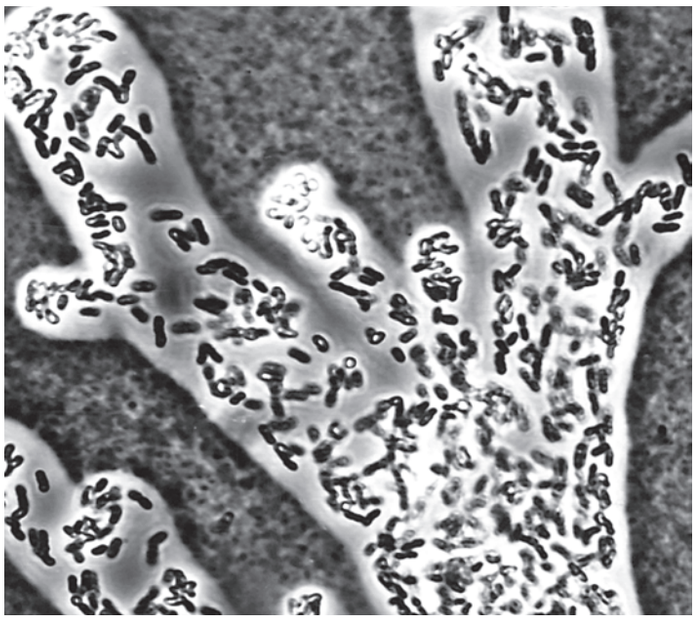


Figure 22.18 A wastewater floc formed by the bacterium *Zoogloea ramigera*. Floc formed in the activated sludge process consists of a large number of small, rod-shaped cells of *Z. ramigera* surrounded by a polysaccharide slime layer and arranged in characteristic fingerlike projections in this phase-contrast photomicrograph of a negatively stained preparation.

not undertaken, the effluent is typically chlorinated or treated with ozone to further reduce the possibility of biological contamination. The treated effluent can then be discharged into streams or lakes.

Check Your Understanding

- What is biochemical oxygen demand (BOD), and why is its reduction important in wastewater treatment?
- How do primary and secondary wastewater treatment methods differ?
- Other than treated water, what are the final products of wastewater treatment? How might these end products be used?

22.7 Tertiary Wastewater Treatment: Further Removal of Phosphorus and Nitrogen

The combination of primary and secondary wastewater treatments removes a large amount of the BOD and some of the inorganic nutrients from wastewaters. However, additional treatment can generate effluent water of much higher quality. Such advanced wastewater treatment is called **tertiary wastewater treatment**. Typical goals of tertiary wastewater treatment include removal of additional BOD, removal of key inorganic nutrients required for microbial growth (in particular, ammonia, nitrate, nitrite, and phosphorus), and degradation of any remaining toxic materials. Tertiary treatment is the most complete method of treating sewage and is common in many European countries but has not been widely adopted in other parts of the world because it is expensive. Here we focus on the biological removal of phosphorus and nitrogen, two key nutrients controlling microbial growth.

Biological Phosphorus Removal

Conventional secondary biological treatment removes only about 20% of phosphorus from wastewater, necessitating additional chemical or biological treatment. Chemical precipitation is the most commonly used process, removing up to 90% of the influent phosphorus. Removal is accomplished by the addition of either Fe or Al as chloride or sulfate salts. At near-neutral pH, Fe^{3+} forms insoluble ferric phosphate ($FePO_4$) or ferric hydroxide-phosphate complexes. These then precipitate and are removed as sludge.

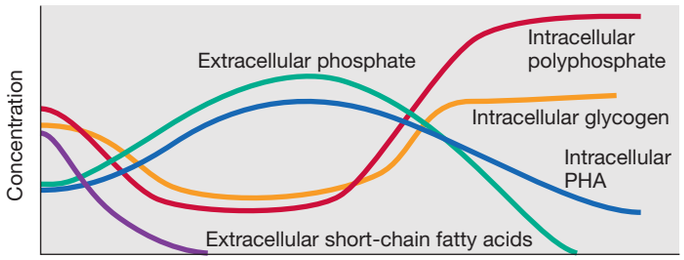
The chemical precipitation process results in a toxic sludge, contributing to additional disposal problems. As an alternative, tertiary treatment that encourages the growth of phosphorus-accumulating bacteria removes up to 90% of phosphorus, a process called *enhanced biological phosphorus removal*, and does not generate a metal-rich sludge. Here the waste stream is processed by sequential passage through anaerobic and aerobic bioreactors (**Figure 22.19**). During the *anaerobic* phase, *phosphorus-accumulating organisms* (PAOs) utilize polyphosphate (a polymer of phosphate, ◀ Section 2.7) and glycogen reserves to generate ATP and reducing power to fuel the uptake and conversion of volatile fatty acids in the wastewater into another storage material, polyhydroxyalkanoate (PHA) (Figures 22.14*b* and 22.19*a*; ◀ Section 2.7). In the subsequent *aerobic* phase, PAOs oxidize the PHA for cell growth and replenish polyphosphate and glycogen reserves. The new biomass (sludge) with high polyphosphate content is then collected for phosphorus removal (Figure 22.19*b*).

The ability to store carbon as PHA under anaerobic conditions and later use this intracellular reserve for aerobic growth allows the PAOs to outcompete other chemoorganotrophs that can only consume carbon aerobically. Two of the principal PAOs, the appropriately named bacterium *Accumulibacter phosphatis* (*Betaproteobacteria*, ◀ Section 16.2) and species of the gram-positive bacterium *Tetrasphaera* (*Actinobacteria*, ◀ Section 16.10), have been used in the laboratory as model systems for the process of enhanced biological phosphorus removal.

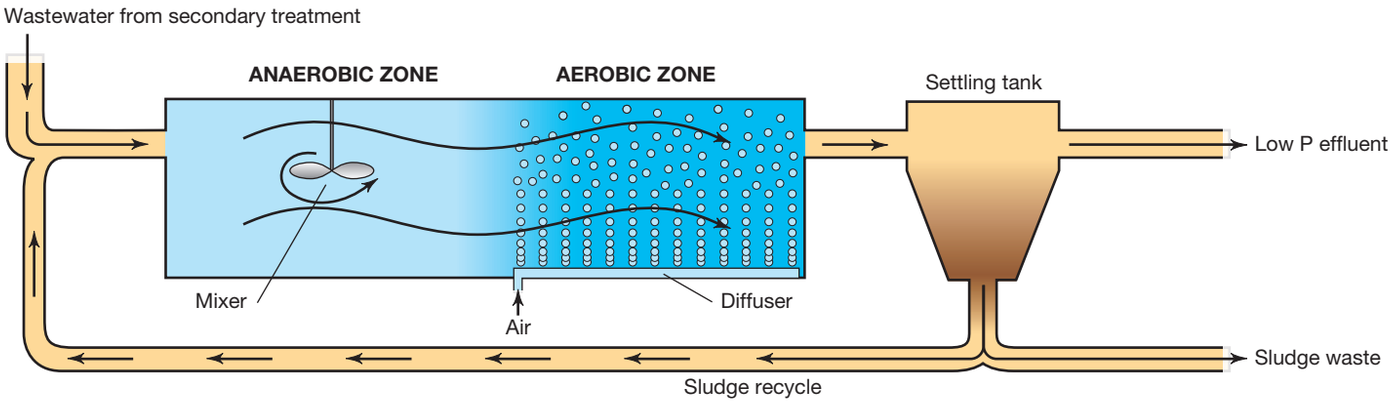
Biological Nitrogen Removal: The Conventional Nitrification–Denitrification Process

The strict regulatory limitations on release of nitrogen as ammonia or nitrate/nitrite, also called *reactive nitrogen* (*Nr*), from wastewater treatment facilities reflects their adverse health effects on human and aquatic life and contribution to eutrophication of receiving water bodies (◀ Section 20.9). Thus, there has been increasing use of tertiary treatment to remove remaining Nr from wastewater following secondary treatment or anaerobic sludge digestion (see Figure 22.22).

Conventional ("classical") treatment converts the Nr to its inert atmospheric form (N_2) using a combination of nitrification and denitrification (**Figure 22.20*a***). Nitrification (◀ Section 14.9) is first used to convert ammonia to nitrate followed by anaerobic conversion of nitrate to N_2 (and some N_2O) by denitrification (◀ Section 14.11). In the following reactions for the conversion of 1 mole of ammonia to 0.5 mole of N_2, organic carbon is represented by its chemical oxygen demand (COD), the mass of oxygen that is reduced by a specific



(a)



(b)

Figure 22.19 Enhanced biological phosphorus removal process. *(a)* Carbon and phosphate transitions during the treatment process. *(b)* Wastewater processing. During the passage of wastewater through the reactor system, the microbial community transitions from anaerobic to aerobic growth. In the anaerobic zone, short-chain fatty acids are taken up, intracellular glycogen is degraded, and internal stores of polyphosphate are released as extracellular orthophosphate. In the aerobic zone, the glycogen reserves are regenerated, extracellular phosphate is reassimilated as polyphosphate, and the intracellular stores of polyhydroxyalkanoates (PHAs) are metabolized. High-phosphorus sludge is harvested for disposal.

amount of organic matter; for example, a COD of 4 g reduces 4 g of O_2 (0.125 moles) to water:

Ammonia oxidation: $NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+$

Nitrite oxidation: $NO_2^- + 0.5\,O_2 \rightarrow NO_3^-$

Denitrification: $NO_3^- + 40\,gCOD + H^+ \rightarrow 0.5\,N_2$

(producing 15 g sludge biomass)

Although the primarily autotrophic ammonia- and nitrite-oxidizing microbes require little or no reduced carbon for growth and also generate very little biomass, a carbon source is required for the subsequent microbial reduction of nitrate to N_2 by denitrification. This may require supplementation with additional carbon for wastewaters having a low ratio of organic carbon to reactive nitrogen (C/N). Supplementation is commonly accomplished by adding a relatively inexpensive carbon source, such as methanol in low concentrations. However, because a typical treatment plant treats well over a million gallons (3.8 million liters) of wastewater a day, organic carbon addition can add significant costs, and this has fueled the development of less costly processes for advanced treatment.

Biological Nitrogen Removal: The Advanced Process of Nitritation–Denitrification

The cost of Nr removal can be reduced through advanced treatment processes that suppress the activity of nitrite-oxidizing bacteria, a process called *nitritation* (Figure 22.20*b*). By stopping the oxidation of ammonia at nitrite, the carbon requirement for denitrification is reduced by ~40%, oxygen by ~25%, and biomass by ~40%.

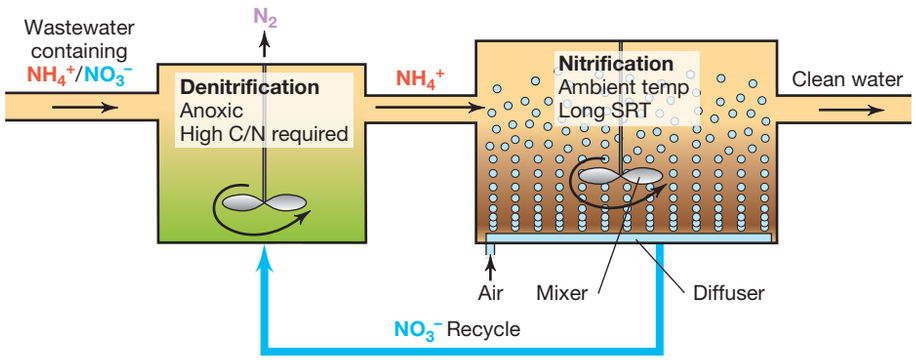
Ammonia oxidation: $NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+$

Denitrification: $NO_2^- + 24\,gCOD + H^+ \rightarrow 0.5\,N_2$

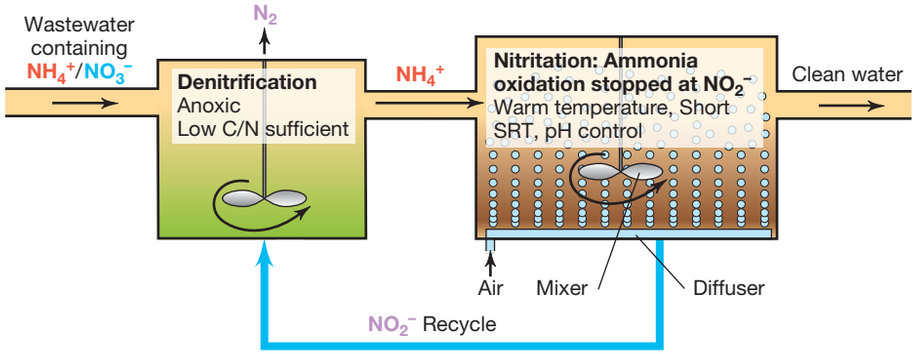
(producing 9 g sludge biomass)

This makes nitrogen removal from low C/N wastewater less costly. The only complication to this treatment strategy is the need to suppress nitrite-oxidizing bacteria (NOB), since they generally have higher substrate utilization rates than the ammonia-oxidizing bacteria (AOB). Achieving cost-effective control of partial ammonia oxidation is therefore key to advanced nitrogen removal treatment systems.

Suppression of nitrite oxidation is achieved by adjusting growth conditions that differentially affect AOB and NOB, primarily those of pH and temperature. AOB grow faster than NOB at temperatures above room temperature, having a specific growth rate (◀Section 4.7) approximately two times that of NOB at 35°C. For example, one widely used process relies on washing out NOB from a reactor operated at 30–40°C and a short sludge retention time (SRT) of 1–1.5 days (Figure 22.20*b*). The SRT can be controlled by the rate the reactor is fed wastewater or by allowing sludge to settle during a period without mixing before withdrawing the treated effluent. Control of



(a) **Classical denitrification**



(b) **Advanced treatment—denitrification**

Figure 22.20 Alternative treatment processes for nitrogen removal from wastewater. *(a)* Classic denitrification process. *(b)* Advanced treatment process for more efficient and economical removal of nitrogen. Wastewater is first treated anaerobically to remove incoming nitrate by denitrification before being nitrified in a second reactor and recycled back to the receiving tank for full nitrogen removal. SRT, sludge retention time.

reactor pH provides another way to achieve partial nitrification, as an alkaline pH (7.5–8.5) favors the growth of AOB over NOB (Figure 22.20*b*).

Aerobic Granular Sludge Treatment

Aerobic granular sludge consists of microbial aggregates of average 0.5 to 3.0 mm diameter having settling velocities at least 10 times greater than the loosely structured microbial flocs in flocculent activated sludge (**Figure 22.21*b, c***). Treatment systems are designed to select for granule formation by repeated removal of suspended floc material that does not settle rapidly. The greater microbial density in granules compared to that in flocs results in much greater nutrient removal capacity on a reactor volume basis than can be achieved using systems that produce flocculent sludge. In addition, the granular architecture provides another unique advantage relative to flocs. As was described for oxygen diffusion into soil aggregates (◀ Section 20.3 and Figure 20.3), the rapid consumption of O_2 by aerobes near the granule surface results in the formation of an anoxic core in larger granules. Thus, both aerobic and anaerobic processes can be sustained within the same granule, providing spatially resolved niches for different functional guilds.

In a granular sludge reactor designed for simultaneous nitrogen and phosphate removal, the reactor is cycled between oxic and anoxic conditions by feeding of wastewater sequentially to the bottom of the reactor (Figure 22.21*a*). The ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) localize near the granule surface (Figure 22.21*d*). Facultative denitrifying PAOs are enriched throughout the granule (Figure 22.21*d*). As was shown for the enhanced biological phosphorus removal process (Figure 22.19), during the anoxic phase, the PAOs consume intracellular reserves of glycogen and polyphosphate to release inorganic phosphate to the bulk wastewater, and at the same time synthesize and store PHAs [Figure 22.21*a*, $T(stage)_2$]. During the oxic phase the AOB and NOB work together to oxidize ammonia to NO_3^-. The PAO populations oxidize their intracellular reserves of PHA and take up phosphate from the bulk liquid (Figure 22.21*a*, T_3). The PAOs near the granule surface use oxygen as their electron acceptor for phosphate uptake, whereas those localized to the anoxic interior use nitrate or nitrite originating from ammonia oxidation for concurrent phosphate uptake and denitrification (Figure 22.21*d*). The granular sludge is then allowed to settle, some of the sludge is removed to recover phosphorus (Figure 22.21*a*, T_4), and the cycle is repeated.

High efficiencies for nitrogen and phosphorus removal have been shown for full-scale aerobic granular sludge facilities treating municipal wastewaters. As application of these treatment systems becomes more widespread, energy savings are significant, and the footprint of the wastewater treatment facility is reduced relative to traditional systems using flocculent activated sludge (Figure 22.17) designed to treat the same wastewater.

Check Your Understanding

- What are the advantages of enhanced biological phosphorus removal relative to traditional chemical removal of phosphorus?
- What are two important advantages of aerobic granular sludge technology relative to traditional activated sludge treatment?

22.8 Sludge Processing and Contaminants of Emerging Concern

The generation of significant microbial biomass (sludge) as a product of secondary and tertiary wastewater treatment requires costly landfill disposal. However, sludge volume can be reduced by **anaerobic treatment**, using the degradative and fermentative reactions of anaerobic microorganisms to lower disposal costs as well as generating methane that can be burned for energy recovery.

Anaerobic Sludge Treatment

As we saw in Section 21.2, under conditions of electron acceptor limitation, fermentation by a community of *Bacteria* and *Archaea* converts organic matter into methane and carbon dioxide (**Figure 22.22*b***; ◀ Figure 21.7). This requires syntrophic processes in

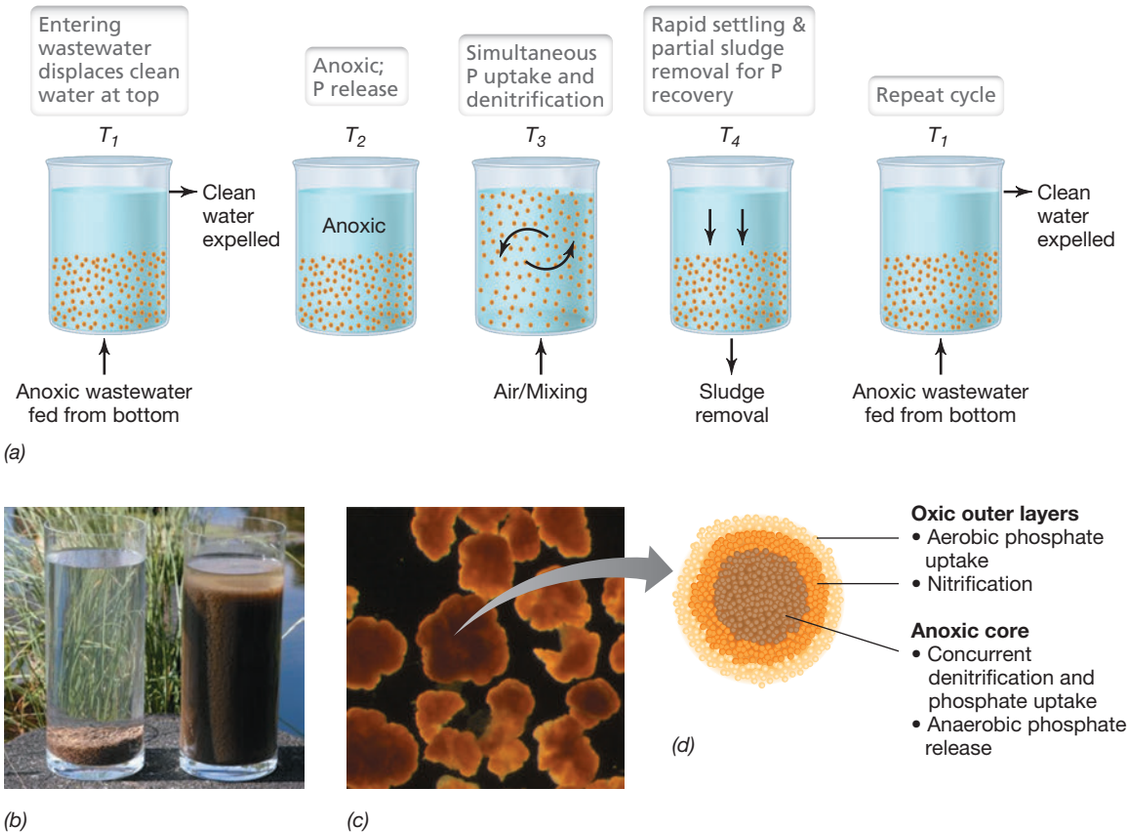


Figure 22.21 Granular sludge wastewater treatment. *(a)* Simultaneous phosphorus removal and denitrification using granular sludge technology (Nereda® process). Stages of phosphate release and uptake by phosphorus-accumulating organisms (PAOs) are shown by treatment stages T_1–T_4. At anoxic stage T_2, glycogen and polyphosphate intracellular reserves are consumed to fuel production of intracellular polyhydroxyalkanoate (PHA) and the release of phosphate. At T_3, PHA is consumed to drive phosphate uptake and synthesis of intracellular polyphosphate, using O_2 or nitrite/nitrate generated by nitrification to fuel PAO denitrification. *(b)* The very rapid settling of granular sludge (left) relative to activated sludge (right) after only a few minutes. *(c)* Granules average 1–3 mm in diameter. *(d)* Schematic shows how the layered structure of a granule separates aerobic and anaerobic processes in the same granule.

which the fermentative organisms supply electron donors (such as H_2 and acetate) for methanogens. The treatment of wastewater sludge is an ideal example of these cooperative processes in action.

The anaerobic degradation process takes place in large, enclosed tanks called *sludge digesters* or *bioreactors* (Figure 22.22*a*, *c*). The process requires the collective activities of many different microbes, and the major reactions are summarized in Figure 22.22*b*. Anaerobes use polysaccharidases, proteases, and lipases to digest suspended solids and large macromolecules into soluble components. These are then fermented to yield a mixture of fatty acids, H_2, and CO_2; the fatty acids are further fermented by the cooperative actions of syntrophic bacteria (◀ Sections 14.22 and 21.2) to produce acetate, CO_2, and H_2. These products are then converted to CH_4, and the CH_4 is either burned off or used as fuel to heat and power the wastewater treatment plant.

Although sludge volume is greatly reduced by anaerobic treatment, significant nitrogen and phosphate remains in the liquid phase, generating a *side stream* of N- and P-rich brine, with N concentrations up to 100-fold greater than that produced by secondary treatment. The N-rich side stream must be further treated, using either conventional tertiary treatment or the anammox process, which we consider now.

Nitrogen Removal by Partial Nitritation–Anammox

Since anammox bacteria are anaerobic chemolithotrophic autotrophs (◀ Section 14.10), they do not depend on organic carbon as an electron donor or oxygen as an electron acceptor. The anammox process is as follows:

$$\text{Anammox: } NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow 1.02\,N_2 + 0.26\,NO_3^- + 2.03\,H_2O + 0.066\,C_{biomass}$$

The key to using the anammox process for nitrogen removal is an initial oxidation of *only half* the ammonia in the side stream brine to nitrite, yielding a 50:50 mixture of ammonia and nitrite. This process is called *partial nitritation* (Figure 22.22*d*). The effluent of the partial nitritation reactor is then fed to an anammox reactor for conversion of ammonia and nitrite to N_2. The partial nitritation reactor exploits the higher growth rate of AOB compared to NOB at the higher temperatures used, and the higher affinity of AOB for oxygen compared to NOB. By keeping reactor temperature warm and oxygen concentration low, ammonia is oxidized to nitrite at a short SRT. Since AOB and NOB are unable to grow in the absence of oxygen, anammox is the only significant process in the anoxic reactor (Figure 22.22*d*).

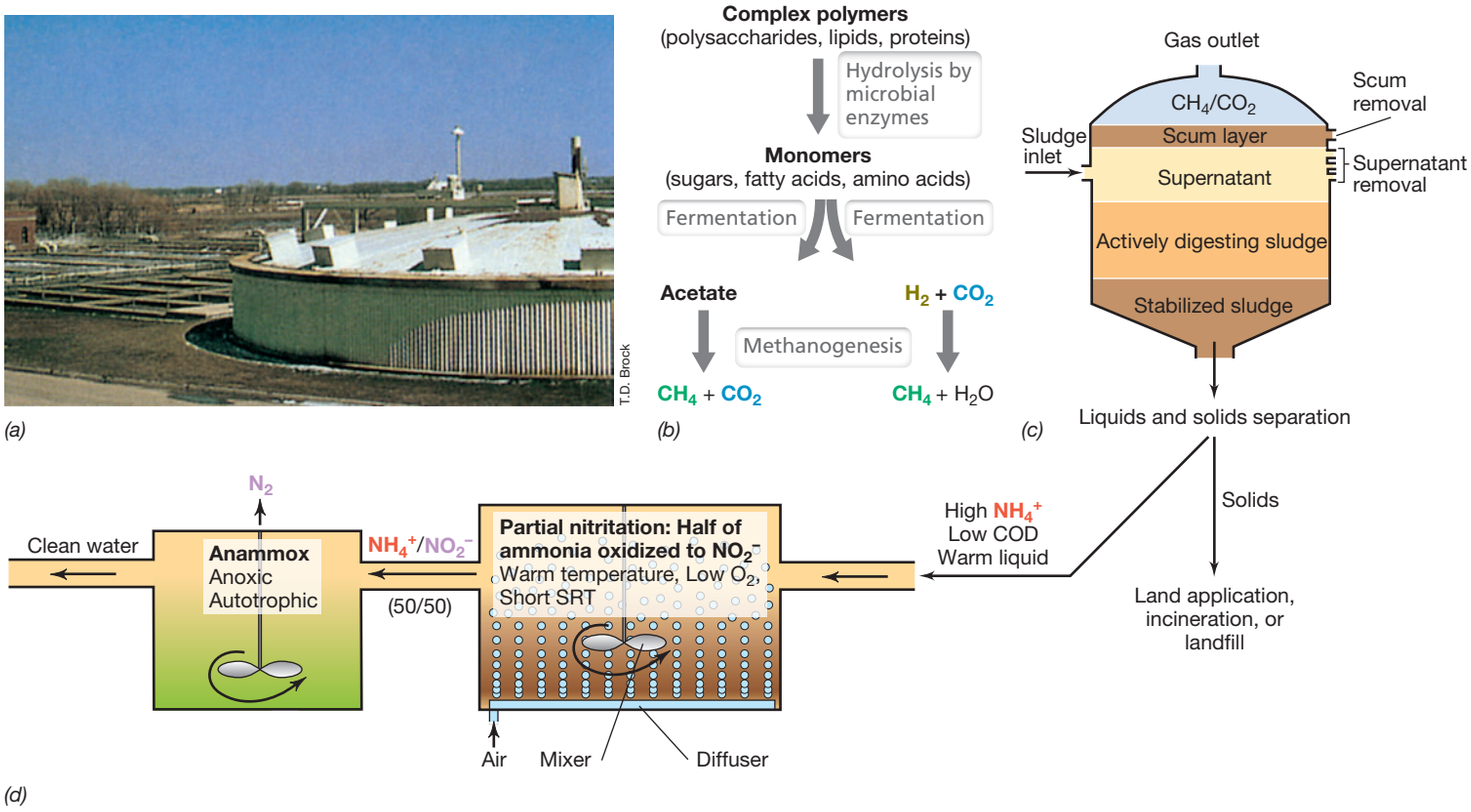


Figure 22.22 **Sludge processing.** *(a)* Anaerobic sludge digester. Only the top of the tank is shown; the remainder is underground. *(b)* Major microbial processes in anaerobic sludge digestion. *(c)* Inner workings of a sludge digester. Methane (CH_4) and carbon dioxide (CO_2) are the major products of anaerobic biodegradation. *(d)* Following separation of solids and liquids, the warm ammonia-rich brine (about 50% each of NH_4^+ and NO_2^-) is treated by the anammox process.

This overall process is called *partial nitritation–anammox* (PNA). Compared with conventional biological nitrogen removal (Figure 22.20*a*), PNA has no requirement for added organic carbon, uses less electricity, generates less sludge, and has lower emissions of greenhouse gases (primarily CO_2 and N_2O). Thus, this process greatly reduces the environmental and energy costs of more conventional nitrogen removal technology.

Contaminants of Emerging Concern

A wide variety of chemicals end up in wastewaters besides the usual organic carbon and inorganic nutrients that processing systems are designed to deal with. These include heavily used agricultural products and chemicals that demonstrate acute toxicity or carcinogenicity. However, bioactive pollutants also enter wastewater streams and pose new challenges for microbial bioremediation. These include pharmaceuticals, active ingredients in personal care products, fragrances, household products, sunscreens, prescription and other medications, and many other uncommon or xenobiotic molecules.

Unlike pesticides, these pollutants are more or less continuously discharged into the environment through the release of treated sewage, which means they do not need to persist to have environmental effects. For example, it is known that low levels of synthetic estrogen compounds, excreted in the urine of women taking birth control pills and eventually discharged in active form from wastewater treatment plants, can activate estrogen response genes in aquatic animals such as fish and contribute to the feminization of males.

The environmental threat from these emerging contaminants has created a need for newly designed wastewater treatment facilities to stimulate bioremediation of these wastes. However, because these substances are often present in very low concentrations and in some cases may be new classes of xenobiotics, they may not actually support microbial growth but rather only be degraded by cometabolism (Section 22.5) or by highly specialized species. Moreover, because wastewaters contain such high concentrations of readily degradable organic materials, structurally complex emerging contaminants may be ignored by microbes until the COD of the wastewater is greatly reduced. We can therefore expect that the bioremediation of emerging contaminants will be an active area of microbiological research in coming years.

Check Your Understanding

- What are the advantages of further treating activated sludge by anaerobic digestion?
- Why is the incomplete oxidation of ammonia essential for effective treatment of sludge brine using the anammox process?
- What advantages does the anammox process have over classical wastewater treatments for nitrogen removal?
- Give an example of an "emerging" contaminant.

22.9 Drinking Water Purification and Stabilization

Wastewater treated by secondary methods can usually be discharged into rivers and streams. However, such water is not **potable** (safe for human consumption). The production of potable water requires further treatment to remove potential pathogens, eliminate taste and odor, reduce nuisance metals such as iron and manganese, and decrease **turbidity**, which is a measure of suspended solids. **Suspended solids** are small particles of solid pollutants that resist separation by ordinary physical means.

In 1900, water purification in the United States was limited to filtration to reduce turbidity. Although filtration significantly decreased the microbial load of water, many microbes still passed through the filters and thus cases of waterborne diseases such as cholera and typhoid (▶ Section 33.3 and Figure 30.7) were common. Not until the practice of **chlorination** became widespread were waterborne diseases controlled. Indeed, major improvements in public health in the United States and other developed countries were largely due to the adoption of water filtration and disinfection treatment procedures. Today, waterborne diseases still occur, but are rarely linked to well-run and properly maintained municipal water facilities.

Physical and Chemical Purification

A typical municipal installation for drinking water treatment is shown in **Figure 22.23*a***. Figure 22.23*b* shows the process that purifies **raw water** (also called **untreated water**) that flows through the treatment plant. Raw water is first pumped from the source, in this case a river, to a sedimentation basin where anionic polymers, alum (aluminum sulfate), and chlorine are added. **Sediment**, including soil, sand, mineral particles, and other large particles, settles out. The sediment-free water is then pumped to a **clarifier** or coagulation basin, which is a large holding tank where **coagulation** takes place. The alum and anionic polymers form large particles from the much smaller suspended solids. After mixing, the particles continue to interact, forming large, aggregated masses, a process called **flocculation**. The large, aggregated particles (flocs) settle out by gravity, trapping microorganisms and adsorbing suspended organic matter and sediment.

After coagulation, flocculation, and sedimentation, the clarified water undergoes **filtration** through a series of filters designed to remove organic and inorganic solutes, as well as remaining suspended particles and microorganisms. The filters typically consist of thick layers of sand, activated charcoal, and ion exchangers. After this step and the previous purification steps, the filtered water is free of particulate matter, most organic and inorganic chemicals, and nearly all microorganisms.

Disinfection

Clarified, filtered water must be disinfected before it is released to the supply system as potable **finished water**. **Primary disinfection** is the introduction of sufficient disinfectant into clarified, filtered water to kill existing microorganisms and inhibit further microbial growth. Chlorination is the most common method of primary disinfection. In sufficient doses, chlorine kills most microorganisms within 30 minutes by physically oxidizing and otherwise destroying the cell and its contents. A few pathogenic protists such as *Cryptosporidium*, however, are not easily killed by chlorine treatment (▶ Section 34.4). In addition to killing microorganisms, chlorine oxidizes and effectively neutralizes many organic compounds. Since most taste- and odor-producing chemicals are organic compounds, chlorination thus improves water taste and smell as well as disinfecting.

Mastering Microbiology
Art Activity: Figure 22.20*b* Water purification plant

Chlorine is added to water from either a concentrated solution of sodium or calcium hypochlorite or as chlorine gas from pressurized tanks. Chlorine gas (Cl_2) is commonly used in large water treatment plants because it is most amenable to automatic control.

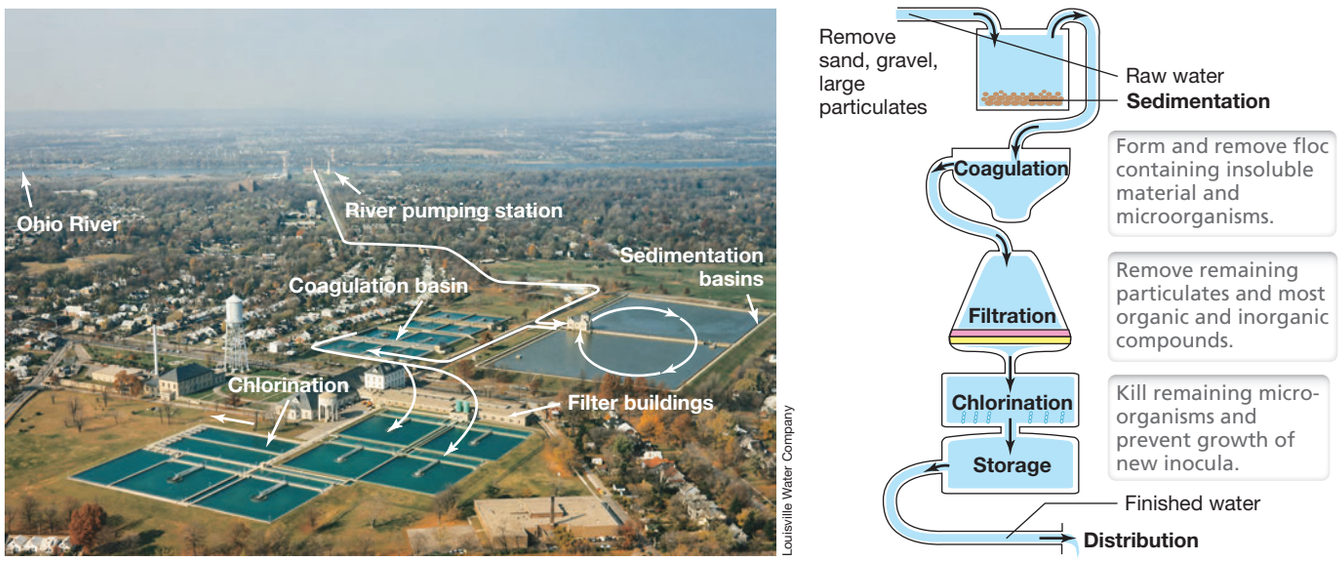


Figure 22.23 Water purification plant. *(a)* Aerial view of a drinking water treatment plant in Louisville, Kentucky, USA; source water is from the Ohio River. The arrows indicate direction of flow of water through the plant. *(b)* Schematic overview of a typical community water purification system.

When dissolved in water, chlorine gas forms hypochlorous acid (HOCl), which dissipates within hours from treated water. To maintain adequate levels of chlorine for primary disinfection, many municipal water treatment plants introduce ammonia gas with the chlorine to stabilize the chlorine by forming **chloramine** (NH_2Cl): $HOCl + NH_3 \rightarrow NH_2Cl + H_2O$.

Chlorine is consumed when it reacts with organic materials. Therefore, sufficient quantities of chlorine must be added to finished water containing organic materials so that a small amount, called the *chlorine residual*, remains. The water plant operator performs chlorine analyses on the treated water to determine the level of chlorine to be added for **secondary disinfection**, the maintenance of sufficient chlorine residual in the water distribution system to inhibit microbial growth. A chlorine residual level of 0.2–0.6 mg/liter is the level achieved in most water supplies. After chlorine treatment, the now potable water is pumped to storage tanks from which it flows by gravity or pumps through a distribution system of storage tanks and supply lines to the consumer. Although residual chlorine inhibits growth of bacteria in the finished water prior to the water reaching the consumer, it does not protect against catastrophic system failures, such as a broken pipe in the distribution system.

Ultraviolet (UV) radiation is also used as an effective means of disinfection. As we discussed in Section 4.18, UV radiation is used to treat secondarily treated effluent from water treatment plants. In Europe, UV irradiation is commonly used for drinking water applications, and it is increasingly used in the United States. For disinfection, UV light is generated from mercury vapor lamps. Their major energy output is near 260 nm, a wavelength that is strongly absorbed by DNA and is mutagenic (◀ Section 9.4) and thus bactericidal; irradiation may also kill cysts and oocysts of protists such as *Giardia* and *Cryptosporidium*, important eukaryotic pathogens in water (▶ Section 34.4). Viruses, however, are more resistant.

UV radiation has several advantages over chemical disinfection procedures like chlorination. First, UV irradiation is a physical process that introduces no chemicals into the water. Second, UV radiation–generating equipment can be used in existing flow systems. Third, no disinfection byproducts (such as chloroform, a possible human carcinogen) are formed with UV disinfection. Especially in smaller systems where finished water is not pumped long distances or held for long periods (reducing the need for residual chlorine), UV disinfection may be preferable to reduce dependence on chlorination.

Check Your Understanding

- What specific purposes do sedimentation, coagulation, filtration, and disinfection accomplish in the drinking water treatment process?
- What general procedures are used to reduce microbial numbers in water supplies?
- What are the advantages of disinfection with ultraviolet radiation versus, or as a complement to, chemical disinfection with chlorine?

22.10 Water Distribution Systems

Mastering Microbiology
Art Activity: Figure 22.21 Drinking water distribution system

Once drinking water leaves the treatment facility, the water often travels long distances through municipal and premise distribution pipes from the facility to the consumer (**Figure 22.24**). In addition to taste and odor problems that may be present in the source water, the long transit and residence times may also contribute to undesirable taste, odors, and color from biological and chemical processes. Although undesirable, taste and odor alone usually do not signal a health threat. However, water distribution systems may also promote the growth of obligate or opportunistic pathogens (▶ Section 25.4), sequester and protect pathogens, or select for more pathogenic and resistant forms of microorganisms. Even though drinking-water-associated disease often goes unreported, disease outbreaks do occur and are occasionally fatal (Chapter 33).

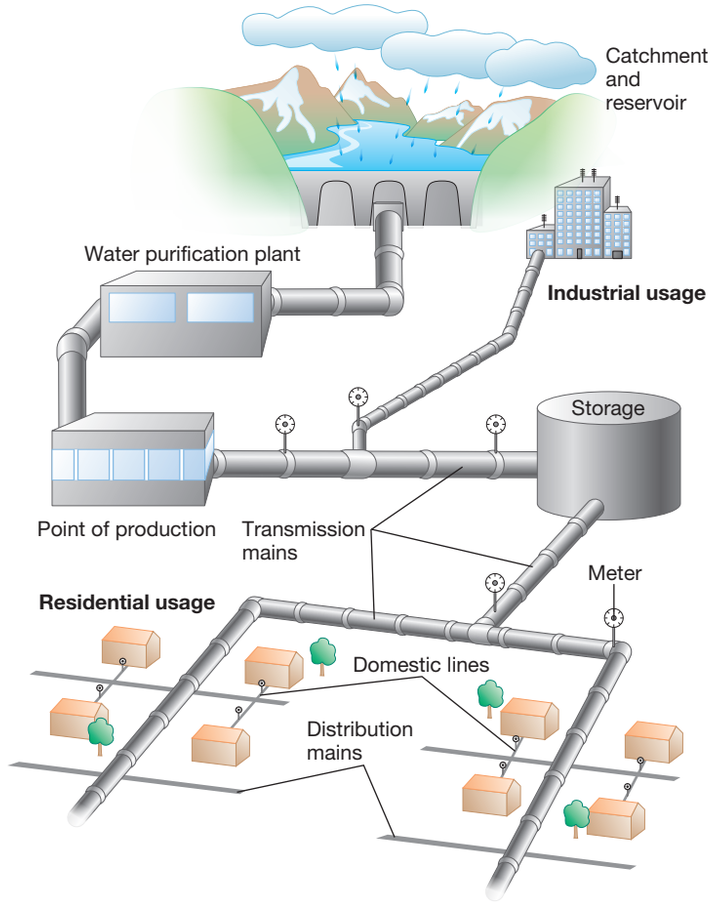


Figure 22.24 Drinking water distribution system. A municipal distribution system includes a surface reservoir, water purification plant, distribution mains, and domestic lines that encompass many miles of pipes in a typical community.

The Microbiology of Municipal Water Distribution Systems

Microbial growth in drinking water distribution systems can be eliminated only through complete nutrient removal (elimination of growth substrates originating from the source water and from distribution system structural materials) or by maintaining appropriate residual chlorine levels throughout the distribution system. Neither of these is realistically attainable. Growth is unavoidable as a consequence of reduction in chlorine concentration with increasing distance from the point of production together with the tendency for microorganisms to form biofilms on the pipe walls. Microorganisms

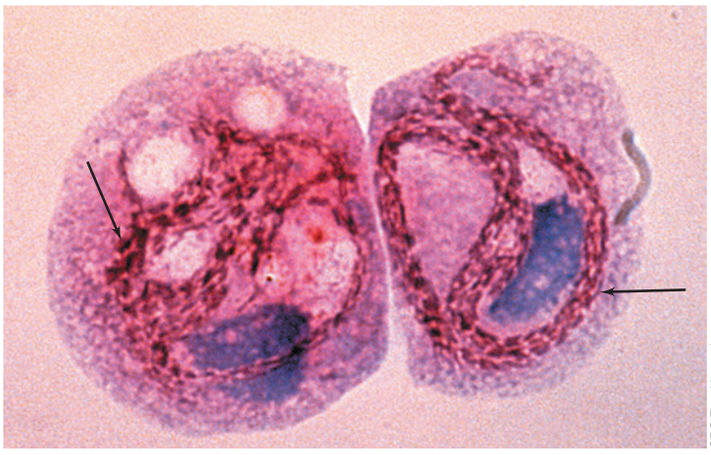
in biofilms are more resistant to disinfection (◀ Sections 4.9, 8.10, and 20.4), and significant microbial accumulation is found in all distribution systems, over 90% of which is in the form of biofilms that coat the pipe walls.

Culture-independent molecular techniques, in particular 16S rRNA sequence analysis (◀ Section 19.6), have been used to explore biofilms in water distribution pipes. Although these studies are showing that pathogenic species are rare, some opportunistic pathogens (▶ Section 25.4) are present and can infect susceptible humans, including infants, the elderly, and individuals with compromised immune systems. Opportunistic pathogens found in water distribution systems include (1) nontuberculous mycobacteria (including *Mycobacterium avium*, *M. intracellulare, M. kansasii*, and *M. fortuitum*) associated with many thousands of clinical cases each year in the United States; (2) *Legionella pneumophila* (the causative agent of Legionnaires' disease, ▶ Section 33.4); (3) *Pseudomonas aeruginosa* (which can infect the eyes, ears, skin, and lungs); and (4) opportunistic protozoan pathogens such as *Naegleria* and *Acanthamoeba* (▶ Section 34.3) that can cause keratitis and encephalitis.

Water distribution systems also support numerous grazing protists that subsist by consuming bacteria. Bacteria that survive and replicate following ingestion by these protists are potentially also less susceptible to clearance by the mammalian immune system. An example is *Legionella*, which is able to establish residence and replicate in protists inhabiting water-handling systems (**Figure 22.25**), including premise plumbing, shower heads, and air conditioning systems. The basic mechanisms *Legionella* uses to gain entry and replicate in a broad variety of protists (including *Acanthamoeba, Hartmannella, Naegleria*, and *Tetrahymena*) also allow it to more easily infect human cells.

In addition to *Legionella*, opportunistic pathogens that can survive or grow within protists include *Pseudomonas* and *Mycobacterium* species. Nontuberculous mycobacteria (*M. avium, M. intracellulare, M. kansasii*, and *M. fortuitum*), also called "environmental mycobacteria," cause a variety of lung infections distinct from that of tuberculosis. These bacteria are more resistant to chlorine disinfection and protozoal grazing and have been found to be enriched in showerheads receiving municipal water that still maintains a chlorine residual. It is possible that aerosols generated in the showering process could transmit these pathogens to humans. Thus, although water purification has made great strides in eliminating waterborne infections on a grand scale, the architecture of water distribution systems coupled with the aging condition of many systems can still compromise human health (see Figure 22.26*a*).

We transition now to the final part of this chapter where we examine the microbiology and microbial activities that occur in buildings, homes, and other structures.



CDC/Don Howard

Figure 22.25 Protists as reservoirs of *Legionella*. Two cells of the protist *Tetrahymena* contain chains of the rod-shaped pathogen *Legionella pneumophila* (arrows). In premise water systems, protists can persist and be reservoirs of bacterial pathogens.

Check Your Understanding

- Trace the treatment of water through a drinking water treatment plant, from the inlet to the final distribution point (faucet).
- How could your household water system transmit disease?

IV • Indoor Microbiology and Microbially Influenced Corrosion

Indoor air, both in private dwellings and public places, as well as surfaces in the structures themselves, can be teeming with microbes. In addition, costly losses occur yearly from corrosion catalyzed by microbial activities on metal, stone, and concrete infrastructure.

Although one might think that being indoors protects a person from the microbial world, nothing could be farther from the truth. Microbes are everywhere life can be found. Moreover, microbial metabolism accelerates corrosion through the changes in pH or redox that accompany the metabolism, production of corrosive metabolites, and creation of corrosive microenvironments in biofilms. Here we examine the microbial ecosystems of the built environment and a few cases in which the microbial contribution to corrosion is well understood. Recall that we have previously seen how surfaces are favorable microbial habitats (◀ Section 20.4); virtually any surface that provides nutrients will be colonized by microorganisms.

22.11 The Microbiology of Homes and Public Spaces

Humans in urban environments spend the majority of their life indoors. They share this indoor environment with a microbiota that inhabit the air, dust, surfaces, and ventilation and water systems (**Figure 22.26**). The health effects of indoor microbial exposure may be positive or negative (Figure 22.26*a*). Pathogens, such as antibiotic-resistant *Staphylococcus aureus* and other *Staphylococcus* species, may be elevated in the indoor environment as a consequence of shedding from human skin. In contrast, the increased incidence of allergies and autoimmune disorders in children in developed countries has been attributed in part to the "too-clean" indoor environments that

(a) (b)

Figure 22.26 Sources of airborne and surface-associated microorganisms in the built environment. *(a)* Sources of microorganisms in a typical household, including surfaces, humans, pets, plumbing systems, and outdoor air. The colors correspond to microorganisms or spores that may be beneficial or not harmful (green) or potentially detrimental (red) to human health. *(b)* Air particle collector (arrow) deployed in the New York City subway system for surveying the diversity of airborne bacteria by 16S rRNA gene sequencing.

reduce exposure to microbes important for "training" the immune system early in life. Thus, a microbially depleted indoor environment may actually be detrimental to human health.

In addition to living with microbes in our home environment, what microbial exposures does a person experience in major public spaces, such as subway systems, the supermarket, or even the classroom? These questions can now be addressed using 16S (or 18S) rRNA gene sequencing of DNA isolated from samples collected from different parts of the indoor environment (Figure 22.26*b*; ◀ Section 19.6).

Microbiology of the Indoor Air in Private Dwellings

Studies of the microbiology of indoor air and surfaces have become increasingly popular. For example, dust collected from upper trims of inside and outside doors of over a thousand homes in the United States revealed distinct indoor and outdoor microbial communities composed of fungi and bacteria. The outdoor fungi closely resemble the outdoor fungal populations found in the same geographical region, whereas the indoor bacterial communities more strongly reflect the type and number of occupants, including pets. Local geography also influences the indoor microbiome. For example, rural and farmland households harbor a greater abundance of microorganisms associated with the guts of farm animals, including the opportunistic pathogens *Bartonella*, *Enterococcus*, and *Ruminococcus*, than do urban dwellings.

In general, the overall indoor diversity of bacteria and fungi is greater than that found outdoors, reflecting a mixing of indoor and outdoor sources. A small number of fungal species are more abundant inside the house, including common molds such as *Aspergillus, Penicillium*, *Alternaria*, and *Fusarium*. In addition, the incidence of these fungal populations increases with the age of the dwelling and whether or not a basement is present (basements are often damp, which increases mold abundance).

Bacteria that are characteristic of the human skin (gram-positive bacteria such as *Staphylococcus, Streptococcus, Corynebacterium*, and *Propionibacterium*), feces (*Bacteroides, Faecalibacterium, Ruminococcus*), or the vagina (*Lactobacillus, Bifidobacterium, Lactococcus*) are much more commonly found inside a house than outdoors. The increased incidence of species of the bacteria *Prevotella*, *Porphyromonas*, *Moraxella*, and *Bacteroides* is typically associated with the presence of domestic animals—in particular dogs or cats—in a home. In fact, it is possible to predict with near certainty whether a home has these pets based only on a molecular analysis of household microbiota.

In addition to the surveys of dust described above, molecular surveys of home surfaces show that the microbial communities of human hands, noses, and feet (◀ Section 10.10 and Figure 10.28; ▶ Section 24.5) closely resemble the organisms found on household surfaces, including floors, light switches, countertops, and door knobs (Figure 22.26*a*). Moreover, the surface-associated microbiota of a home is highly predictive of a specific family, and the composition of the microbial community has been found to shift within days of a change in home occupants.

Diagnostic microbial populations in an individual are also highly correlated with the objects they touch, such as desks, computers, phones, and chairs. For example, in a study that combined 16S rRNA gene sequencing and metabolomics (◀ Sections 10.10 and 19.8) of the surfaces of commonly touched objects, residues from medicines and personal care products such as shampoos, deodorants, and lotions were detected. The presence or absence of theophylline, a by-product of caffeine, identified coffee drinkers or nondrinkers. When information of microbial species composition and chemical profiles were combined, all the individuals working in the same office could be linked to specific office furniture and equipment at high confidence. Studies such as this point to the likely development in the not-too-distant future of microbiology-based forensics.

The common use of antimicrobials in cleansers and soaps is now known to significantly alter the indoor microbiome. Although now banned in the United States, the chlorinated antimicrobials triclosan and triclocarban were widely used and can still be found in older

household products such as soaps, cosmetics, deodorants, and kitchen cutting boards. Because gram-positive bacteria are less susceptible to these substances than gram-negative bacteria, gram-positives, including some drug-resistant species, become predictably enriched in households using these products. Thus, practices once thought to be a measure of a healthy household and good personal hygiene may actually select for, rather than eliminate, potentially harmful organisms.

Microbiology of Public Places

Public buildings, office spaces, and transit systems are another important component of the built environment. The heavily trafficked New York City municipal subway system (Figure 22.26*b*) alone moves over 1.5 billion passengers a year. Similar to homes, subways and offices contain a mixture of airborne microorganisms sourced from humans and the outdoor air. Because air exchange in a subway system must be extensive, most airborne microorganisms in subway systems are typical of those found outdoors. In addition, however, about 5% of the microbial population in subway air is composed of microbes that normally reside on the feet, hands, arms, and heads of humans; these are most likely shed from the more exposed areas of subway riders.

Indoor plumbing of public and private buildings is another well-recognized point of microbial exposure. Each flush of a toilet generates over 100,000 small (<5-μm) aerosol particles. Since aerosolized bacteria and viruses can remain viable for hours after they deposit on bathroom surfaces, flushing is a potential mechanism of enteric pathogen transmission as well as a means of transmitting harmless saprophytes from person to person. Transmission of human diseases caused by direct contact (such as sexually transmitted diseases) is not a major issue with bathroom fixtures because pathogens such as *Neisseria gonorrhoeae* (gonorrhea) and *Treponema pallidum* (syphilis) are very sensitive to drying. However, because of the enormous numbers of bacteria shed in feces, transmission of enteric bacteria by bathroom aerosols is a distinct possibility.

Studies of indoor microbiology continue to reveal the types and origins of microorganisms we spend much of our day in contact with, providing information that has several applications. Besides identifying potential hot spots of infectious disease, knowledge of indoor microbiology can inform the design of future construction such that new dwellings enhance beneficial exposures and limit detrimental exposures to microorganisms. Although microbe-free dwellings are impossible because humans and pets carry microbes, controlling the microbial content of recirculated air transmitted through dwellings and the development of environmentally friendly yet still effective antimicrobials for indoor use are worthwhile goals.

Check Your Understanding

- How can a microbial inventory reveal information about the presence or absence of household pets?
- Which room(s) in a private dwelling are potentially the most dangerous from a microbiology perspective, and why?
- How might microbiology be used to develop a new type of forensic science?

22.12 Microbially Influenced Corrosion of Metals

Iron is the most commonly used metal in the built environment. Water, gas, and oil distribution pipelines—especially those laid decades ago—are made of metal, and their corrosion contributes to the greatest loss of infrastructure in the built environment. Corrosion of iron by oxygen in air is thought to be solely an electrochemical process. However, much critical iron-containing infrastructure is buried or submerged, restricting exposure to oxygen, and these are subject to microbial attack. At near-neutral pH and anoxic conditions, corrosion of iron and steel is significantly accelerated by **microbially influenced corrosion (MIC)**. Microbial groups implicated in MIC include sulfate-reducing bacteria (◀ Sections 14.12 and 15.11), ferric-iron-reducing bacteria (◀ Sections 15.13 and 21.5), ferrous-iron-oxidizing bacteria (◀ Sections 14.8, 15.14, and 21.6), and methanogens (◀ Sections 14.15, 17.2, and 21.2).

Metal Corrosion by Sulfate-Reducing Bacteria

Metal structures submerged in the marine environment and pipelines used for transmission of low-grade oil are particularly subject to MIC through the activities of sulfate-reducing bacteria. Corrosion by sulfate-reducing bacteria is partly attributable to the chemically corrosive nature of hydrogen sulfide (H_2S), the product of their metabolism. Crude oils containing more than about 0.5% sulfur by weight are called "sour" and may be naturally corrosive because of the H_2S that is present. In oil fields near the ocean, such as in the Middle East and Alaska, seawater is injected to maintain reservoir pressure and force oil into the producing well. Since seawater contains nearly 30 mM sulfate, an undesirable consequence of injection is further souring by stimulating the growth of sulfate-reducing bacteria, some species of which can oxidize hydrocarbons anaerobically using sulfate as electron acceptor (Section 22.4; ◀ Section 14.24).

A strategy now used by the petroleum industry to control souring is the addition of nitrate (NO_3^-) in the injection water, stimulating the growth of nitrate-reducing bacteria. Since nitrate respiration is energetically more favorable than sulfate respiration (◀ Sections 14.11 and 14.12), the nitrate reducers outcompete sulfate reducers for usable organic electron donors in the oil. Nitrate also stimulates the growth of sulfide-oxidizing, nitrate-reducing chemolithotrophs (◀ Sections 14.7 and 15.12), thereby reversing souring by oxidizing the sulfide.

Mechanisms of Metal Corrosion

At least two mechanisms have been proposed for how sulfate reducers corrode iron. In the first mechanism, H_2 consumption by the sulfate reducer accelerates electrochemical pitting of the iron surface (**Figure 22.27*a***). This model is based on the capacity of many sulfate reducers to use hydrogen (H_2) as an electron donor, thereby accelerating the energetically favorable but kinetically slow H_2 production originating from the chemical oxidation of elemental iron ($Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2$). The overall stoichiometry of this reaction shows that Fe^{2+} formed from pitting reacts with sulfide from sulfate reduction in an energetically favorable reaction.

A second mechanism, such as that carried out by the bacterium *Desulfopila corrodens*, has the capacity to take up electrons directly from Fe^0 (Figure 22.27*b*). In this mechanism, cells attached to the metal

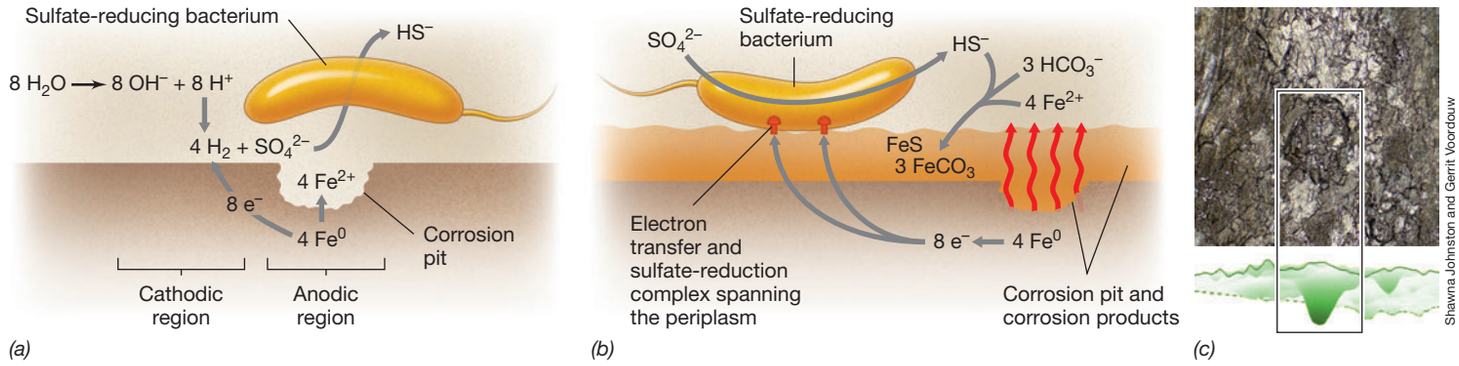


Figure 22.27 Corrosion of iron by sulfate-reducing bacteria. Two models for the activities of sulfate-reducing bacteria in metal corrosion. *(a)* Oxidation of metallic iron is accelerated by bacterial consumption of H_2 produced abiotically by proton reduction at the metal surface. *(b)* Direct electron transfer from the metal occurs via electron-conductive outer cell wall structures connecting to an electron transfer system spanning the periplasm. *(c)* Top: photo of a model iron surface undergoing sulfidic corrosion. Bottom: scan of a side view of the metal surface in the photo shows in dark green the areas where corrosion and pitting of the metal surface is greatest.

surface engage in direct (cathodic) electron uptake from Fe^0 through an electroconductive sulfidic corrosion layer (Figure 22.27*b*), likely facilitated by conductive cellular structures like those used in the oxidation or reduction of insoluble electron donors or acceptors (◀ Sections 15.13 and 21.5). A similar ability to take up electrons directly from Fe^0 has been observed for a *Methanobacterium* species that produces methane (CH_4) from CO_2 reduction rather than sulfide from sulfate reduction during growth on Fe^0. The Fe^{2+} resulting from methanogenic Fe^0 oxidation then complexes with HCO_3^- to form $FeCO_3$ (Figure 22.27*b*). However, regardless of mechanism and the organism oxidizing the Fe^0, the end result is metal corrosion (Figure 22.27*c*).

Check Your Understanding

- How does a nitrate addition prevent sulfide souring of crude oil?
- Why is accelerated microbial corrosion of iron metal thought to require a direct interaction between the sulfate reducers and the metal surface?

22.13 Biodeterioration of Stone and Concrete

In the same way that microorganisms contribute to soil formation through the dissolution of mineral and rock surfaces by their physical and metabolic activities (◀ Section 20.6), buildings or other structures composed of natural stone or concrete are also subject to microbial colonization and may undergo a slow loss of structural integrity through microbial metabolic activities. This degradative process is called **biodeterioration**.

Mastering Microbiology
Art Activity: Figure 22.25 Crown corrosion of concrete sewer pipes

Biodeterioration of Stone Building Materials

Microbial colonization of natural and structural stone building material is ubiquitous. Microbes can colonize the surface and penetrate several millimeters into rocky material depending on its physical characteristics (e.g., surface roughness, porosity, light penetration). Microbes can also grow on and within the facades of buildings constructed of limestone, sandstone, granite, basalt, and soapstone. These "within stone," or *endolithic*, communities are phylogenetically diverse, comprised of chemoorganotrophic and chemolithotrophic *Bacteria* and *Archaea*, microbial eukaryotes including fungi and algae, and cyanobacteria (◀ Figure 18.35). The cyanobacteria and algae primarily nourish the community, living in close or symbiotic association with other microbial members.

Life on and within stone building materials requires adaptation to multiple extreme conditions, including intense solar radiation, desiccation, temperature and moisture fluctuations, and lack of nutrients. Protection from solar radiation is conferred by production of UV-absorbing pigments (for example, melanin, mycosporines, and carotenoids) by fungi and other microbial community members. The fungi also play a central role in this process of slow biodeterioration through the production of oxalic acid, which dissolves and mobilizes mineral constituents of the stone. Mineral dissolution and mobilization provide the communities with nutrients and increase habitability by enlarging pore spaces within the stone and thereby accelerating deterioration.

Crown Corrosion of Wastewater Distribution Systems

A rapid form of microbial biodeterioration is observed in the **crown corrosion** of concrete sewer tiles, a process leading ultimately to collapse of the pipe. Crown corrosion is a consequence of interactions between sulfate-reducing bacteria (◀ Sections 14.12 and 15.11) and chemolithotrophic sulfur-oxidizing bacteria (◀ Sections 14.7 and 15.12) in these underground wastewater transmission systems (**Figure 22.28**).

The first step in crown corrosion is the reduction of sulfate in the sewage to H_2S by sulfate reducers, using primarily organic electron donors available in the waste stream water. The H_2S is then released into the headspace of the pipe where conditions are oxic. The sulfide, or partially oxidized intermediates such as thiosulfate or sulfur, is then oxidized by neutrophilic thiobacilli such as *Thiobacillus thioparus*. As the pH drops to 4–5 with continued microbial production of sulfuric acid, acidophilic sulfur-oxidizing species such as *Acidithiobacillus thiooxidans* displace the neutrophilic species. Destruction and ultimate structural failure of the concrete results from the reaction of sulfuric acid with lime in the concrete, producing $CaSO_4 \cdot 2H_2O$ (gypsum) that penetrates into the concrete. The gypsum subsequently reacts

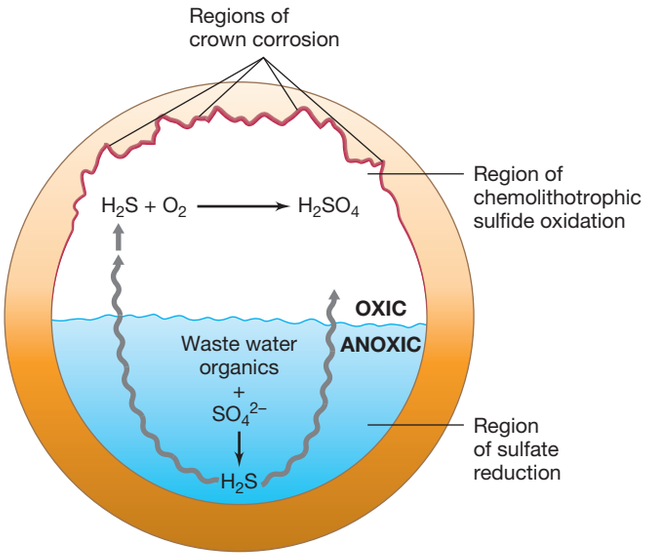


Figure 22.28 Crown corrosion of concrete sewer pipes. Corrosion is the result of a microbial sulfur cycle that develops within the transmission pipe. Sulfate-reducing bacteria consume organic material in the anoxic wastewater, producing H_2S. The latter is oxidized by sulfur-oxidizing chemolithotrophic bacteria that attach to the oxic upper (crown) pipe surface, accelerating corrosion from the production of H_2SO_4 (sulfuric acid).

with natural calcium–aluminum minerals present in the concrete, forming a new mineral that increases internal pressure and contributes to cracking and further acceleration of the corrosion process.

A series of steps and microbial participants similar to those of crown corrosion can corrode concrete holding tanks and cooling towers, particularly those in or near the marine environment where sulfate levels are typically high. Such deterioration can cause slow structural damage, such as concrete chipping, or even lead to a catastrophic rupture with threats to both the environment and humans.

Check Your Understanding

- How does the production of oxalic acid by fungi contribute to the deterioration of stone building materials?
- Which two major microbial physiologies are required to achieve crown corrosion? Which is an aerobic process and which is anaerobic?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Mineral Recovery and Acid Mine Drainage

22.1 The capacity of bacteria to oxidize Fe^{2+} aerobically at acidic pH is used to mine metals, principally copper-, uranium-, and gold-containing low-grade ores, through the process of microbial leaching. Bacterial oxidation of Fe^{2+} to Fe^{3+} is the key reaction in most microbial leaching processes because Fe^{3+} can extract metals in the ores under either oxic or anoxic conditions.

Q Which crucial step in the oxidation of copper ores is carried out by *Acidithiobacillus ferrooxidans*? How is copper recovered from copper solutions produced by leaching?

22.2 Spontaneous microbial oxidation of ferrous iron in pyritic ore or coal that has been exposed to air and water, such as occurs during some coal-mining operations, causes a type of pollution called acid mine drainage.

Q Which *Bacteria* and *Archaea* play a major role in acid mine drainage? Why do they carry out the reactions that they do? Why is air necessary for this process?

II • Bioremediation

22.3 Although an inorganic pollutant such as uranium cannot be destroyed, containment is possible by reducing its mobility. For example, metal-reducing microorganisms in a region of uranium contamination can be stimulated to reduce U^{6+} to U^{4+}, forming the immobile mineral uraninite that does not move into the groundwater.

Q What could thwart microbial bioremediation of a site that contains buried nuclear weapons that are leaking uranium?

22.4 Hydrocarbons are excellent carbon sources and electron donors for bacteria and are readily oxidized when O_2 is available. Hydrocarbon-oxidizing bacteria bioremediate spilled oil, and their activities can be assisted by addition of inorganic nutrients.

Q What physical and chemical conditions are necessary for the rapid microbial degradation of oil in aquatic environments?

22.5 Some xenobiotics persist, whereas others are readily degraded, depending on their chemistries. Chlorinated xenobiotics can be degraded both aerobically and anaerobically. Highly chlorinated xenobiotics can be detoxified by organohalide-respiring bacteria that reductively remove the chlorides. Some oil-based plastics such as PET can be completely degraded by bacteria such as *Ideonella*.

Q Why are some bacterial transformations of xenobiotics possible only through cometabolism? How might *Ideonella sakaiensis* or its enzymes be used in the recycling industry?

III • Wastewater and Drinking Water Treatment

22.6 Sewage and industrial wastewater treatment reduces the organic and nutrient load of wastewater. Primary and secondary wastewater treatment employs physical, biological, and physicochemical processes. Following treatment, the effluent water has significantly reduced BOD and is suitable for release into the environment.

Q Trace the treatment of wastewater in a typical plant from incoming water to release. What is the overall reduction in the BOD for typical household wastewater? What is the overall reduction in the BOD for typical industrial wastewater?

22.7 Tertiary wastewater treatment for nitrogen and phosphorus removal is used to improve the quality of the wastewater previously subjected to primary and secondary treatment. The increasing use of granular sludge treatment technology offers significant reductions in the energy, space, and chemical requirements of traditional wastewater treatment.

Q Why is tertiary wastewater treatment desirable from an environmental point of view?

22.8 Sludge processing is used to reduce the amount of sludge for disposal and produce methane for energy. The anammox process is an effective and efficient way to remove nitrogen from high-ammonia sludge brine. Of increasing concern are pharmaceuticals and ingredients in personal care products that are not degraded by conventional treatment systems and that can have adverse environmental effects, even at very low concentrations.

Q Which of the following are aerobic treatment processes and which are anaerobic: nitrification, anammox, denitrification, sludge digestion to methane?

22.9 Drinking water purification plants employ industrial-scale physical and chemical systems that remove or neutralize biological, inorganic, and organic contaminants from water sources. Water purification plants employ clarification, filtration, and chlorination processes to produce potable water.

Q Identify (stepwise) the process of purifying drinking water. What important contaminants are targeted by each step in the process?

22.10 The pipes that carry municipal drinking water and the premise plumbing that provide it to the consumer have created new microbial habitats. By forming biofilms on water pipe walls, microbial communities are more resistant to chlorine, can sustain or sequester opportunistic pathogenic bacteria, and its members can be distributed by household water usage.

Q What features of municipal and premise water distribution systems might contribute to a microbial health hazard? Why might showering increase your exposure to opportunistic pathogens?

IV • Indoor Microbiology and Microbially Influenced Corrosion

22.11 The indoor air and surfaces of dwellings and other buildings contain a diversity of mostly harmless saprophytic microbes that are typically a reflection of the humans and animals that reside there. However, the microbiota of certain parts of the indoor built environment, such as bathrooms and toilets, may contain enteric pathogens from aerosols generated there.

Q From the perspective of a child's health, can a home be "too clean"? Explain.

22.12 Corrosion of metal structures exposed to the environment can be accelerated by microbial activity during microbially influenced corrosion. Structures in or near seawater are particularly prone to corrosion as a consequence of the direct and indirect activities of sulfate-reducing bacteria.

Q How does microbial metabolism accelerate the corrosion of various metals?

22.13 Complex microbial communities colonize stone and concrete and produce substances that dissolve and mobilize its mineral constituents. Crown corrosion of concrete sewer lines results from the concerted activities of sulfate-reducing and sulfur-oxidizing bacteria growing within the wastewater and the headspace of sewer pipes, respectively. The resulting sulfuric acid is primarily responsible for the destruction of the concrete.

Q How have fungi adapted in order to survive harsh conditions on stone building materials?

APPLICATION QUESTIONS

1. Acid mine drainage is in part a chemical process and in part a biological process. Discuss the chemistry and microbiology that lead up to acid mine drainage and point out the key reactions that are biological. What ways can you think of to prevent acid mine drainage? How might you prevent further generation of acid drainage?
2. Why is reduction of BOD in wastewater a primary goal of wastewater treatment? What are the consequences of releasing wastewater with a high BOD into local water sources such as lakes or streams?
3. Discuss the microbial ecology contributing to crown corrosion of concrete sewer lines. In consideration of this ecology, what intervention strategies might be useful in reducing or eliminating corrosion?

CHAPTER GLOSSARY

Acid mine drainage acidic water containing H_2SO_4 derived from the microbial and spontaneous oxidation of iron sulfide minerals released by coal mining

Anaerobic treatment degradative and fermentative reactions carried out by microorganisms under anoxic conditions to treat sludge solids or wastewater containing high levels of insoluble organic materials

Biochemical oxygen demand (BOD) the relative amount of dissolved oxygen consumed by microorganisms for complete oxidation of bioavailable organic and inorganic material in a water sample

Biodeterioration microbially catalyzed processes that degrade stone or concrete

Bioremediation the cleanup of oil, toxic chemicals, and other pollutants by organisms, usually microorganisms

Chloramine a disinfectant chemical manufactured on-site by combining chlorine and ammonia at precise ratios

Chlorination disinfection of water with Cl_2 at a sufficiently high concentration that a residual level is maintained throughout the distribution system

Clarifier a reservoir in which suspended solids in raw water are coagulated and removed through precipitation

Coagulation the formation of large, insoluble particles from much smaller, colloidal particles by the addition of aluminum sulfate and anionic polymers

Crown corrosion the destruction of the upper half, or crown, of concrete wastewater pipes by sulfuric acid produced through the concerted activities of sulfate-reducing and sulfur-oxidizing bacteria

Effluent water treated wastewater discharged from a wastewater treatment facility

Filtration the removal of suspended particles from water by passing it through one or more permeable media (e.g., sand or activated charcoal) and ion exchangers

Finished water water delivered to the distribution system after treatment

Flocculation the water treatment process after coagulation that uses gentle stirring to cause suspended particles to form larger, aggregated masses (flocs)

Microbial leaching the extraction of valuable metals such as copper from sulfide ores by microbial activities

Microbially influenced corrosion (MIC) the contribution of microbial metabolic activities to accelerating the corrosion of metal structures

Potable drinkable; safe for human consumption

Primary disinfection the introduction of sufficient chlorine or other disinfectant into clarified, filtered water to kill existing microorganisms and inhibit further microbial growth

Primary wastewater treatment physical separation of wastewater contaminants, usually by separation and settling

Pyrite a common iron-containing ore, FeS_2

Raw water surface water or groundwater that has not been treated in any way (also called untreated water)

Reductive dechlorination an anaerobic respiration in which a chlorinated organic compound is used as an electron acceptor, usually with the release of Cl^-

Secondary disinfection the maintenance of sufficient chlorine or other disinfectant residual in the water distribution system to inhibit microbial growth

Secondary wastewater treatment oxidative reactions carried out by microorganisms under aerobic conditions to treat wastewater containing low levels of organic materials

Sediment in treatment of water for drinking, the soil, sand, minerals, and other large particles found in raw water

Sewage liquid effluents contaminated with human or animal fecal material

Suspended solid a small particle of solid pollutant that resists separation by ordinary physical means

Tertiary wastewater treatment any treatment process in which unit operations are added for the further processing of the secondary treatment effluent or solids

Turbidity a measurement of suspended solids in water

Untreated water surface water or groundwater that has not been treated in any way (also called raw water)

Wastewater domestic sewage or liquid industrial waste, which cannot be discarded in untreated form into lakes or streams

Xenobiotic a synthetic compound never produced in nature

23 Microbial Symbioses with Microbes, Plants, and Animals

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MICROBIOLOGY**NOW**

Coral Fluorescence Provides the Guiding Light for Their Symbiotic Algae

Although many symbiotic microorganisms are transferred directly between generations of their host (vertical transfer), others are acquired from the environment (horizontal transfer) and must find their way to their host. Chemical signaling is one attraction mechanism. However, in at least one symbiosis, light from the green fluorescent protein (GFP) controls the process.

GFP is widely distributed among marine invertebrates, including jellyfish, anemones, and corals. Although often used in protein tagging experiments to measure gene expression and localize specific proteins in the cell, GFP and other proteins that fluoresce in different colors have natural functions that remain unclear. Some possibilities include shifting the color spectrum of bioluminescent organisms for prey attraction or avoidance or functioning in light sensory systems. However, GFP is now known to guide the symbiosis between stony corals (cnidarians) and *Symbiodinium*, the algal symbiont that nourishes the coral.

A significant fraction of *Symbiodinium* acquisition is by horizontal transfer, mostly during the coral larval and juvenile stages. How does the alga find the coral? The answer lies in the phototaxis of *Symbiodinium* toward light emitted from the coral's GFP (photo). Researchers demonstrated the importance of phototaxis by painting GFP on traps placed in laboratory aquaria containing *Symbiodinium* and in natural reef communities. *Symbiodinium* cells migrated to the painted traps, but not to unpainted traps. The GFP light emission directing *Symbiodinium* phototaxis matched the spectrum optimal for its photosynthesis. Thus, the coral is simply exploiting the natural capacity of *Symbiodinium* to use phototaxis for positioning optimally in the marine light gradient. Phototaxis may also be important for coral reacquisition of their symbionts following a bleaching event. As coral bleaching becomes a more frequent occurrence with climate change, it is essential to better understand mechanisms of repair. Without recovery from bleaching, the major marine ecosystems supported by corals could soon be lost.

Source: Aihara, Y., Maruyama, S., Baird, A. H., Iguchi, A., Takahashi, S., and Minagawa, J. 2019. Green fluorescence from cnidarian hosts attracts symbiotic algae. *Proc. Natl. Acad. Sci. (USA) 116*: 2118.

Many microbial species form intimate and often mutually beneficial associations, called **symbioses**, with other microorganisms, plants, or animals. In a **mutualism**, both symbiotic partners benefit; in **parasitism**, one symbiotic partner benefits and the other is harmed; in **commensalism**, one symbiotic partner benefits and the other is unaffected. Symbioses typically develop through prolonged association and **coevolution** of the partners, evolution that proceeds jointly in a pair of intimately associated species owing to the effects each has on the other. In this chapter we explore some well-studied examples of these microbial symbioses as a prelude to our coverage in the following chapter of microbial symbioses with humans—symbioses that are not only extensive, but in many instances, absolutely essential for our well-being.

I • Symbioses Between Microorganisms

Microorganisms can form intimate associations of two or more species to use resources more efficiently. Common associations form between phototrophic and chemotrophic partners, such as in lichens and some aquatic bacteria. Other associations exploit conductive surface structures to electrically couple two species for the oxidation of organic carbon.

Many microbial species form mutualisms with other microbial species. Direct microscopic observations of natural samples show that many microbes are not solitary entities but are associated with other microbes on surfaces or as suspended aggregates of cells. In most cases, the advantages conferred by an association are unknown. Because microbial ecologists have recognized that *communities* of interacting microbial populations—not individual organisms—control environmental processes, research to discover the nature of strictly microbial symbioses has increased. In Part I we present three microbial mutualisms where the advantages to both partners are clear.

23.1 Lichens

Lichens are readily visible, leafy or encrusting microbial symbioses often found growing on bare rocks, tree trunks, house roofs, man-made surfaces, and bare soils—surfaces where other organisms typically do not grow (Figure 23.1). The lichen symbiosis originates from an ancient mutualistic association between two dominant microorganisms: a fungus, usually an ascomycete (◀ Section 18.13), and either an alga or a cyanobacterium. This association is one of the earliest forms of terrestrial plant life and can be traced back 400 million years to the Devonian period. The alga or cyanobacterium is the phototrophic partner and produces organic matter that feeds the fungus. The fungus is a chemotroph and provides a firm anchor within which the phototrophic partner can grow. The fungus also releases *lichen acids* that promote the dissolution and chelation of inorganic nutrients from the rock or other surface that are needed by the phototroph. The fungus facilitates the uptake of water and sequesters some for the phototroph, a characteristic that enables lichens to colonize almost all terrestrial environments, including deserts and other arid environments. Cells of the phototroph (also called the *photobiont*) are embedded in defined layers or clumps among cells of the fungus, together forming the *thallus* (Figure 23.2), and are protected from erosion by rain or wind.

(a)

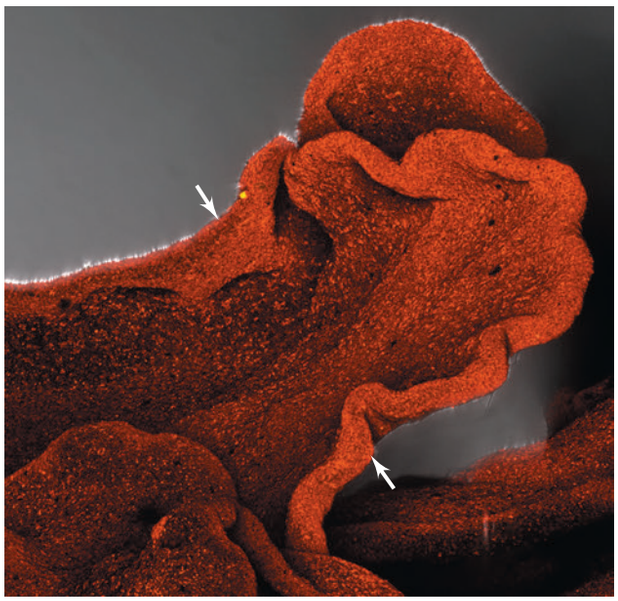
(b)

(c)

Figure 23.1 Lichens. Characteristic forms of lichens associated with *(a)* living and *(b)* dead tree branches, and *(c)* coating the surface of a large rock. Lichens are generally categorized by morphology and nearly all can be classified into one of the three types shown here, *(a)* foliose, *(b)* fruticose, and *(c)* crustose.

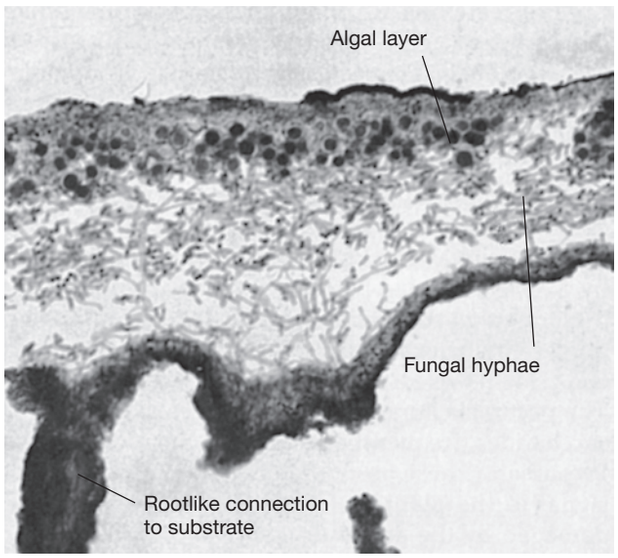
Lichen Components

Lichen photobionts are mostly green algae (◀ Section 18.16) but are sometimes species of brown algae (◀ Section 18.5). When cyanobacteria partner with lichens, they are often nitrogen-fixing species, organisms such as *Anabaena* or *Nostoc* (◀ Sections 3.12 and 15.3). In a few lichens, two phototrophic species are present within a single thallus, and molecular studies have revealed that additional low-abundance species of photobionts can also be co-residents. Variation in the lichen physical structure is primarily determined by the fungus (Figure 23.1), and more than 18,000 species of fungi are able to form lichen associations with a variety of photobionts. Their habitat range spans tropical to polar climatic zones, and coastal to high-altitude habitats. Lichens typically grow quite slowly, with



Pierre Philippe Laissue

(a)



T.D. Brock

(b)

Figure 23.2 Lichen structure. *(a)* Magnified image of a lichen thallus. The distance between the two arrows is approximately 6 mm. *(b)* Photomicrograph of a cross section through lichen thallus. The algal layer is positioned within the lichen structure near the top so as to receive the most sunlight. The fungal partners form the structural layers (cortex) positioned above and below the algal layer.

growth rates varying from 1 mm or less per year to over 3 cm per year, depending on the organisms composing the symbiosis and the temperature and amount of rainfall and sunlight received.

Diversity among the photobionts is lower than that of the fungi, and thus many different kinds of lichens can have the same phototrophic partner. The partner photobiont is primarily determined by lichen reproduction, which may be either asexual or sexual depending on the fungal species. During asexual reproduction, clonally derived fungal propagules co-disperse with the photobiont, preserving the specific symbiotic association. In contrast, fungal spores released by sexual reproduction lack the photobiont and require independent acquisition of a new symbiotic partner after dispersal. Such transmission provides an opportunity for the fungus to form new symbiotic associations that may have locally adaptive features. In contrast, lichens that disperse by propagules have less adaptive flexibility.

Molecular Studies of Lichens

The conventional view that lichens are simple two-partner assemblages has been challenged by culture-independent molecular studies (◀ Section 19.6) showing that in addition to multiple photobionts, lichens may have more than one fungus and also host bacteria that may benefit the association. The cortex of many lichens, formed by fungal structural tissue bounding the phototroph layer (Figure 23.2), can also contain a yeast (basidiomycete, ◀ Section 18.14) in addition to the ascomycete. Similarly, a common feature of many lichens is association with *Alphaproteobacteria* of the order *Rhizobiales*—known N_2-fixing bacteria—suggesting that in addition to cyanobacteria, other organisms may contribute fixed nitrogen to the symbiosis.

Metagenomic and metabolomic studies (◀ Sections 10.7, 10.10, and 19.8) have identified other metabolic benefits of the bacterial association, including the production of vitamins and protection from toxic compounds. A striking example of the latter is the increased abundance of genes encoding arsenic resistance detected in lichens inhabiting environments contaminated with arsenic. These examples demonstrate how culture-independent methods can reveal exciting new information about even well-studied symbioses.

Check Your Understanding

- What two types of microbes form a partnership in the lichen symbiosis? What are the benefits to both partners?
- Besides organic compounds, of what benefit to the fungus is a mutualism with *Anabaena*?
- What might be advantages of having bacteria present in the lichen symbiosis?

23.2 *"Chlorochromatium aggregatum"*

Microbial mutualisms called **consortia** form in freshwater environments. A commonly observed consortium develops between nonmotile green sulfur bacteria (phototrophs that are colored either green or brown) and certain motile, nonphototrophic bacteria. These consortia are found worldwide in stratified sulfidic freshwater lakes and can account for up to 90% of the green sulfur bacteria present and nearly 70% of the bacterial biomass in these lakes. The basis of the mutualism of these consortia is in the phototrophic production of organic matter by the green sulfur bacterium and the motility and organic matter consumption of the chemotrophic partner organism. Each consortium has been given a genus and species name, but since these names do not denote true species (because they are not a single organism), the names are enclosed in quotation marks. We examined the general biology of these consortia in Section 15.6.

Nature of the Consortium

The morphology of a green sulfur bacterial consortium depends on its species composition. The consortium generally consists of 13–69

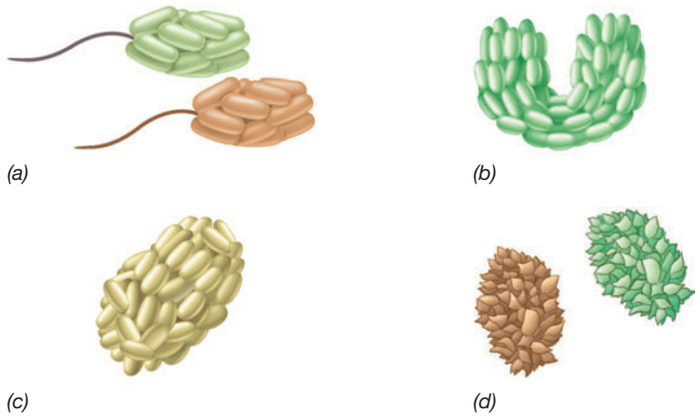


Figure 23.3 Drawings of some motile phototrophic green bacterial consortia found in freshwater lakes. Green epibionts: *(a)* "*Chlorochromatium aggregatum*," *(b)* "*C. glebulum*," *(c)* "*C. magnum*," *(d)* "*C. lunatum*." Brown epibionts: *(a)* "*Pelochromatium roseum*," *(d)* "*P. selenoides*." The epibionts are 0.5–0.6 μm in diameter. Adapted from Overmann, J., and H. van Gemerden. 2000. *FEMS Microbiol. Rev. 24*: 591.

green sulfur bacteria, called *epibionts*, surrounding and attached to a central, colorless, flagellated, rod-shaped bacterium (**Figure 23.3**). Several distinct motile phototrophic consortia have been recognized based on the color, morphology, and presence or absence of gas vesicles (◀ Section 2.7) of the epibionts. For example, in "*Chlorochromatium aggregatum*" the central bacterium is surrounded by rod-shaped green bacteria, whereas in "*Pelochromatium roseum*" the epibiont is brown. The consortium "*Chlorochromatium glebulum*" is bent and includes green epibionts that contain gas vesicles (Figure 23.3).

Green sulfur bacteria are obligately anaerobic phototrophs that form a distinct phylum (*Chlorobi*, ◀ Section 15.6). The green and brown species differ in the types of bacteriochlorophyll and carotenoids they contain. Both green and brown species are found in stratified lakes where light penetrates to depths at which the water contains hydrogen sulfide (H_2S), the primary electron donor for photosynthetic CO_2 fixation by the phototroph. In stratified lakes, the motile consortia reposition rapidly to remain in regions where conditions are most favorable for photosynthesis in the constantly changing gradients of light, oxygen, and sulfide that occur throughout the course of a day. Water samples collected from depths where these conditions are most favorable are enriched in this morphologically conspicuous consortium (**Figure 23.4**). The consortia show dark aversion (scotophobotaxis, ◀ Section 2.12) and positive chemotaxis toward sulfide.

Some free-living green sulfur bacteria, such as *Pelodictyon* (*Chlorobium*) *phaeoclathratiforme*, produce gas vesicles that regulate buoyancy and vertical position in the water column. However, because they are so small, the time they require for repositioning in the water column is from one to several days, which is not fast enough for tracking the more rapidly changing gradients. By contrast, motile consortia move up and down in the water column fast enough to follow the gradients of light and sulfide as they change on a diel basis.

Although green bacterial consortia were discovered almost a century ago, only with the advent of molecular techniques and newer culture methods has it become possible to study certain aspects of these remarkable associations. Sequencing of 16S ribosomal RNA (rRNA) genes revealed a significant geographic distribution of epibionts in lakes of Europe and the United States, many of which were genetically distinct. Epibionts in neighboring lakes have identical 16S rRNA gene sequences, whereas the sequences of morphologically similar epibionts in widely separated lakes differ. Phylogenetic analysis indicates that mechanisms of cell–cell recognition between epibionts and their central bacterium arose through coevolution of each specific partnership.

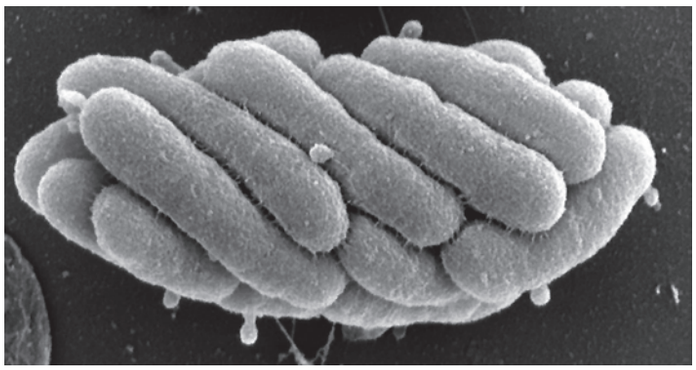
Phylogeny and Metabolism of a Consortium

The epibiont of "*Chlorochromatium aggregatum*" has been isolated and grown in pure culture. Although this green sulfur bacterium, named *Chlorobium chlorochromatii*, can be grown in pure culture, no naturally free-living variant has been observed, supporting the view that in nature a symbiotic lifestyle is obligate for epibionts. The central bacterium of "*Chlorochromatium aggregatum*" is a species of *Betaproteobacteria* (◀ Section 16.2). This bacterium requires α-ketoglutarate (an intermediate of the citric acid cycle, ◀ Figure 3.12) for growth, and this is presumably supplied to it by the epibiont. However, the central cell only assimilates fixed carbon in the presence of light and sulfide—conditions in which the epibionts are active and can transfer nutrients to the central bacterium. Genomic analysis of the central bacterium of one consortium revealed massive gene loss, indicating that this organism is unable to grow independently of the green sulfur bacterium.

Recent studies comparing the transcriptome and proteome (◀ Sections 10.8 and 10.9) of *C. chlorochromatii* growing alone or in association with the central rod bacterium have identified some features specifically related to the symbiosis. Approximately 50 *C. chlorochromatii* proteins are unique to the symbiotic state. Most of approximately 350 differentially regulated genes are repressed when the organism is symbiotically associated, whereas only 19 genes are more highly expressed. Many of the more highly expressed genes encode proteins of amino acid metabolism and nitrogen regulation. These include the enzyme glutamate synthase (◀ Section 3.14) and a transport protein for branched amino acids, suggesting that in addition to α-ketoglutarate, the metabolic coupling between the epibiont and central bacterium involves the exchange of amino acids as well.

J. Overmann and H. van Gemerden

Figure 23.4 Phase-contrast micrograph of "*Pelochromatium roseum*" from Lake Dagow (Brandenburg, Germany). The preparation was compressed between a coverslip and microscope slide to reveal the central rod-shaped bacterium (arrow). A single consortium is about 3.5 μm in diameter. Used with permission from J. Overmann and H. van Gemerden. 2000. *FEMS Microbiol. Rev. 24*: 591.



J. Overmann, with permission from J. Bacteriol

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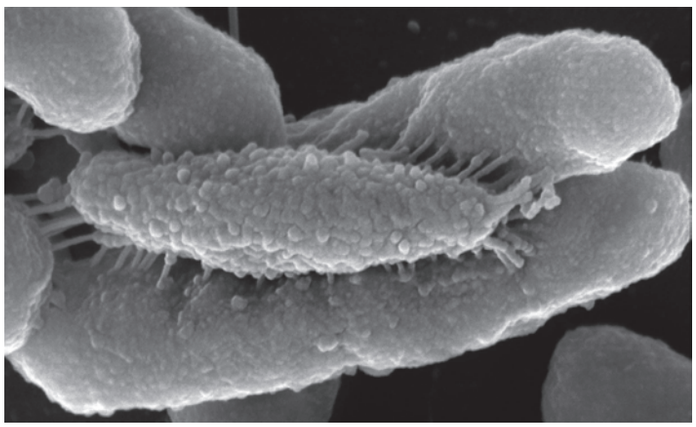


J. Overmann, with permission from J. Bacteriol

(b)

Figure 23.5 **Scanning electron micrographs of "*Chlorochromatium aggregatum*."** *(a) Chlorobium chlorochromatii* epibionts tightly clustered around a flagellated central bacterium. *(b)* The central bacterium exhibits numerous protrusions of its outer membrane that make intimate contact with the epibionts, possibly fusing the periplasms of the two gram-negative organisms. Cells of the epibiont are about 0.6 μm in diameter. Used with permission from G. Wanner et al. 2008. *J. Bacteriol. 190*: 3721.

Scanning electron microscopy of the consortium (Figure 23.5) has revealed that tubular extensions of the central bacterium's periplasm (◀ Section 2.4) cover much of its surface and appear to fuse with the periplasm of the epibiont. If the two bacterial partners actually share a common periplasmic space, this would facilitate the transfer of nutrients from phototroph to chemotroph and vice versa (however, whether the chemotroph reciprocates and transfers nutrients to the phototroph is unknown). The fact that the central bacterium is unable to grow without its phototrophic partner (while the phototroph can be grown in pure culture), and that organic compounds are only assimilated by the chemotroph in the light, is strong evidence that nutrients flow from the phototroph to feed the chemotroph and that the chemotroph is obligatorily dependent on its phototrophic partner.

Check Your Understanding

- What is the evidence that "*Chlorochromatium aggregatum*" is a stable product of evolution?
- What advantage does motility offer a phototrophic consortium?
- How might nutrients be shuttled between phototroph and chemotroph in the consortium?

23.3 Methanotrophic Consortia: Direct Interspecies Electron Transfer

Methanotrophic consortia that couple the activities of two anaerobic microbes effectively oxidize methane to CO_2 in anoxic marine sediments. The biochemistry of this process was discussed in Section 14.16.

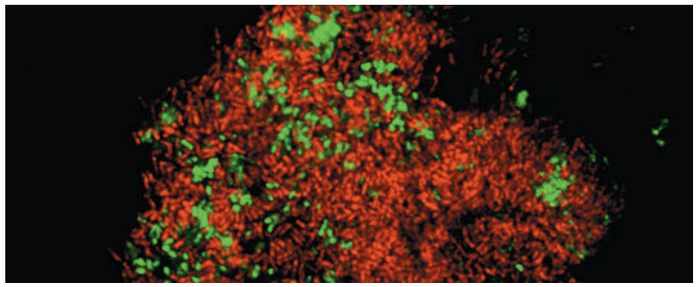
Anaerobic Oxidation of Methane and Short-Chain Alkanes

It has long been known that methane is oxidized in anoxic marine sediments. Subsequent culture-independent studies showed that specific methane-oxidizing (methanotrophic) *Archaea* form intimate associations with sulfate-reducing bacteria (Figure 23.6*a*) to do this, and working together, the organisms carry out the following reaction:

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2H_2O \quad \Delta G^{0\prime} = -18\,kJ$$

The methanotrophic *Archaea* comprise three different groups of *an*aerobic *me*thane (ANME)-oxidizing *Euryarchaeota*. Members of the ANME-1 cluster belong to the *Methanomicrobia*, ANME-2 the order *Methanosarcinales*, and ANME-3 to *Methanococcoides* species (◀ Section 17.2).

All ANME *Archaea* employ part of the metabolic pathway used by methanogens to generate methane, but ANME run the pathway in the opposite direction. The first step of anaerobic oxidation is activation of methane by methyl-coenzyme M reductase (MCR), producing coenzyme M–bound methyl groups that are further oxidized by



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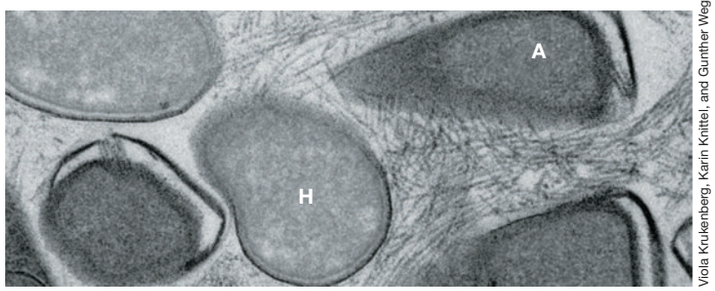


Viola Krukenberg, Karin Knittel, and Gunther Wegener

(b)

Figure 23.6 **Anaerobic methane-oxidizing consortia.** *(a)* A methanotrophic consortia enriched from geothermally heated sediments (Guaymas Basin, Gulf of California, Mexico) by cultivation with methane as sole electron donor and stained by CARD-FISH (◀ Section 19.5) using probes selective for the ANME methanotroph (red fluorescence) and its sulfate-reducing bacterial partner (green fluorescence). *(b)* Electron micrograph of a thin section through the consortia, showing the electrically conductive "nanowires" produced by the sulfate reducer (H), connecting it electrically to cytochrome-rich proteins on the surface of the ANME methanotroph (A).

the C_1 pathway (◀ Section 14.15). Some species of ANME belonging to the *Methanomicrobia* can completely oxidize short-chain alkanes (ethane, propane, and butane) using variants of MCR that activate the alkanes for further oxidation in a similar fashion to how MCR activates methane in ANME species. For example, ethane-oxidizing enrichment cultures produce ethyl-coenzyme M (ethyl-CoM) as an initial intermediate. Despite this variation in substrates, short-chain alkane oxidizers depend on syntrophic interactions with a sulfate reducer or other appropriate electron-accepting partner bacteria.

Sulfate-Reducing Bacteria as Partner Organisms in Anoxic Hydrocarbon Oxidations

A variety of sulfate-reducing *Bacteria* are syntrophic partners, including the *Deltaproteobacteria Desulfosarcina* and *Desulfococcus* species, and species of the family *Desulfobulbaceae* (◀ Section 15.11). These bacteria accept electrons from hydrocarbon-oxidizing methanogens and dispose of them by reducing sulfate to sulfide. The transfer of electrons occurs by direct electron transfer between the two species, using electrically conductive appendages similar to those described for metal-reducing bacteria (◀ Sections 14.13, 15.13, and 21.5 and Figures 14.31 and 21.14). The conductive structures (also called "nanowires") bridge the intercellular space between the organisms (Figure 23.6*b*).

Anaerobic methane oxidation may not depend on syntrophic association in all cases. For example, enrichment cultures dominated by certain ANME *Archaea* established from freshwater samples were shown to couple methane oxidation to the reduction of nitrate to nitrite, rather than to the reduction of sulfate. In addition, some evidence suggests that redox-active metals such as ferric iron may also function as electron acceptors to support anoxic methanotrophy. However, in both of these cases the presence of a second organism (the electron consumer) to form a syntrophic association cannot be ruled out, as pure cultures of ANME organisms have not yet been obtained.

Direct Interspecies Electron Transfer (DIET)

Direct interspecies electron transfer (abbreviated DIET) of the type that drives the ANME–sulfate reducer association is much more common than was earlier suspected. For example, apart from the direct reduction of insoluble metals by *Geobacter* species (◀ Section 21.5), species of *Geobacter* also form partnerships with methanogens. When paired in an association with *G. metallireducens*, the normally acetate-utilizing (acetoclastic) methanogen *Methanosaeta harundinacea* reduces carbon dioxide to methane using electrons delivered by *G. metallireducens* from acetate oxidation. In this partnership, *Geobacter* benefits because it uses the acetate as its carbon and energy source. And, because abiotic electron acceptors are limiting or absent, *Geobacter* uses the methanogen as its electron acceptor. The methanogen benefits because instead of splitting acetate to form methane, it reduces CO_2 to CH_4 instead, a more energetically favorable reaction (◀ Section 14.15). As we learn more about organisms participating in DIET, greater metabolic variety will almost surely be revealed.

Check Your Understanding

- What observations suggest that direct interspecies electron transfer may be more widely distributed among *Bacteria* and *Archaea* than now recognized?
- Which of the two partner organisms, the ANME or the sulfate-reducing bacterium, may have the capacity to live independently? How might this influence the specificity of interaction?

II • Plants as Microbial Habitats

Symbiotic associations between microorganisms and plants can be mutualistic, where the microbe and the plant exchange nutrients, or parasitic, where the plant produces nutrients only for the bacterium. Prime examples of the former exist in the *Rhizobium*–root nodule symbiosis and of the latter in crown gall disease.

Plants interact closely with microbes through their roots and leaf surfaces and even more intimately within their vascular tissue and cells. Most mutualisms between plants and microorganisms increase nutrient availability to the plants or defend them against pathogens. We consider three examples in the next three sections: (1) a mutualism where the nature of the symbiosis is understood in exquisite detail (root nodules), (2) a mutualism in which plants expand and interconnect their root system through association with a fungus (mycorrhizae), and (3) a symbiosis that is harmful to the plant (crown gall disease).

23.4 The Legume–Root Nodule Symbiosis

A plant–bacterial mutualism of great importance to humans is that of leguminous plants and nitrogen-fixing bacteria. *Legumes* are flowering plants that bear their seeds in pods and include such agriculturally important members as soybeans, clover, alfalfa, beans, and peas. These crops are key commodities for the food and agricultural industries, and the ability of legumes to grow without added nitrogen saves farmers millions of dollars in fertilizer costs yearly and reduces the polluting effects of fertilizer runoff.

The partners in a symbiosis are called *symbionts*, and most nitrogen-fixing bacterial symbionts of plants are collectively called *rhizobia*, derived from the name of a major genus, *Rhizobium*. Species of rhizobia are *Alpha-* or *Betaproteobacteria* (**Figure 23.7**; ◀ Sections 16.1

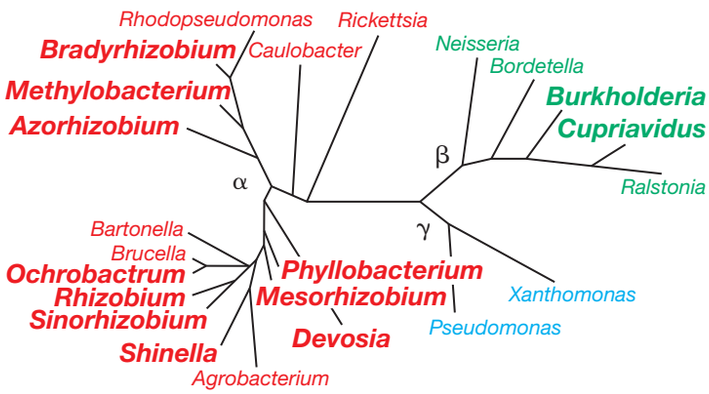


Figure 23.7 Phylogeny of rhizobial (genus names in boldface) and related genera inferred from analysis of 16S rRNA gene sequences. More than 70 species of rhizobia are found in 12 genera of *Alpha-* and *Betaproteobacteria*.

Mastering Microbiology
Art Activity: Figure 23.10 Steps in the formation of a root nodule in a legume infected by *Rhizobium*

Joe Burton

Figure 23.8 Soybean root nodules. The nodules developed from infection by *Bradyrhizobium japonicum*. The main stem of this soybean plant is about 0.5 cm in diameter.

and 16.2) that can grow freely in soil or infect leguminous plants and establish a symbiotic relationship. The same bacterial genus (or even species) of legume can contain both rhizobial and nonrhizobial strains of nitrogen fixers. Infection of legume roots by rhizobia leads to the formation of **root nodules** (Figure 23.8) in which the bacteria convert N_2 to NH_3 (◀ Sections 3.12 and 15.9). Nitrogen fixation in root nodules accounts for a fourth of the N_2 fixed annually on Earth and is of enormous agricultural importance, as it increases the fixed nitrogen content of soil. Nodulated legumes can grow well on unfertilized bare soils that are nitrogen deficient, while other plants grow only poorly on them (Figure 23.9).

Leghemoglobin and Cross-Inoculation Groups

In the absence of its bacterial symbiont, a legume cannot fix N_2. Rhizobia, on the other hand, can fix N_2 when grown in pure culture under microaerophilic conditions (a low-oxygen environment is necessary because the key nitrogen-fixing enzyme *nitrogenase* is inactivated by high levels of O_2, ◀ Section 3.12). In the nodule, O_2 levels are precisely controlled by the O_2-binding protein **leghemoglobin**. Production of this iron-containing protein in healthy N_2-fixing nodules (Figure 23.10) is induced through the interaction of the plant and bacterial partners. Leghemoglobin functions as an "oxygen buffer," cycling between the oxidized (Fe^{3+}) and reduced (Fe^{2+}) forms of iron to supply sufficient O_2 for bacterial respiration while keeping unbound O_2 within the nodule low. The ratio of leghemoglobin-bound O_2 to free O_2 in the root nodule is thus maintained on the order of 10,000:1.

There is a marked specificity between the species of legume and rhizobium that can establish a symbiosis. A particular rhizobial species is able to infect certain species of legumes but not others. A group of related legumes that can be infected by a particular rhizobial species is called a *cross-inoculation group*. Each group consists of all the legume species that will develop nodules when inoculated with rhizobia obtained from any other legume of the group (Table 23.1). If legumes are inoculated with the correct rhizobial strain, leghemoglobin-rich, N_2-fixing nodules develop on their roots (Figures 23.8–23.10).

Ben B. Bohlool

Figure 23.9 Effect of nodulation on plant growth. A field of unnodulated (left) and nodulated (right) soybean plants growing in nitrogen-poor soil. The yellow color is typical of chlorosis, the result of nitrogen starvation.

Steps in Root Nodule Formation

How root nodules form is well understood, and the steps are as follows (Figure 23.11):

1. Recognition of the correct partner by both plant and bacterium and attachment of the bacterium to the root hairs
2. Secretion of oligosaccharide signaling molecules (Nod factors) by the bacterium
3. Bacterial invasion of the root hair
4. Movement of bacteria to the main root by way of the infection thread
5. Formation of modified bacterial cells (bacteroids) within the plant cells, development of the N_2-fixing state, and continued plant and bacterial cell division forming the mature root nodule

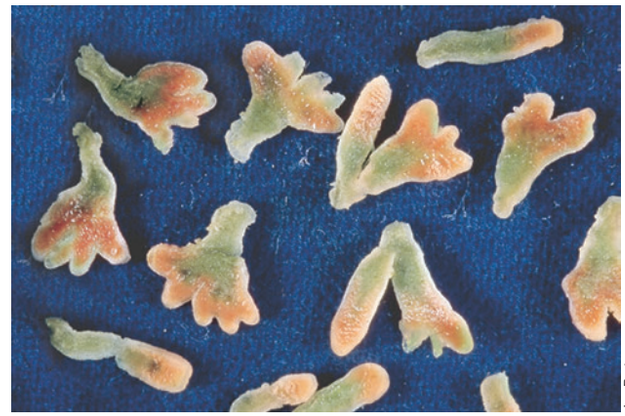

Joe Burton

Figure 23.10 Root nodule structure. Sections of root nodules from the legume *Coronilla varia*, showing the reddish pigment leghemoglobin.

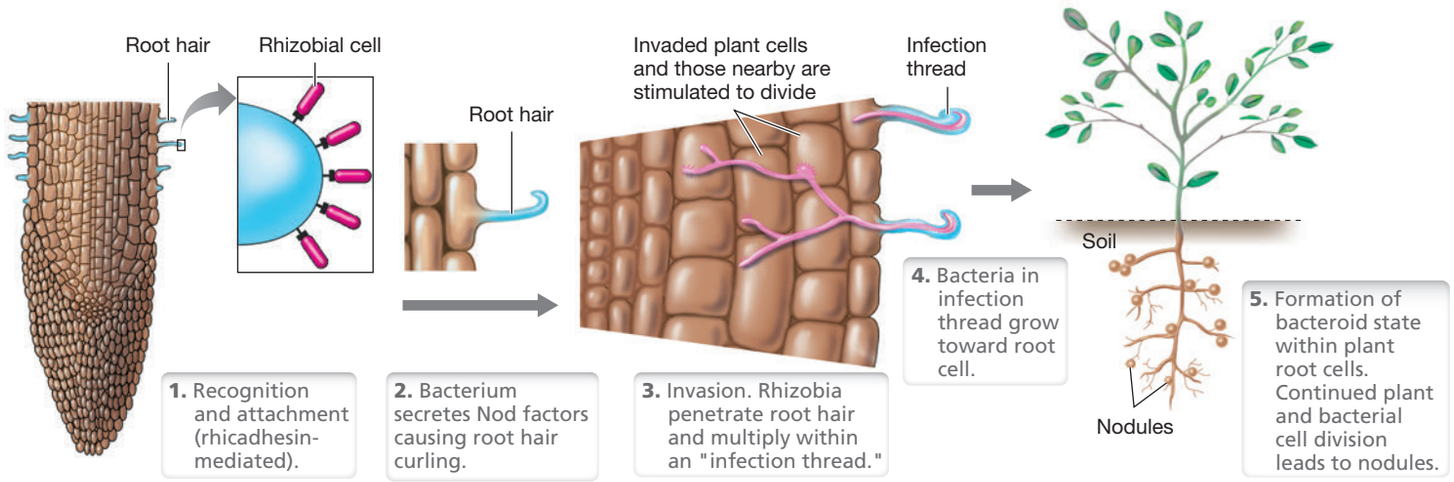


Figure 23.11 Steps in the formation of a root nodule in a legume infected by *Rhizobium*. Formation of the bacteroid state is a prerequisite for nitrogen fixation. The time course of nodulation events from infection to effective nodule is about 1 month for soybeans. See Figure 23.16 for physiological activities in the nodule.

Attachment and Infection

The roots of leguminous plants secrete organic compounds that stimulate the growth of a diverse rhizosphere microbial community. If rhizobia of the correct cross-inoculation group are in the soil, they will form large populations and eventually attach to the root hairs that extend from the roots of the plant (Figure 23.11). An adhesion protein called *rhicadhesin* is present on the cell surfaces of rhizobia. Other substances, such as carbohydrate-containing proteins called *lectins* and specific receptors in the plant cytoplasmic membrane, also play roles in plant–bacterium attachment.

After attaching, a rhizobial cell penetrates into the root hair, which curls in response to substances (Nod factors) secreted by the bacterium. The bacterium then induces the plant to form a cellulosic tube, called the **infection thread** (Figure 23.12*a*), which spreads down the root hair. Root cells adjacent to the root hairs subsequently become infected by rhizobia, and plant cells divide. Continued plant cell division forms the tumorlike nodule (Figure 23.12*a*) consisting of plant cells filled with bacteroids (discussed below and shown in Figure 23.12*b*, *c*). A different mechanism of infection is used by some rhizobia adapted to aquatic or semiaquatic tropical legumes (see Figure 23.17). These rhizobia enter the plant at the loose cellular junctions of roots emerging perpendicular from an established root (*lateral roots*), or on a stem in or just above the water. Following entry into the plant, some of the rhizobia develop infection threads, whereas others do not.

TABLE 23.1 Major cross-inoculation groups of leguminous plants

Host plant group	Nodulated by
Pea	*Rhizobium leguminosarum* biovar *viciae*[a]
Bean	*Rhizobium leguminosarum* biovar *phaseoli*[a]
Bean	*Rhizobium tropici*
Lotus	*Mesorhizobium loti*
Clover	*Rhizobium leguminosarum* biovar *trifolii*[a]
Alfalfa	*Sinorhizobium meliloti*
Soybean	*Bradyrhizobium japonicum*
Soybean	*Bradyrhizobium elkanii*
Soybean	*Sinorhizobium fredii*
Sesbania rostrata (a tropical legume)	*Azorhizobium caulinodans*

[a]Several varieties (biovars) of *Rhizobium leguminosarum* exist, each capable of nodulating a different legume.

Bacteroids

The rhizobia multiply rapidly within the plant cells and become transformed into swollen, misshapen, and branched cells called **bacteroids**. A microcolony of bacteroids becomes surrounded by portions of the plant cytoplasmic membrane to form a structure called the *symbiosome* (Figure 23.12*c*), and only after the symbiosome forms does N_2 fixation begin. Nitrogen-fixing nodules can be detected experimentally by the reduction of acetylene to ethylene (◀ Section 19.9). When the plant dies, the nodule deteriorates, releasing bacteroids into the soil. Although bacteroids are incapable of division, a small number of dormant rhizobial cells are always present in the nodule. These now proliferate, using some of the products of the deteriorating nodule as nutrients. The bacteria can then initiate infection the next growing season or maintain a free-living existence in the soil.

Nodule Formation: *nod* Genes, Nod Proteins, and Nod Factors

Rhizobial genes that direct the steps in nodulation of a legume are called *nod genes*. It is thought that the ability to form nodules has independently emerged multiple times through the horizontal transfer of such genes as *nod* and *nif* that are located on plasmids or

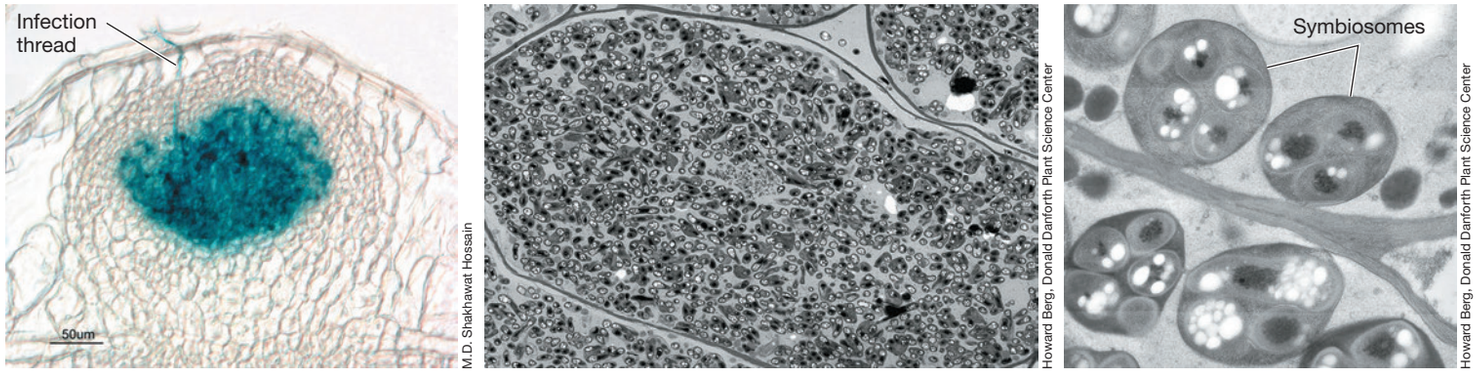


Figure 23.12 The infection thread and formation of root nodules. *(a)* Light micrograph of an early-stage nodule from a legume (*Lotus japonicus*) infected with a rhizobium strain containing the *lacZ* gene. The nodule was sectioned and bacterial distribution (blue) determined using an activity stain (X-gal) that turns blue when cleaved by the enzyme β-galactosidase (◀ Section 12.2 and Figure 12.10). An infection thread, consisting of a cellulosic tube through which bacteria move to root cells, is clearly visible extending from the surface to the interior. *(b)* Transmission electron micrograph through a soybean nodule infected with *Bradyrhizobium japonicum*, showing bacteroid-filled plant cells. The plant cell is approximately 50 μm long. *(c)* Higher-magnification micrograph showing individual symbiosomes, each filled with several bacteroids. The clear areas in each bacteroid are the storage polymer poly-β-hydroxybutyrate (◀ Section 2.7). Bacteroids are about 2 μm long.

transferable regions of chromosomal DNA. In *Rhizobium leguminosarum* biovar *viciae*, which nodulates peas, ten *nod* genes have been identified. The *nodABC* genes encode proteins that produce oligosaccharides called **Nod factors**; these induce root hair curling and trigger cell division in the pea plant, eventually leading to formation of the nodule (see Figure 23.16 for a description of root nodule biochemistry).

Nod factors are lipochitin oligosaccharides to which various substituents are bonded (**Figure 23.13**) that function as primary rhizobial signaling molecules triggering legumes to develop new plant organs: root nodules that host the bacteria as nitrogen-fixing bacteroids (Figure 23.12). Resolving the details of the signaling pathway triggered by Nod binding to cell surface receptors (NFR1 and NFR2) and leading to the induction of organogenesis (nodule formation) is an active area of research (**Figure 23.14**). Interestingly, many elements of the signaling pathway leading to nodulation are also used by the mycorrhizal fungi for infection of plant roots (Figure 23.14 and Section 23.5).

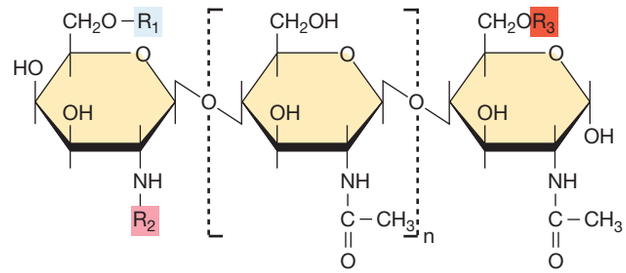
Which plants a given rhizobial species can infect is in part determined by the structure of the Nod factor it produces. Besides the *nodABC* genes, which are universal and whose products synthesize the Nod backbone, each cross-inoculation group contains *nod* genes that encode proteins that chemically modify the Nod factor backbone to form its species-specific molecule (Figure 23.13). In *R. leguminosarum* biovar *viciae*, *nodD* encodes the regulatory protein NodD, which controls transcription of other *nod* genes. After interacting with inducer molecules, NodD *promotes* transcription and is thus a *positive* regulatory protein (◀ Section 7.3). NodD inducers are plant flavonoids, organic molecules that are widely secreted by plants. Some flavonoids that are structurally very closely related to *nodD* inducers in *R. leguminosarum* biovar *viciae* inhibit *nod* gene expression in other rhizobial species (**Figure 23.15**). This indicates that part of the specificity observed between plant and bacterium in the rhizobia–legume symbioses is controlled by the chemistry of the flavonoids secreted by each species of legume.

UNIT 5

Mastering Microbiology
Art Activity: Figure 23.13 Nod and Myc signalling pathways in root nodule formation and mycorrhizal arbuscule formation

Biochemistry of Root Nodules

As discussed in Section 3.12, N_2 fixation is catalyzed by *nitrogenase*. Nitrogenase from bacteroids shows the same biochemical properties as the enzyme from free-living N_2-fixing bacteria, including O_2 sensitivity and the ability to reduce acetylene as well as N_2. Bacteroids are dependent on the plant for the electron donor for N_2 fixation.



(a)

Rhizobial or AM fungus species	R_1	R_2	R_3
Sinorhizobium meliloti (alfalfa)	Ac	C16:2 or C16:3	SO_3H
Rhizobium leguminosarum biovar *viciae* (pea)	Ac	C18:1 or C18:4	H or Ac
Glomus intraradices (many agricultural crops)	H	C16 or C16:1 or C16:2 or C18 or C18:1Δ9Z	H or SO_3H

(b)

Figure 23.13 Nod and Myc factors. *(a)* General structure of Nod factors produced by two rhizobial species and the Myc factor produced by an arbuscular mycorrhizal (AM) fungus (Section 23.5). The central hexose unit can repeat up to three times for different Nod factors and repeat either two or three times for the different Myc factors. *(b)* Table of the structural differences (R_1, R_2, R_3) that define the precise signaling factors of each species. C16:1, C16:2, and C16:3, palmitic acid with either one, two, or three double bonds, respectively; C18:1, oleic acid with one double bond; C18:1Δ9Z, the *trans* isomer of oleic acid with one double bond at the 9th C–C bond; C18:4, oleic acid with four double bonds; Ac, acetyl.

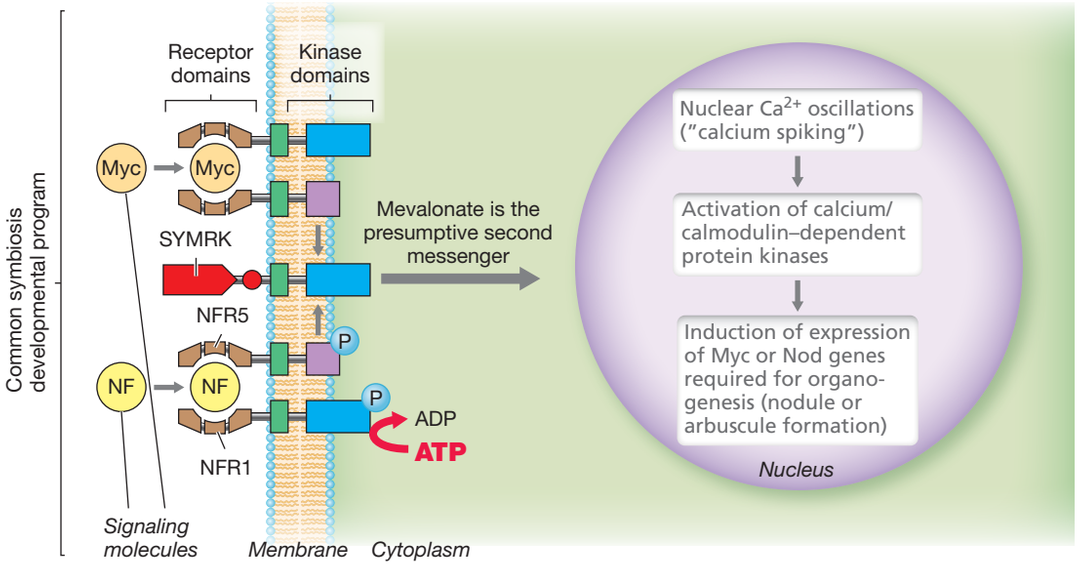


Figure 23.14 Nod and Myc signaling pathways in root nodule and mycorrhizal arbuscule formation. Nod factor (NF) signaling involves at least three membrane-associated receptors (NFR1, NFR5, and SYMRK) that together initiate nodulation via protein phosphorylation. NFR1 and SYMRK have active kinase domains (blue), whereas NFR5 kinase is inactive. The direct binding of NF to a complex of NFR1 and NFR5 at the plant cell cytoplasmic membrane initiates signal transduction by activation of the NFR1 kinase. The resulting phosphorylation of the NFR cytoplasmic domains triggers events leading to formation of the infection thread. Signal transduction to the SYMRK by the NFR1–NFR5–Nod factor complex (or by an unidentified receptor for the Myc factor) is part of a conserved symbiosis program in which induction of calcium signaling in the plant cell nucleoplasm triggers gene expression changes and production of plant growth hormones (cytokinins) required for nodule or arbuscule formation. See Section 23.5 for a discussion of mycorrhizae.

The major organic compounds transported across the symbiosome membrane (Figure 23.12*c*) and into the bacteroid proper are citric acid cycle intermediates, in particular, the C_4 organic acids *succinate*, *malate*, and *fumarate* (◀ Figure 3.12; **Figure 23.16**). These are used as electron donors for ATP production and, following conversion to pyruvate, as the ultimate source of electrons for the reduction of N_2.

The product of N_2 fixation is ammonia (NH_3), and the plant assimilates most of this NH_3 by forming organic nitrogen compounds. The NH_3-assimilating enzyme glutamine synthetase is present in high levels in the plant cell cytoplasm and can convert glutamate and NH_3 into glutamine (◀ Section 3.14). Glutamine and a few other organic nitrogen compounds transport bacterially fixed nitrogen throughout the plant.

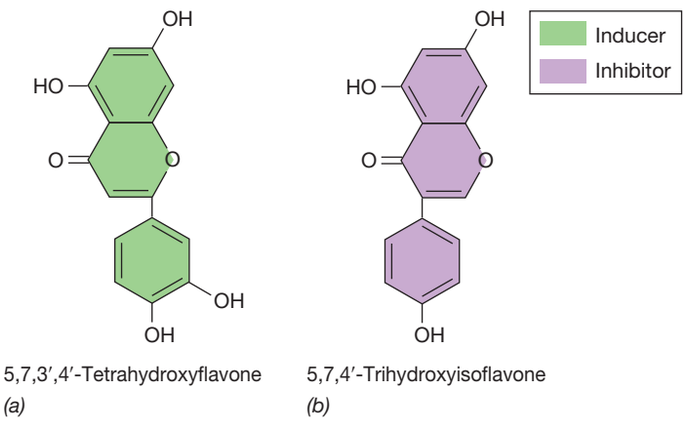


Figure 23.15 Plant flavonoids and nodulation. Structures of flavonoid molecules that are *(a)* an inducer of *nod* gene expression and *(b)* an inhibitor of *nod* gene expression in *Rhizobium leguminosarum* biovar *viciae*. Note the similarities in the structures of the two molecules. The common name of the structure shown in part *a* is *luteolin*, and it is a flavone derivative. The structure in part *b* is called *genistein*, an isoflavone derivative.

Stem-Nodulating Rhizobia

Although most leguminous plants form N_2-fixing nodules on their *roots*, a few legume species bear nodules on their *stems*. Stem-nodulated leguminous plants are widespread in tropical regions where soils are often nitrogen deficient because of leaching and intense biological activity. The best-studied system is the tropical aquatic legume *Sesbania*, which is nodulated by the bacterium *Azorhizobium caulinodans* (**Figure 23.17**). Stem nodules typically form in the submerged portion of the stems or just above

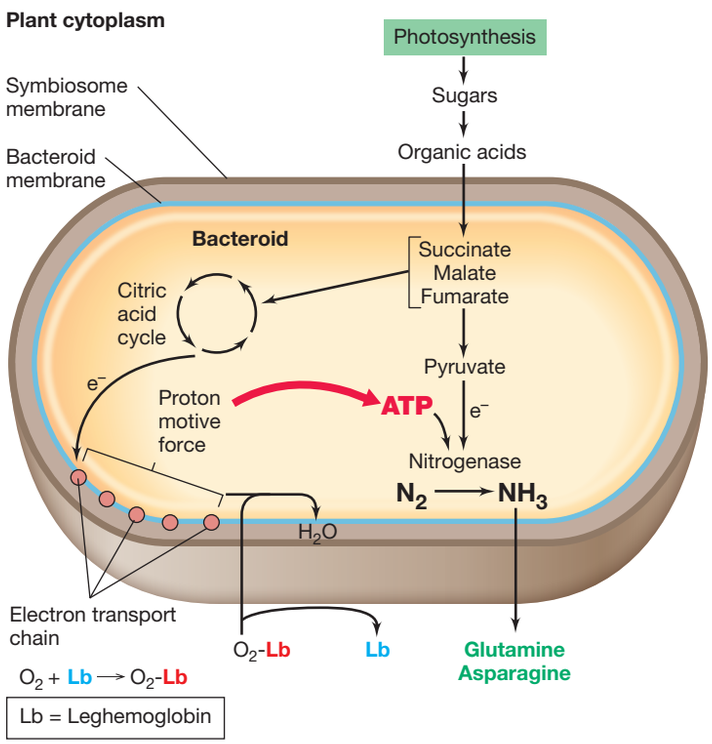


Figure 23.16 The root nodule bacteroid. Schematic of major metabolic reactions and nutrient exchanges in the bacteroid. The symbiosome is a collection of bacteroids surrounded by a membrane originating from the plant (see Figure 23.12*c*).

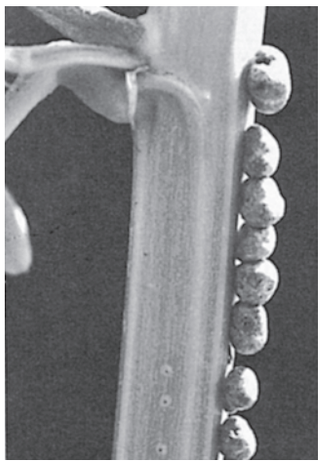


Figure 23.17 Stem nodules formed by stem-nodulating *Azorhizobium*. The right side of this stem of the tropical legume *Sesbania rostrata* was inoculated with *Azorhizobium caulinodans*, but the left side was not.

the water level. The general sequence of events by which stem nodules form in *Sesbania* resembles that of root nodules: attachment, formation of an infection thread, and bacteroid formation.

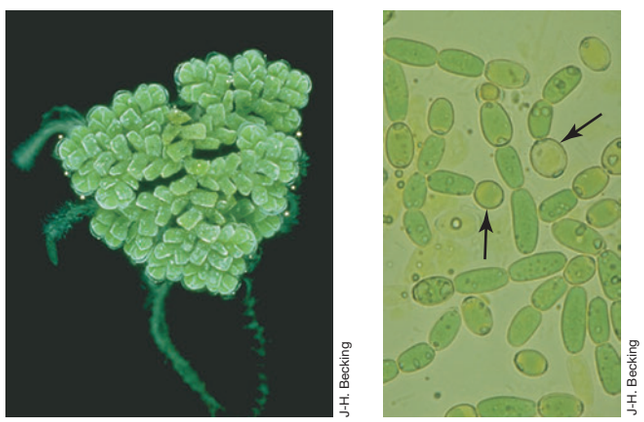
Some stem-nodulating rhizobia produce bacteriochlorophyll *a* and thus have the potential to carry out anoxygenic photosynthesis (◀ Section 14.5) in their illuminated habitat. Bacteriochlorophyll-containing rhizobia, called "photosynthetic *Bradyrhizobium*," are widespread in nature, particularly in association with tropical legumes. In these species, light energy converted to chemical energy (ATP) in photosynthesis probably supplies part of the energy source needed by the bacterium to support N_2 fixation.

Mastering Microbiology
Art Activity: Figure 23.15 The root nodule bacterioid

UNIT 5

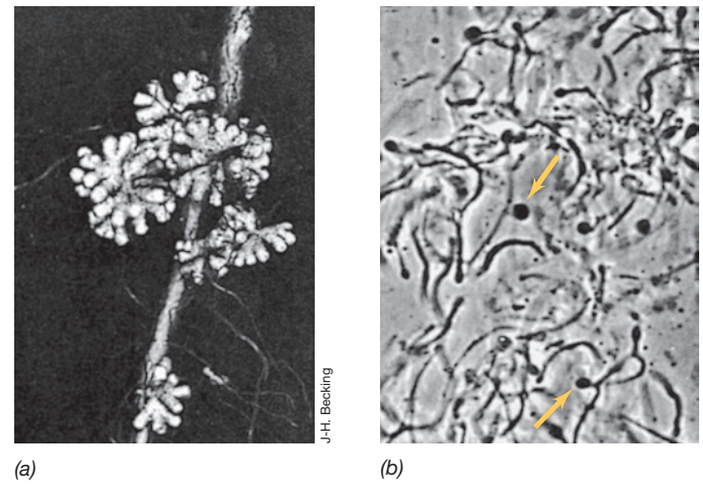
Nonlegume N_2-Fixing Symbioses: *Azolla–Anabaena* and *Alnus–Frankia*

Various nonleguminous plants form N_2-fixing symbioses with bacteria other than rhizobia. For example, the water fern *Azolla* harbors within small pores of its fronds a species of heterocys-tous N_2-fixing cyanobacteria (◀ Section 15.3) called *Anabaena azollae* (**Figure 23.18**). *Azolla* has been used for centuries to enrich Asian rice paddies with fixed nitrogen. Before planting rice, the farmer allows the surface of the rice paddy to become densely covered with *Azolla*. As the rice plants grow, they eventually crowd out the *Azolla*, causing its death and the release of its nitrogen, which is assimilated by the rice plants. By repeating this process each growing season, rice farmers can obtain high yields of rice without applying nitrogenous fertilizers.



(a) (b)

Figure 23.18 *Azolla–Anabaena* symbiosis. *(a)* Intact association showing a single plant of *Azolla pinnata*. The diameter of the plant is approximately 1 cm. *(b)* Cyanobacterial symbiont *Anabaena azollae* as observed in crushed leaves of *A. pinnata*. Single cells of *A. azollae* are about 5 μm wide. Vegetative cells are oblong; the spherical heterocysts (lighter color, arrows) are differentiated for nitrogen fixation.



(a) (b)

Figure 23.19 *Frankia* nodules and *Frankia* cells. *(a)* Root nodules of the common alder *Alnus glutinosa*. *(b) Frankia* culture purified from nodules of *Comptonia peregrina*. Note vesicles (arrows) on the tips of hyphal filaments.

The alder tree (genus *Alnus*) has N_2-fixing root nodules (**Figure 23.19*a***) that harbor filamentous, N_2-fixing actinomycetes of the genus *Frankia*. When assayed in cell extracts the nitrogenase of *Frankia* is sensitive to O_2, but cells of *Frankia* fix N_2 at full oxygen tensions. This is because *Frankia* protects its nitrogenase from O_2 by localizing the enzyme in terminal swellings on the cells called *vesicles* (Figure 23.19*b*). The vesicles contain thick walls that retard O_2 diffusion, thus maintaining the O_2 tension within vesicles at levels compatible with nitrogenase activity. In this regard, *Frankia* vesicles resemble the heterocysts produced by some filamentous cyanobacteria as localized sites of N_2 fixation (◀ Sections 8.9 and 15.3).

Alder is a characteristic pioneer tree able to colonize nutrient-poor soils, probably because of its ability to enter into a symbiotic N_2-fixing relationship with *Frankia*. A number of other small or bushy, woody plants are nodulated by *Frankia*. As is the case for the rhizobial symbionts of leguminous plants, a single strain of *Frankia* can form nodules on several different species of plants.

Check Your Understanding

- How do rhizobial root nodules benefit a plant?
- What are Nod factors and what do they do?
- What is a bacteroid, and what occurs within it? What is the function of leghemoglobin?
- What are the major similarities and differences between rhizobia and *Frankia*?

23.5 Mycorrhizae

Mycorrhizae are mutualisms between plant roots and fungi in which nutrients are transferred in both directions. The fungus transfers inorganic nutrients—in particular, phosphorus and nitrogen—from the soil to the plant, and the plant in turn transfers primarily carbohydrates to the fungus. These mutualisms are harnessed in agricultural applications. From fungal spores produced in culture or from root scrapings of infected plants, soil inoculants are produced that enhance plant growth.

Classes of Mycorrhizae

There are two classes of mycorrhizae. In *ectomycorrhizae*, fungal cells form an extensive sheath (*fungal mantle*) around the *outside* of the root with only a slight penetration into the root cellular structure (**Figure 23.20**). As rootlets emerge, they are rapidly colonized by the fungi (Figure 23.20*a*). In *endomycorrhizae*, a part of the fungus becomes deeply embedded within cells comprising the root tissue. Ectomycorrhizae are found mainly on the roots of forest trees, especially conifers, beeches, and oaks, and are most highly developed in boreal and temperate forests. In such forests, almost every root of every tree is mycorrhizal. The root system of a mycorrhizal tree such as a pine (genus *Pinus*) is composed of both long and short roots (Figure 23.20*b*, *c*). The short roots, which are characteristically dichotomously branched in *Pinus* (Figure 23.20*b*, *c*), show typical fungal colonization, and long roots are also frequently colonized.

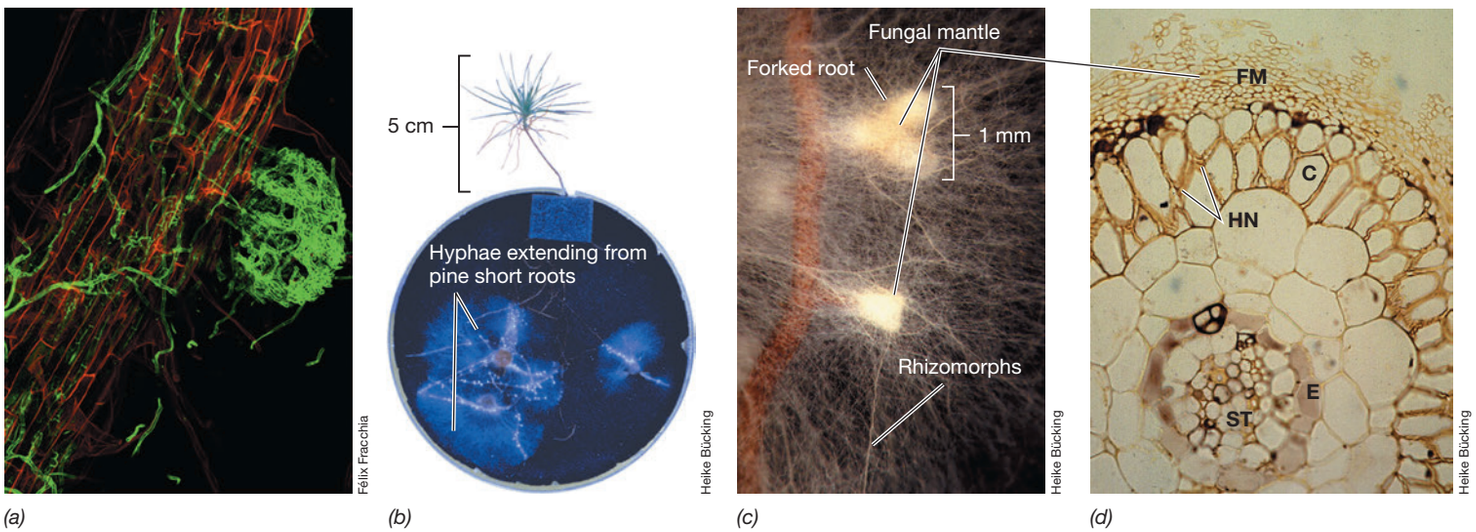
Ectomycorrhizal hyphae extending from the fungal mantle and penetrating between the epidermal and cortical cells form a network called the *Hartig net* (Figure 23.20*d*) where nutrient exchange between the fungus and the host plant occurs that benefit the plant (see Figure 23.23). Most mycorrhizal fungi do not catabolize cellulose and other leaf litter polymers. Instead, they catabolize simple carbohydrates and typically have one or more vitamin requirements. They obtain their carbon from root secretions and obtain inorganic minerals from the soil. Mycorrhizal fungi are rarely found in nature except in association with roots, and most are probably obligate symbionts.

Despite the close symbiotic association between fungus and root, a single species of tree can form multiple mycorrhizal associations. One pine species can associate with over 40 species of fungi. This relative lack of host specificity allows ectomycorrhizal mycelia to interconnect trees, providing linkages for transfer of carbon and other nutrients between trees of the same or different species. Nutrient transfer from well-illuminated overstory plants to shaded trees is thought to help equalize resource availability, subsidizing young trees and increasing biodiversity by promoting the coexistence of different species.

Arbuscular Mycorrhizae

Although ectomycorrhizal fungi play a significant role in the ecology of forests, there is a greater diversity of endomycorrhizae. Most are *arbuscular mycorrhizae* (*AM*) that comprise a phylogenetically distinct fungal division, the *Glomeromycota* (◀ Section 18.12), of which all or most species are obligate plant mutualists (the word *arbuscular* means "little tree"). AM colonize 70–90% of all terrestrial plants, including most grassland species and many crop species. The association between plants and the *Glomeromycota* is thought to be the ancestral type of mycorrhizae, established about 450 million years ago and an important evolutionary step in the successful invasion of dry land by terrestrial plants.

AM fungi produce lipochitin oligosaccharide signaling factors (**Myc factors**) closely related to the Nod factors in the rhizobium–legume symbiosis (Section 23.4), and Myc factors initiate formation



(a) (b) (c) (d)

Figure 23.20 Ectomycorrhizal colonization of pine and beech tree roots. *(a)* Confocal image of an ectomycorrhizal fungus (stained green) aggregating on an emerging tree rootlet (stained red) and then "running" on the surface as it begins to further colonize the root. *(b)* Colonization of pine (*Pinus sylvestris*) roots with the ectomycorrhizal fungus *Suillus bovinus*. *(c)* Ectomycorrhizal root tips of pine roots enclosed by a mantle of ectomycorrhizal fungi (white) and associated hyphae extending into the soil matrix. The ectomycorrhizal fungus *Suillus bovinus* also forms rhizomorphs, hyphal aggregations that are involved in the long-distance transport from the soil to the mycorrhizal root. *(d)* Cross section of a thin beech root showing the fungal mantle (FM) and Hartig net (HN) within the root cortex (C). The Hartig net is the location of nutrient exchange between the plant and fungus. Also shown are root vascular tissue (ST) and endodermis (E).

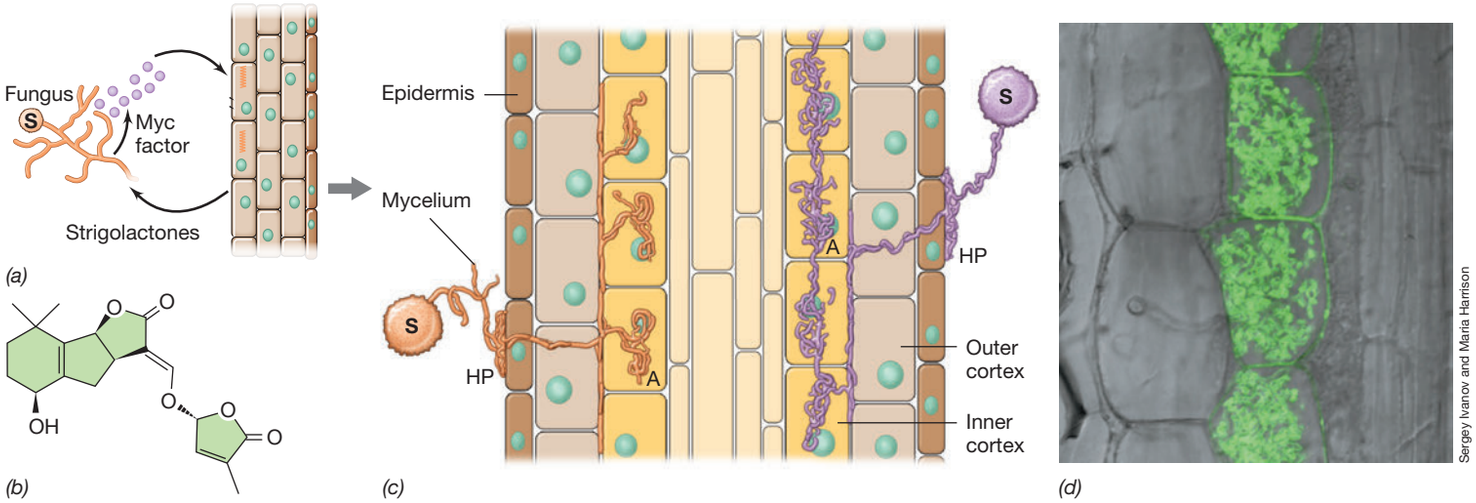


Figure 23.21 Arbuscular mycorrhizal root colonization. *(a)* A spore (S) recognizes the presence of a nearby root by sensing strigolactones released by the plant root. *(b)* The structure of strigol, one type of strigolactone. Strigolactones stimulate spore germination and mycelial branching. Myc factor (see Figure 23.13) produced by the growing fungal mycelium then initiates the infection process. *(c)* The mycelium forms an attachment structure called the hyphopodium (HP) and then enters the inner cortex region of the root by penetrating epidermal cells and cells of the outer cortex. Arbuscules (dichotomously branched invaginations, A) are formed by mycelia spreading either *inter*cellularly (left) or *intra*cellularly (right). *(d)* Image of arbuscules in the root cells of *Medicago truncatula* (barrel clover) colonized by the fungus *Rhizophagus irregularis* engineered to express the green fluorescent protein.

Mastering Microbiology
Art Activity: Figure 23.20*c* Arbuscular mycorrhizal root colonization

of the mycorrhizal state (Figures 23.13 and 23.14). Root colonization by an AM fungus begins with germination of a soilborne spore, producing a branched germination mycelium that recognizes the host plant through reciprocal chemical signaling. Spore germination and mycelial branching is induced by *strigolactones* (**Figure 23.21*a*, *b***), plant hormones released by the roots that also play a key role in plant development. When a plant is nutrient-limited, this hormone represses aboveground plant growth (suppressing formation of secondary shoots) and stimulates the growth of the root system, enhancing the production of lateral roots and root hairs. These developmental changes help the plant secure nutrients before using them later for aboveground growth.

The Myc factor produced by the AM fungal mycelium signals the plant to initiate the developmental process (Figure 23.14). The fungus then forms a contact structure called the *hyphopodium* with root epidermal cells (Figure 23.21*c*). Penetrating hyphae extend into the plant from each hyphopodium, usually forging an intracellular path through epidermal and outer cortical cell layers of the root before forming dichotomously branched or coiled hyphal structures called **arbuscules** within plant inner cortex cells, near vascular tissues (Figure 23.21*c*, *d*). However, the arbuscular hyphae remain separated from plant protoplasm by an extensive plant cytoplasmic membrane that forms a region called the *apoplast* (**Figure 23.22**), and this structure

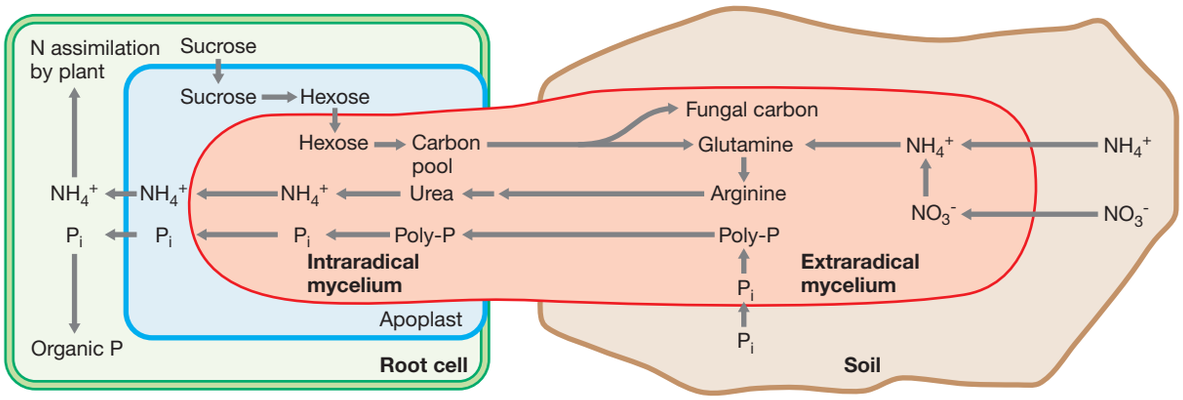


Figure 23.22 Pathways of N, P, and C exchange between plant and arbuscular mycorrhizal fungi. Inorganic nitrogen (NH_4^+ and NO_3^-) and phosphorus (P_i) mined from the soil by the extraradical (soil-associated) mycelia are translocated to the plant as arginine and polyphosphate (poly-P) through the mycelial network and delivered to the plant at the intraradical (plant cell–associated) mycelium. Ammonia and phosphate are regenerated in the intraradical mycelium for transfer to the plant cell. In exchange for the N and P, the plant provides organic carbon to the fungus.

functions to increase the surface area of contact between plant and fungus. Inorganic nitrogen and phosphorus are then "mined" from the soil by the fungi, converted to arginine and polyphosphate, and translocated through the hyphae to the plant (Figure 23.22).

Myc factors are very similar to the rhizobial Nod factors and only relatively minor modifications of the chitin backbone structure confer specificity (Figure 23.13). It is likely that the basic signaling and developmental systems used in the legume–root nodule symbiosis (Section 23.4), which arose about 60 million years ago, first evolved in the much more ancient AM fungi–plant symbiosis and were recruited and adapted for the legume–root nodule symbiosis (Figure 23.14).

Although the arbuscular mycorrhizae are a much more ancient and widely distributed microorganism–plant symbiosis, understanding of their signaling and developmental program has been slower to develop because AM fungi cannot be grown in pure culture. AM fungi are obligately *biotropic* (meaning that they obtain their nutrients only from living cells of their symbiotic partner), and unlike rhizobia, they have no supporting genetic system that can be exploited to unravel the developmental steps leading to the plant–fungus association.

Benefits for the Plant

The beneficial effect of the mycorrhizal fungus on the plant is best observed in poor soils where plants that are mycorrhizal thrive, but nonmycorrhizal ones do not. For example, if trees planted in prairie soils, which ordinarily lack a suitable fungal inoculum, are artificially inoculated at the time of planting, they grow much more rapidly than uninoculated trees (**Figure 23.23**). The mycorrhizal plant can absorb nutrients from its environment more efficiently (Figure 23.22) and thus has a competitive advantage. This improved nutrient absorption is due to the greater surface area provided by the fungal mycelium (see Figure 23.23 inset). In the pine seedling shown in Figure 23.20*b* and *c*, the ectomycorrhizal fungal mycelium makes up the overwhelming part of the absorptive capacity of the plant root system. The mycorrhizal plant is better able to function physiologically and compete successfully in a species-rich plant community, and the fungus benefits from a steady supply of organic nutrients.

In addition to helping plants absorb nutrients, mycorrhizae also play a significant role in supporting plant diversity. Field experiments clearly show a positive correlation between the abundance and diversity of mycorrhizae in a soil and the extent of the plant diversity that develops in it. However, although most mycorrhizae are true mutualistic symbioses, there are also parasitic mycorrhizae. In these less common mycorrhizal symbioses, either the plant parasitizes the fungus or, in some cases, the fungus parasitizes the plant.

There is much more to learn about fungal–plant symbioses, and such discoveries will be important not only in agriculture but also in facilitating the restoration of damaged ecosystems.

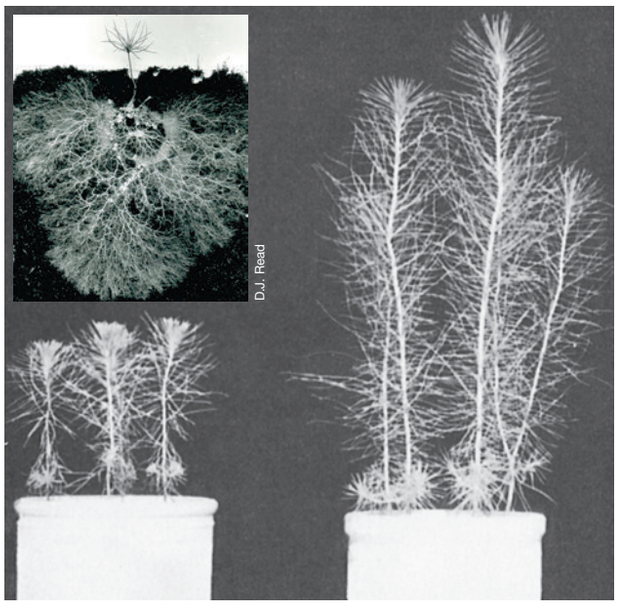


Figure 23.23 Effect of mycorrhizal fungi on plant growth. Six-month-old seedlings of Monterey pine (*Pinus radiata*) growing in pots containing prairie soil: left, nonmycorrhizal; right, mycorrhizal. Inset: A *Pinus* seedling showing the extensive fanlike development of its fungal partner. Also see Figure 23.20*b*, *c*.

Check Your Understanding

- How do endomycorrhizae differ from ectomycorrhizae?
- What features of mycorrhizal fungi might have assisted in colonization of dry land by plants?
- How do mycorrhizal fungi promote plant diversity?

23.6 *Agrobacterium* and Crown Gall Disease

Some microorganisms develop parasitic symbioses with plants. The genus *Agrobacterium*, a relative of the root nodule bacterium *Rhizobium* (Section 23.4), is such an organism, causing the formation of tumorlike growths on diverse plants. The two species of *Agrobacterium* most widely studied are *Agrobacterium tumefaciens* (also called *Rhizobium radiobacter*), which causes *crown gall disease*, and *Agrobacterium rhizogenes* (also called *Rhizobium rhizogenes*), which causes *hairy root disease*.

The Ti Plasmid

Although wounded plants often form a benign accumulation of tissue called a *callus*, the growth in crown gall disease (**Figure 23.24**) is different in that it is uncontrolled growth, resembling an animal tumor. *A. tumefaciens* cells induce tumor formation only if they contain a large plasmid called the **Ti plasmid** (Ti for *t*umor *i*nducing). In *A. rhizogenes*, a similar plasmid called the *Ri plasmid* is necessary for induction of hairy root disease. Following infection, a part of the Ti plasmid called the *transferred DNA* (T-DNA) is integrated into the plant's genome. T-DNA carries the genes for tumor formation and also for the synthesis of a number of modified amino acids called *opines*. *Octopine* [N^2-(1,3-dicarboxyethyl)-L-arginine] and *nopaline* [N^2-(1,3-dicarboxypropyl)-L-arginine] are two common opines. Opines are produced by plant cells transformed by T-DNA and are a source of carbon and nitrogen, and sometimes phosphate, for the parasitic *A. tumefaciens* cells. These nutrients are the benefits for the bacterial symbiont.

Figure 23.24 Crown gall. Photograph of a crown gall tumor (arrow) on a tobacco plant caused by *Agrobacterium tumefaciens*. The disease usually does not kill the plant but may weaken it and make it more susceptible to drought and diseases.

Recognition and T-DNA Transfer

To initiate the tumorous state, *A. tumefaciens* cells attach to a wound site on the plant. Following attachment, the synthesis of cellulose microfibrils by the bacteria helps anchor them to the wound site, and bacterial aggregates form on the plant cell surface. This sets the stage for plasmid transfer from bacterium to plant. The general structure of the Ti plasmid is shown in **Figure 23.25**. Only the T-DNA is actually transferred to the plant. The T-DNA contains genes that induce tumorigenesis. The *vir* genes on the Ti plasmid encode proteins that are essential for T-DNA transfer. Transcription of *vir* genes is induced by metabolites synthesized by wounded plant tissues such as the phenolic compounds acetosyringone and ferulate. The transmissibility genes on the Ti plasmid (Figure 23.25) allow the plasmid to be transferred by conjugation (◀ Sections 9.8 and 9.9) from one bacterial cell to another.

The *vir* genes are the key to T-DNA transfer. The *virA* gene encodes a protein kinase (VirA) that interacts with inducer molecules and then phosphorylates the product of the *virG* gene (**Figure 23.26**). VirG is activated by phosphorylation and functions to activate other *vir* genes. The product of the *virD* gene (VirD) has endonuclease activity and nicks DNA in the Ti plasmid in a region adjacent to the T-DNA. The product of the *virE* gene is a DNA-binding protein that binds the single strand of T-DNA in the plant cell to protect it from destruction by nucleases. The *virB* operon encodes 11 different proteins that form a type IV secretion system (◀ Section 6.13) for single-strand T-DNA and protein transfer between bacterium and plant (Figure 23.26) and thus resembles bacterial conjugation. Laboratory studies of *A. tumefaciens* have shown that it can transfer T-DNA into many types of eukaryotic cells, including fungi, algae, protists, and even human cell lines.

Mastering Microbiology
Art Activity: Figure 23.25 Mechanism of transfer of T-DNA to plant cells by *Agrobacterium tumefaciens*

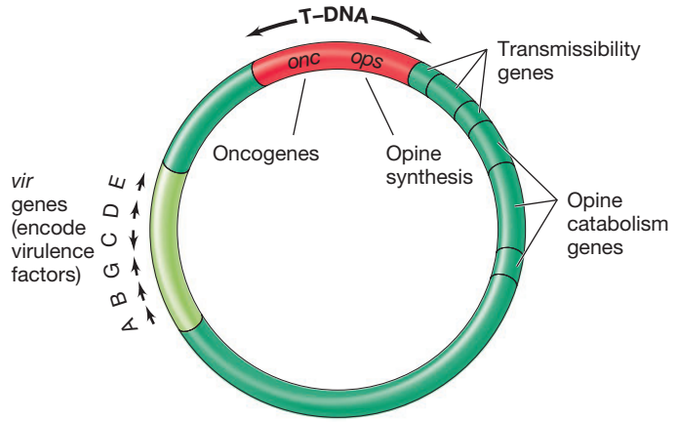


Figure 23.25 Structure of the Ti plasmid of *Agrobacterium tumefaciens*. T-DNA is the region transferred to the plant. Arrows indicate the direction of transcription of each gene. The entire Ti plasmid is about 200 kilobase pairs (kbp) of DNA and the T-DNA is about 20 kbp.

Once inside the plant cell, T-DNA becomes inserted into the genome of the plant. Tumorigenesis (*onc*) genes on the Ti plasmid (Figure 23.25) encode enzymes for plant hormone production and at least one key enzyme of opine biosynthesis. Expression of these genes leads to tumor formation and opine production. The Ri plasmid responsible for hairy root disease also contains *onc* genes. However, in this case the genes confer increased auxin responsiveness on the plant, and this promotes overproduction of root tissue and the symptoms of the disease. The Ri plasmid also encodes several opine biosynthetic enzymes.

Genetic Engineering with the Ti Plasmid

From the standpoint of microbiology and plant pathology, crown gall disease and hairy root disease both require intimate interactions that lead to genetic exchange from bacterium to plant. In other words, tumor induction in these diseases is the result of a natural plant-transformation system. Because of this, recent interest in the Ti–crown gall system has shifted away from the disease itself toward applications of this natural genetic exchange process in plant genetic engineering and biotechnology.

Several modified Ti plasmids that lack disease genes but that can still transfer DNA to plants have been developed by genetic engineering. These have been used for the construction of genetically modified (transgenic) plants. Many transgenic plants have been constructed thus far, including crop plants carrying genes for resistance to herbicides, insect attack, and drought. We discuss the use of the Ti plasmid as a vector in plant biotechnology in Section 12.7.

Now we transition away from plants and look at some remarkable symbioses between bacteria and insects.

Check Your Understanding

- What are opines, and whom do they benefit?
- How do the *vir* genes differ from T-DNA in the Ti plasmid?
- How has an understanding of crown gall disease benefited plant agriculture?

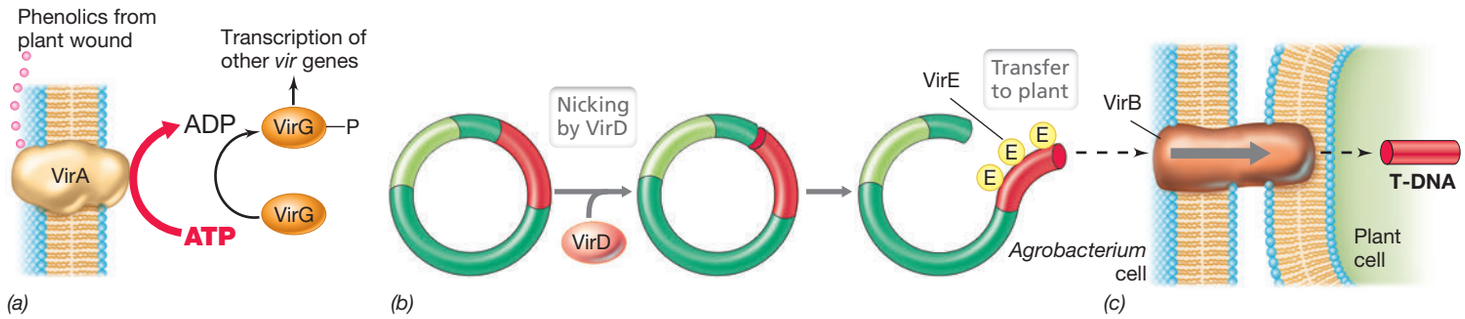


Figure 23.26 **Mechanism of transfer of T-DNA to the plant cell by *Agrobacterium tumefaciens*.** *(a)* VirA activates VirG by phosphorylation, and VirG activates transcription of other *vir* genes. *(b)* VirD is an endonuclease that nicks the Ti plasmid, exposing the T-DNA. *(c)* VirB functions as a conjugation bridge between the *A. tumefaciens* cell and the plant cell, and VirE is a single-strand binding protein that assists in T-DNA transfer. Plant DNA polymerase produces the complementary strand to the transferred single strand of T-DNA.

III • Insects as Microbial Habitats

Mutualisms between microorganisms and insects are common, contributing nutritional advantages or protection from pathogens and predators. Bacterial symbionts can reside within specialized insect cells, associate with specialized surface structures, or colonize the insect gut.

Insects are the most abundant class of animals living today, with over 1 million species known. As many as 20% of all insects are thought to support symbiotic microbes in a mutually beneficial way. The symbioses contribute to the insects' ecological success by providing them either nutritional advantages or protection from parasites. Some symbionts are found on insects' outer surfaces or in their digestive tracts. *Endosymbionts* are intracellular bacteria and are typically localized at specialized organs within the insect.

23.7 Heritable Symbionts of Insects

How symbionts are transferred from one generation to the next determines how a mutualism functions and how stable it is. Microbial symbionts can either be acquired by a host from an environmental reservoir (*horizontal* transmission) or be transferred directly from the parent to the next generation (*heritable* or *vertical* transmission). The mode of symbiont transmission is related to the specificity and persistence of an association. In general, less specificity is associated with horizontal transmission. In this section we focus only on mutualisms in which the microbial symbiont has no free-living form; that is, the symbionts are transmitted in a vertical fashion.

Types of Heritable Symbionts

All known heritable microbial symbionts of insects lack a free-living replicative stage. Thus, they are *obligate* symbionts. However, although these bacteria require the host for replication, not all hosts are dependent upon the symbiont. Relative to host dependence, heritable symbionts are either *primary* or *secondary symbionts*. Primary symbionts are required for host reproduction. They are restricted to a specialized region called the **bacteriome** present in several insect groups; within the bacteriome the bacterial cells reside in specialized cells called **bacteriocytes**. Secondary symbionts are not required for host reproduction. Unlike primary symbionts, secondary symbionts are not always present in every individual of a species and are not restricted to particular host tissues.

Secondary symbionts are broadly distributed among insect groups. Like pathogens, they invade different cell types and may live extracellularly within the insect's *hemolymph* (the fluid bathing the body cavity). In insects with bacteriomes, such as the cedar aphid (Figure 23.27*a*), secondary symbionts can invade the bacteriocytes, co-residing with or sometimes displacing the primary symbionts (Figure 23.27*b*). However, in order to persist in the insect host, the secondary symbiont must confer some advantage, such as a nutritional advantage or protection from environmental stresses such as heat. For example, whiteflies infected with the bacterium *Rickettsia* (◀ Section 16.1) produce offspring at about twice the rate of uninfected flies, and more offspring survive to adulthood. Secondary symbionts may also provide protection against invasion by pathogens or predators. A *Spiroplasma* species (◀ Section 16.9), which was first observed in *Drosophila neotestacea* in the 1980s, provides protection against a parasitic nematode worm. In most instances the basis for increased fitness or protection is unknown,

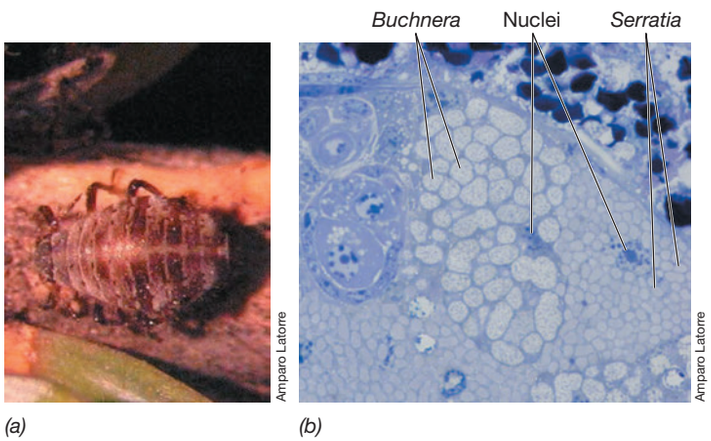


Figure 23.27 **Primary and secondary symbionts of an aphid.** *(a)* The cedar aphid *Cinara cedri*, a model organism for studies of symbioses. *(b)* Transmission electron micrograph of the bacteriome of *C. cedri* showing two bacteriocytes. Packed within each bacteriocyte are cells of *Buchnera aphidicola* (the primary symbiont) or *Serratia symbiotica*, the smaller, secondary symbiont. The nucleus of each bacteriocyte is identified. The bacteriocyte containing *Buchnera* cells is about 40 μm wide.

but in one case a toxin encoded by a lysogenic bacteriophage (◀ Section 5.6) carried by the symbiont is known to confer protection on the insect from infection by a parasitic wasp.

There are heritable parasitic symbionts that manipulate the host's reproductive system, increasing the frequency of female progeny (sex-ratio skewing, ◀ Figure 16.27). Because most heritable symbionts are transmitted maternally, the suppression of male progeny serves to expand the number of infected individuals and increase the rate of spread through an insect population. Since symbiont-conferred functions can spread rapidly within a population, acquisition of symbiont-encoded traits provides a mechanism for much more rapid adaptation than is possible through mutations in insect genes. *Rickettsia* infection of the whitefly population provides one example of how rapidly symbiont-conferred traits can spread through a population. Only 1% of whiteflies tested in the southwestern United States were infected with *Rickettsia* in 2000. In 2006, 97% of flies were infected.

An important applied benefit of improved basic understanding of insect symbionts is the increased use of symbionts in insect pest management and the control of vectorborne diseases, such as malaria and filariasis in humans (▶ Sections 34.5 and 34.7). For example, symbiotic *Wolbachia*, which are reproduction manipulators, are widely distributed among insect species (possibly infecting as many as 60–70% of all insect species). The sperm of *Wolbachia*-infected males can sterilize uninfected females. Although the mechanism for sterilization is not fully understood, the phenomenon is being tested as a means to suppress mosquito-borne human viral disease transmission (see Explore the Microbial World, "Combating Mosquito-Borne Viral Diseases with an Insect Symbiont").

Nutritional Significance of Obligate Intracellular Symbionts of Insects

The association of bacteria and insects has allowed many insects to use food resources that are rich in some nutrients, but poor in others. To achieve adequate nutrition, some insects exploit the metabolic potential of their symbionts. For instance, aphids feed on the carbohydrate-rich but otherwise nutrient-poor sap of phloem vessels in plants. Early on it was suspected that obligate symbionts might benefit the insect by providing nutrients not provided by their primary diet, and this is now known to be true.

Molecular analyses have shown that most families of aphids harbor the bacterium *Buchnera* in their bacteriomes. The role of *Buchnera* in host nutrition was first indicated by experiments using defined diets to examine the nutrient requirements of aphids. Compared with infected controls, symbiont-free aphids required a diet containing all amino acids that are either lacking or rare in phloem sap. Subsequent genomic studies documented the presence in *Buchnera* of genes encoding the biosynthesis of nine amino acids missing from the sap. There are also examples of synergy between host and symbiont where the synthesis of certain amino acids becomes a joint venture. For example, *Buchnera* lacks the enzyme needed for the last step in leucine biosynthesis, but the necessary gene is present in the aphid's genome. Presumably, this enzyme is made by the aphid and participates in the leucine biosynthetic pathway along with the bacterial enzymes.

A secondary symbiont can also contribute to a joint venture. For example, the *Buchnera* symbiont of the cedar aphid is unable to supply tryptophan to the aphid. Two genes in the tryptophan biosynthetic pathway are present in *Buchnera*, but the remaining genes for the pathway are located on the chromosome of a secondary endosymbiont (Figure 23.27). Thus, different parts of a required metabolic pathway can be encoded by different endosymbionts present in the same insect. The fungus-cultivating ants provide yet another example of a complex symbiosis that has formed between an insect and multiple microorganisms (as we review in the next section).

Mealybugs (*Planococcus citri*) present one of the most unusual examples of a partnership between two symbionts infecting the same insect. Mealybugs have two stable bacterial symbionts, "*Candidatus* Tremblaya princeps" (a *Betaproteobacterium*) and "*Candidatus* Moranella endobia" (a *Gammaproteobacterium*). (The term "*Candidatus*" means that these organisms are not yet in pure culture, ◀ Section 13.12, but we refer to them in the usual style below.) These symbionts cooperate in providing their host with essential amino acids missing in its diet, as is true for the symbionts of many sap-feeding insects. However, *Moranella* actually lives *inside of Tremblaya*, the only known example of a bacterium-within-a-bacterium symbiosis. The highly reduced *Tremblaya* genome has lost all genes for tRNA synthetases, an essential function supplied either by the host or by the *Moranella* residing within the cytoplasm of *Tremblaya*.

Genome Reduction and Gene Transfer Events

Common features of primary insect symbionts are extreme genome reduction (◀ Table 10.1), high adenine plus thymine content DNA, and accelerated rates of mutation. Genomes of most

TABLE 23.2 Genome features of some endosymbiotic *Bacteria* of animals[a]

Host	Symbiont (genus)	Genome size (Mbp)	G + C (%)	Genes
Aphid	Heterotroph (*Buchnera*)	0.42–0.62	20–26	362–574
Tsetse fly	Heterotroph (*Wigglesworthia*)	0.70	22	617
Carpenter ant	Heterotroph (*Blochmannia*)	0.71–0.79	27–30	583–610
Sharpshooter	Heterotroph (*Sulcia*)	0.25	22	227
Mealybug	Heterotroph ("*Candidatus* Moranella endobia," *Gammaproteobacteria*)	0.54	43.5	406
Mealybug	Heterotroph ("*Candidatus* Tremblaya princeps," *Betaproteobacteria*)	0.14	58.8	121
Clam (*Calyptogena okutanii*)	Sulfur oxidizer (unnamed)	1.0	32	975
Clam (*Calyptogena magnifica*)	Sulfur oxidizer (*Ruthia*)	1.2	34	1248
Tube worm (*Riftia pachyptila*)	Sulfur oxidizer (unnamed)	3.3[b]	NA	NA

[a] All listed symbionts are obligately associated with their hosts, with the exception of the symbiont of *Riftia*, which also has a free-living stage. For a comparison with the genomes of free-living *Bacteria*, see Table 10.1.
[b] The free-living sulfur-oxidizing bacterium *Thiomicrospira crunogena* has a genome significantly smaller (2.4 Mb) than this symbiont.

Combating Mosquito-Borne Viral Diseases with an Insect Symbiont

The global reemergence of mosquito-borne viral disease is correlated with rapid human population growth and urbanization, particularly in the tropics. Dengue (▶ Section 32.5) is now the world's most common mosquito-borne viral disease. In addition, major outbreaks of Zika virus (**Figure 1*a, b***) have occurred in the South Pacific and the Americas, with infection in the Americas associated with congenital abnormalities. Urbanization has created greater opportunities for mosquito reproduction and transmission, and subtropical cities are the preferred habitat of the primary disease vector, *Aedes aegypti* (Figure 1*c*), which lives and breeds in building structures, urban waste, and pools of standing water.

With the tremendous resurgence of mosquito-borne disease, conventional mosquito control methods—eliminating breeding sites or applying insecticides—are no longer effective. In response to what is becoming a global crisis, novel technologies based on the rearing and releasing of large numbers of modified mosquitos into wild populations have been developed and tested in the field. The basic concept is to introduce mosquitoes modified in such a way that their mating with the native population yields nonviable offspring, thus reducing the size of the vector population. Some attempts have been made using sterilized males or genetically modified males that carry lethal genes. However, the most successful and cost-effective approach to date, and one that does not require genetic engineering or repeated releases of altered males, is the use of *Wolbachia*, a bacterium that naturally infects 40–60% of all insects.

Wolbachia is an insect endosymbiont that uses a mechanism called *cytoplasmic incompatibility* to promote its spread through insect populations. Infected females can mate successfully with both infected and uninfected males, which enables the rapid spread of *Wolbachia* throughout a naive population. However, when infected males mate with uninfected females, the progeny are not viable, thus effectively sterilizing those females. Cytoplasmic incompatibility appears to be conferred by two incompatibility factors (*cifA* and *cifB*) encoded by a bacteriophage integrated into the *Wolbachia* genome (a prophage, ◀ Section 5.6). Infection with *Wolbachia* also inhibits virus replication in the insect. In fact, mosquito transmission of dengue, Zika, and chikungunya viruses (▶ Section 32.5) is greatly reduced when *A. aegypti* mosquitoes are infected with *Wolbachia*.

The potential for effective pathogen control by *Wolbachia* was shown by the release of a large number of *Wolbachia*-infected male *Culex quinquefasciatus* mosquitoes in locations in Myanmar. This insect is the vector of the filarial nematode that causes elephantiasis (▶ Section 34.7), and the release effectively eliminated the local mosquito population. Later release studies in Australia showed that the introduction of *Wolbachia*-infected mosquitoes into an uninfected population can achieve an infection rate of over 90% for years, thus requiring far fewer mosquitoes and releases than other control methods.

The success of these early studies was the genesis for the World Mosquito Program. This program is conducting large-scale release studies of *Wolbachia*-infected *A. aegypti* in Brazil, Colombia, and Indonesia that cover more than 2 million inhabitants in each study. These studies will yield the epidemiological evidence (disease tracking, Chapter 30) essential to fully document the efficacy of *Wolbachia* control of vectorborne diseases. However, as for all new technologies that affect natural systems, caution about unintended consequences is also essential. For example, whiteflies infected with the bacterium *Hamiltonella* (a symbiont related to enteric bacteria) are more likely than uninfected flies to transmit tomato yellow leaf curl virus to plants.

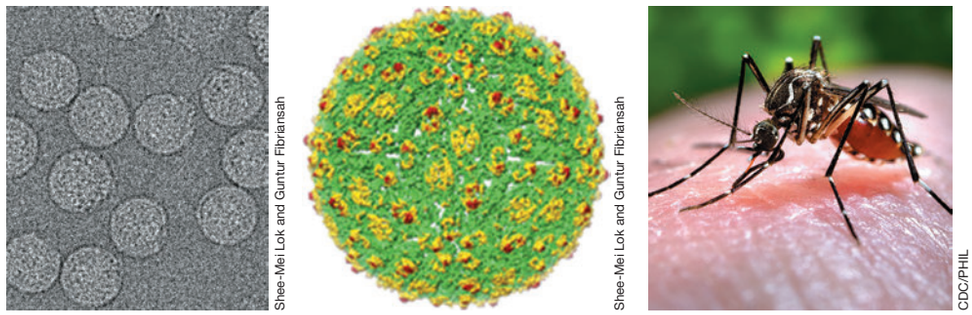


Figure 1 Zika virus and its mosquito vector. *(a)* Transmission electron microscopic image of Zika viruses. Each virion is approximately 40 nm in diameter. *(b)* Zika virus structural model. *(c)* The *Aedes aegypti* mosquito, the principal vector for transmission of Zika fever, yellow fever, and dengue fever.

insect symbionts fall within a range from 0.12 to 0.80 megabase pairs (Mbp) and 16.5 to 33% G + C (**Table 23.2**). The 0.14-Mbp (140 kilobase-pair) genome of *Tremblaya princeps* is among the smallest genomes known for any cell. In contrast, the genomes of related free-living bacteria range from 2 to 8 Mbp with a base composition closer to 50% G + C. Two common types of spontaneous mutation, cytosine deamination and the oxidation of guanosine, if not repaired, change a GC pair to an AT pair (◀ Section 9.4). Symbionts with reduced genomes have fewer DNA repair enzymes (◀ Section 9.4), and this likely facilitates a shift over time to genomes of lower G + C content.

The streamlined genomes of insect symbionts have lost genes from most functional categories (Chapter 10) and tend to retain only genes required for host fitness and essential molecular processes, such as translation, replication, and transcription. Genome reduction implies that the symbionts are reliant on the host for many functions no longer encoded in the symbiont genome (◀ Section 10.3). For example, in many cases genes needed for the biosynthesis of cell wall components are missing, including lipid A and peptidoglycan, suggesting that the host supplies these functions or that the structures are not required to form stable cells within the bacteriocyte.

There is an interesting genomic contrast between primary symbionts and typical disease-causing bacteria (pathogens). While primary symbionts tend to lose genes encoding proteins required in *catabolic* pathways, pathogenic bacteria typically retain these, but lose genes for *anabolic* pathways. This reflects their differing relationships with their hosts; the insect symbiont provides the host with essential biosynthetic nutrients while the pathogen obtains important biosynthetic nutrients from the host.

Because genome sequences for a large number of insects and their symbionts are now appearing, microbiologists can begin to evaluate the frequency of gene transfer between them. Horizontal gene transfer is the movement of genetic information across normal mating barriers (Chapter 9 and 13). Although early research demonstrated that DNA of *Wolbachia* has been transferred to the nuclear genomes of their insect and nematode hosts, inspection of other insect mutualisms for which both host and symbiont genome sequences are available (e.g., aphid and body louse) indicate that DNA transfer is very rare. Even though transfer of genes between insect symbiont and host genomes appears to be very rare, as we will see in the next section, there can be extensive movement of genetic information between symbionts that associate with a specific insect host.

Check Your Understanding

- What factors stabilize the presence of a secondary insect symbiont?
- What are the consequences of symbiont genome reduction?
- How could it be determined if a symbiont and its host have experienced a long period of coevolution?

23.8 Defensive Symbioses

In addition to symbioses that provide nutritional benefits, other symbioses between insects and microbes provide the insect with a defense weapon. A widespread defensive strategy used by insects to deter pathogens and predators is the production of toxic and antimicrobial chemicals. The chemical deterrent is sometimes produced by the insect. For example, a toxic mix of hydroquinone and hydrogen peroxide is prepared in a specialized abdominal chamber of the bombardier beetle before being squirted at an approaching predator. However, in many more instances, the defensive chemical is the product of microorganisms symbiotically associated with the insect.

The Rove Beetle

An example of this defensive strategy can be found in the *Paederus* beetle (commonly called the rove beetle), which deters predators using the chemical *pederin* (**Figure 23.28**) synthesized by an endosymbiotic *Pseudomonas* species. This cytotoxic chemical (inhibiting mitosis in eukaryotes) accumulates in the insect's hemolymph and is deposited in its eggs, effectively deterring arthropod predation on the eggs. The beetle is better known in the tropics for causing severe dermatitis and blistering of the human skin if the insect is inadvertently crushed when brushed away, releasing the pederin-containing hemolymph.

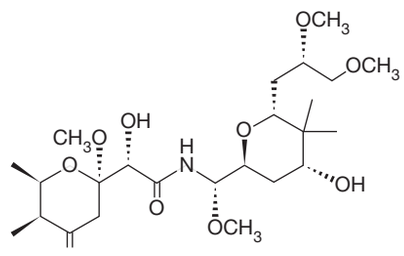
Many such defensive symbioses are now recognized, protecting the insect from predation, parasitism, and microbial pathogens. Defensive chemicals are made by a wide variety of bacterial symbionts, including members of the *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Some are intracellular, others reside within the gut, and others are associated with specialized anatomical structures on the insect exoskeleton. Comparative metagenomic analyses (◀ Sections 10.7 and 19.8) of symbiont gene content has shown that the array of defensive chemicals deployed by individual symbionts is primarily determined by horizontal gene transfer. Pathways for chemical synthesis commonly reside on plasmids and other mobile genetic elements (◀ Sections 6.2 and 9.11), such that even closely related symbionts can express very different defensive chemicals. Specific associations between insects and defensive symbionts are maintained by a variety of transmission mechanisms, including strictly vertical inheritance from the mother through the germ line and horizontal recruitment from the environment in each generation.

The Leafcutter Ants

The attine ants, more generally called leafcutter ants, are an example of an elaborate symbiotic association between multiple microbial species and insect. These ants have established an obligate mutualism with a fungus they cultivate, using small leaf fragments to feed the fungus. A close symbiotic relationship between ant and fungus was first indicated by the observation that one specific fungus was cultivated by each ant lineage. In addition to this close mutualistic relationship, the symbioses include four other microbial symbionts: a small fungus that is parasitic on the cultivated fungus, nitrogen-fixing bacteria (◀ Sections 3.12 and 15.9) associated with the cultivated fungus, an actinobacterium that antagonizes the parasitic fungus, and a black yeast that interferes with the actinobacterium.

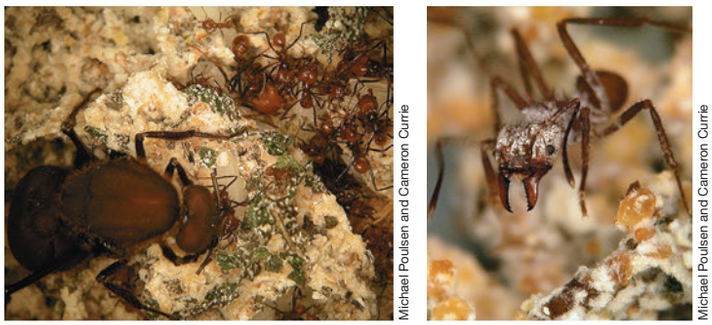
The fungus is vertically transmitted between ant generations by colony-founding queens. The queen collects a pellet of fungus and, after mating, uses the fungus pellet to establish a new nest containing the cultivated fungus (**Figure 23.29**). Nitrogen-fixing *Klebsiella* and *Pantoea* species associated with the fungus enrich the nutritional quality of the garden by adding new nitrogen to the nitrogen-poor leaf growth substrate. The cultivated fungus is susceptible to a parasitic fungus of the genus *Escovopsis*, and to repel this parasite, the ant has formed another symbiotic association with an actinobacterium (genus *Pseudonocardia*) that appears as a "waxy bloom" growing on the cuticle of the ant (Figure 23.29*b*). These bacterial cells, housed in specialized cuticular modifications on the ant's body, secrete highly selective antimicrobial agents that inhibit the growth of *Escovopsis* but not the cultivated fungus.

(a)



(b)

Figure 23.28 **Defensive chemical of insects.** *(a)* The rove beetle. *(b)* The insect's bacterial symbiont produces large amounts of pederin, a highly cytotoxic defensive chemical that accumulates in the hemolymph of the beetle.



(a) (b)

Figure 23.29 Leafcutter attine ants. *(a)* Queen and worker ants cultivating fungi. *(b)* Mutualism with the symbiont *Pseudonocardia* covers much of the exoskeleton of workers (white areas). The antifungal agent produced by the symbiont kills parasitic fungi but not the cultured fungi.

The *Pseudonocardia* cells likely receive nourishment from the ant in glandular secretions through pores localized in regions of cuticular modification. Comparative genomic sequencing has revealed good congruence between the phylogenies of the ants, fungal cultivars, *Escovopsis*, and *Pseudonocardia*, pointing to very specific interactions among microbes and ants in this complex symbiosis. The fourth and final microorganism identified in this symbiosis is a yeast that grows in the same cuticular regions colonized by the bacterium *Pseudonocardia*. This black-pigmented yeast interferes with chemical protection of the cultivated fungi by stealing nutrients from *Pseudonocardia*, thereby reducing its ability to suppress *Escovopsis* growth. The attine ant–microbial symbiosis is thus a complex maze of interactions between a macroorganism—the ant—and two groups of microbes, fungi and bacteria.

The Gut Microbiome of Bees

Bees and other animal pollinators provide vital ecosystem services. Thus, the loss of nearly 60% of honeybee colonies in the United States since 1947 is a major concern. Several factors are thought to contribute to this decline including fragmentation of habitats, pesticides, pollution, land use changes, and climate change. The decline of bee populations has also drawn attention to the importance of the bee gut microbiome for combating bee pathogens and environmental stressors.

The gut communities of bumblebees and honeybees (**Figure 23.30**) are surprisingly simple and consist of a core community of only five dominant and culturable bacterial species in adult worker bees. These include two gram-negative *Proteobacteria* (*Snodgrassella alvi* and *Gilliamella apicola*), two *Lactobacillus* species, and a *Bifidobacterium* species. *S. alvi* and *G. apicola* pack the lumen of the honeybee gut (Figure 23.30). Occasionally, up to four other species are present. Although different strains of each species in the core microbial community of the honeybee gut are closely related in 16S rRNA gene sequence, their genomes have diverged significantly through coevolution with bees over millions of years. As a result, strains have become host-specific. For example, *S. alvi* isolated from honeybees will not colonize bumblebees, and vice versa.

Metabolic interdependencies exist among microbes and the bee. For instance, *S. alvi* oxidizes the fermentation products of *G. apicola*, and *G. apicola* has genes for the degradation of pectin, a polysaccharide in

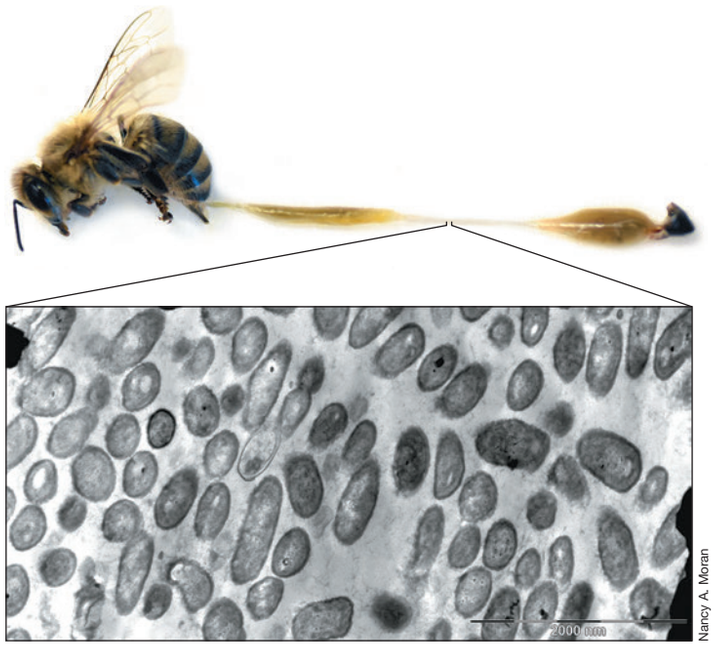


Figure 23.30 Scanning electron micrograph of honeybee gut microbiome. Five dominant bacterial species pack the lumen of the honeybee midgut as shown in the thin sectioned transmission electron micrograph.

the cell wall of pollen grains. Because bees do not produce pectinase and pollen is a key component of their diet, bee gut microbiota are essential for utilization of this important food source. Some bee gut microbes also carry genes for the utilization of uncommon sugars such as arabinose and raffinose that are indigestible and potentially toxic to the bee. This finely tuned microbiome plays a defensive role for the insect in that it inhibits invasive microorganisms through direct competition for space and resources and by stimulating the insect to produce antimicrobial peptides that target pathogenic bacteria.

Check Your Understanding

- How does the leafcutter ant prevent its cultivated fungus from being parasitized by another fungus?
- What is unusual about the diversity of the honeybee gut microbiome?
- How does the rove beetle use its *Pseudomonas* symbiont for protection?

23.9 Termites

Microorganisms are primarily responsible for the degradation of wood and cellulose in nature. However, the activities of free-living microbial species have been exploited by certain groups of insects that have established microbial symbioses in order to digest lignocellulosic materials. Like the rumen of many herbivorous animals (see Section 23.15), the insect gut provides a protective niche for microbial symbionts, and in return, the insect gains access to nutrients derived from an otherwise indigestible carbon source. Termites are among the most abundant representatives of this type of symbiotic alliance.

Termite Natural History and Biochemistry

Microbial symbionts in termites decompose the greater part of cellulose and hemicellulose in the plant material termites ingest. In contrast to the heritable insect symbionts discussed in Section 23.7, most termites do not harbor *intracellular* bacteria. Instead, the symbiotic bacteria are present in digestive organs (guts) as in the case of mammals. Termite diets include lignocellulosic plant materials (either intact or at various stages of decay), dung, and soil organic matter (humus). About two-thirds of the terrestrial environment supports one or more termite species, with the greatest representation in tropical and subtropical regions, where termites may constitute as much as 10% of all animal biomass and 95% of soil insect biomass.

Termites are categorized as higher or lower based on their phylogeny, and this classification correlates with different symbiotic strategies. The posterior alimentary tract of *higher* termites (family *Termitidae*, comprising about three-fourths of termite species) contains a dense and diverse community of mostly anaerobic bacteria, including cellulolytic species. In contrast, the *lower* termites harbor diverse populations of both anaerobic bacteria and cellulolytic protists. Bacteria of lower termites participate little or not at all in cellulose digestion; only the protists phagocytize and degrade the wood particles ingested by the termites.

The termite gut consists of a foregut (including the crop and muscular gizzard), a tubular midgut (site of secretion of digestive enzymes and absorption of soluble nutrients), and a relatively large hindgut of about 1 microliter volume (**Figure 23.31**). In lower termites, the hindgut consists primarily of a single chamber, the *paunch* (Figure 23.31*a*). The hindgut of higher termites is more complex, being divided into several compartments (Figure 23.31*b*). For both higher and lower termites, the hindgut harbors a dense and diverse microbial community and is a major site of nutrient absorption. Acetate and other organic acids are produced during microbial fermentation of carbohydrate in the hindgut, and these products are primary carbon and energy sources for the termite (Figure 23.31*c*). High O_2 consumption by bacteria near the gut wall keeps the interior of the hindgut anoxic. However, microsensor measurements (◀ Section 19.9) have shown that O_2 can penetrate up to 200 μm into the gut before it is completely removed by microbial respiration (Figure 23.31*c*). Thus, this tiny gut compartment offers distinct microbial niches with respect to O_2 and can support diverse microbial activities.

Bacterial Diversity and Lignocellulose Digestion in Higher Termites

The microbial gut communities differ significantly in termites of different genera. Analysis of 16S rRNA gene sequences from hindgut contents of two genera of wood-eating higher termites (*Nasutitermes* and *Microcerotermes*) and one genus of a wood-eating lower termite (*Reticulitermes*) revealed a high diversity of microbial species from 12 phyla of *Bacteria*, but few *Archaea* (**Figure 23.32**). Spirochetes of the genus *Treponema* (◀ Section 15.17) dominated, with a lesser contribution from thus far uncultured organisms distantly related to the phylum *Fibrobacteres* (◀ Section 16.22), a group also present in the rumen (Figure 23.47).

Metagenomic analysis (◀ Sections 10.7 and 19.8) of the *Nasutitermes* hindgut microbial community has revealed bacterial genes encoding glycosyl hydrolases that hydrolyze cellulose and hemicelluloses. These metagenomic data clearly implicate spirochetes and *Fibrobacteres* in the digestion of lignocellulose, although the corresponding cellulolytic bacteria have not yet been isolated from the higher termites. The abundance of *Bacteroidetes* varies greatly between different species of higher termites, depending on their dietary preferences. In particular, *Bacteroidetes* are enriched in higher termites that digest wood with the help of a lignin-degrading basidiomycete fungus (*Termitomyces* spp.). As do the leafcutter ants

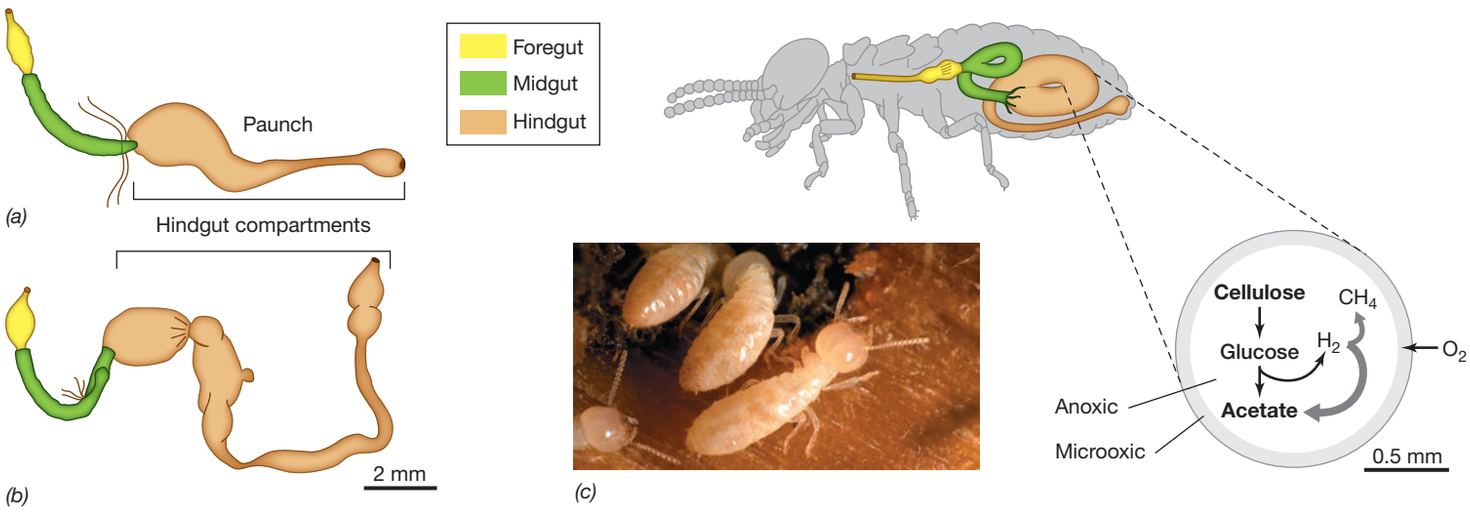


Figure 23.31 Termite gut anatomy and function. Gut architecture of lower *(a)* and higher *(b)* termites, showing the foregut, midgut, and differing complexity of the hindgut compartments. *(c)* Photo of workers, gut architecture, and biochemical activities of the lower termite *Coptotermes formosanus*. Microbial fermentation products, particularly acetate, are assimilated by the termite. Hydrogen produced by fermentation is consumed primarily by CO_2-reducing acetogens, with a smaller amount going to hydrogenotrophic methanogens. Acetogenesis and methanogenesis are discussed in Sections 14.14 and 14.15 respectively. Syntrophic associations between protists and methanogens are discussed in Section 21.2 (◀ Figure 21.8).

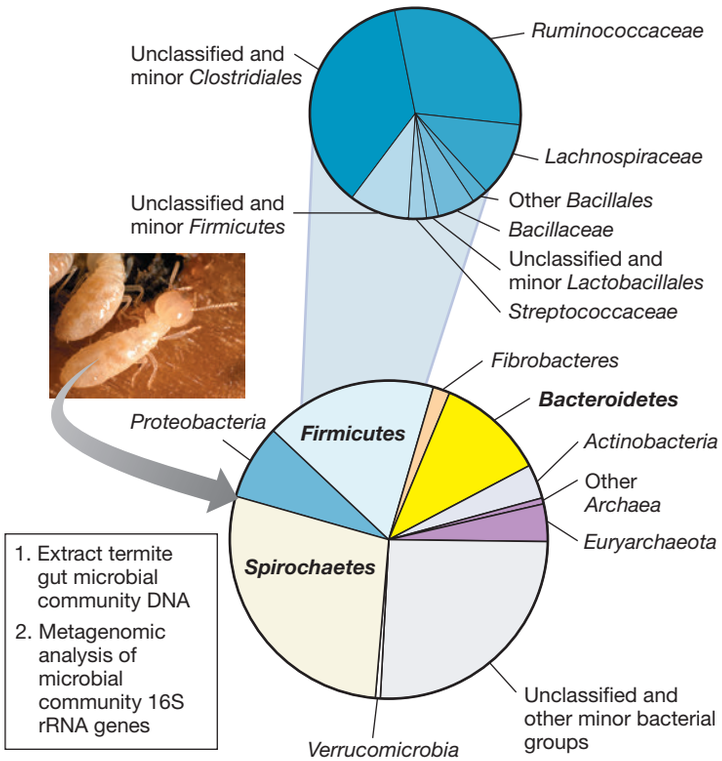


Figure 23.32 Microbial composition of termite hindgut inferred from 16S rRNA sequences. The results are pooled analyses of 5075 sequences from amplified or metagenomic sequencing studies of three genera of wood-feeding higher termites, *Nasutitermes, Reticulitermes*, and *Microcerotermes*. The data provide a picture primarily of diversity, not relative abundance. Data assembled and analyzed by Nicolas Pinel.

(see Section 23.8), these termites cultivate fungi that they feed lignocellulosic plant litter. The termites feed on both the fungus and the partially digested wood. The *Bacteroidetes* in these termites encode many glycosyl hydrolases that assist in the breakdown of plant and fungal cell wall material. In addition to the cellulolytic activities of *Fibrobacteres* and *Spirochaetes* in termites specializing in direct consumption of wood, other cellulolytic species include *Ruminococcaceae*, the latter comprising a significant fraction of *Firmicutes* diversity in the termite gut (Figure 23.32). At every molting of an individual termite, gut symbionts are lost, yet there is good conservation of the gut community within each termite species. Stable horizontal transmission of gut symbionts likely occurs as a result of the intimate social behavior and close contact characteristic of termites.

Acetogenesis and Nitrogen Fixation in the Termite Gut

Genes encoding enzymes of the acetyl-CoA pathway (◀ Section 14.14) are highly represented in the spirochetes of the *Nasutitermes* hindgut, consistent with their function as the major CO_2-reducing acetogens. The termite gut microbial communities have long been recognized as important to host nitrogen metabolism, providing new fixed nitrogen (◀ Sections 3.12 and 15.9) and helping to conserve nitrogen by recycling excretory nitrogen back to the insect for biosynthesis. Consistent with this, metagenomic analyses have revealed that many bacteria, including *Fibrobacteres* and certain spirochetes, contain genes encoding nitrogenase, the enzyme required to fix N_2.

From a simple energetic viewpoint, methanogenesis from H_2 and CO_2 is more favorable than acetogenesis from the same substrates (-34 kJ/mol of H_2 versus -26 kJ/mol of H_2, respectively), and thus methanogens should have a competitive advantage in all habitats in which the two processes compete (◀ Sections 14.14–14.15). However, in termites they do not. There are at least two reasons for this. First, unlike methanogens, acetogens are able to use other substrates such as sugars or methyl groups from lignocellulose degradation as electron donors for energy metabolism. Second, termite acetogens (which seem to consist mostly of spirochetes) can for some reason better colonize the H_2-rich termite gut center, whereas methanogens are largely restricted to the gut wall. On the gut wall, methanogens are located downstream of the H_2 gradient and thus receive only a fraction of the H_2 flux. In addition, the wall likely contains higher O_2 tensions, which may negatively affect the physiology of methanogens. So, despite the fact that termites are methanogenic, producing up to 150 teragrams of CH_4 per year on a global basis (1 teragram $= 10^{12}$ grams), carbon and electron flow in the termite gut favor acetogenesis in this interesting anoxic microbial habitat.

Check Your Understanding

- How are anoxic conditions maintained in the termite hindgut?
- Why does reductive acetogenesis predominate over methanogenesis in many termites?
- Which group of morphologically unusual bacteria, absent from molecular surveys of prokaryotic cells in the rumen, seem to dominate activities in the termite hindgut?

IV • Other Invertebrates as Microbial Habitats

Microbial symbionts of invertebrates perform different behavioral and nutritional functions. Some symbionts provide a light source to their host while others absorb light or consume inorganic chemicals to feed the animal. In some cases the invertebrate uses the symbiont as an offensive weapon, producing toxic compounds to kill its prey.

Thus far in this chapter we have discussed how certain macroorganisms that live in terrestrial environments provide habitats for microbial symbionts. We now consider some additional symbioses occurring in both terrestrial and aquatic environments. We begin in the marine environment as microbial symbioses with marine animals, especially with invertebrates, are common. By finding habitats in marine invertebrates, microorganisms establish a safe residence in a nutritionally rich environment.

23.10 Bioluminescent Symbionts and the Squid Symbiosis

Some bacteria can emit light, and this capacity has been exploited by several marine invertebrates. The light organ of the Hawaiian bobtail squid has become an important model system for such

symbioses, especially for resolving the complex communication between microorganism and animal required for establishing a specific and stable symbiosis.

Key Bioluminescent Genera and Biochemistry of Bioluminescence

Several species of bacteria among the genera *Vibrio, Aliivibrio, Photobacterium*, *Shewanella*, and *Photorhabdus* (all *Gammaproteobacteria*, ◀ Section 16.4) can emit light, a process called **bioluminescence** (**Figure 23.33**). Most bioluminescent bacteria inhabit the marine environment, and some species colonize specialized *light organs* of certain marine fishes and squids, producing light that the animal uses for signaling, avoiding predators, and attracting prey (Figure 23.33*c–f*). When living symbiotically in light organs of fish and squids, or saprophytically, for example on the skin of a dead fish, or parasitically in the body of a crustacean, luminous bacteria can be recognized by the light they produce.

Mechanism and Ecology of Bioluminescence

Although *Photobacterium*, *Aliivibrio*, and *Vibrio* isolates are facultative aerobes, they are bioluminescent only when O_2 is present. Luminescence in bacteria requires the genes *luxCDABE* (◀ Section 7.7) and is catalyzed by the enzyme *luciferase*, which uses O_2, a long-chain aliphatic aldehyde (RCHO) such as tetradecanal, and reduced flavin mononucleotide ($FMNH_2$) as substrates:

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + \text{light}$$

The light-generating system constitutes a metabolic route for shunting electrons from $FMNH_2$ to O_2 directly, without employing other electron carriers such as quinones and cytochromes.

Luminescence in many luminous bacteria only occurs at high population density. The enzyme luciferase and other proteins of the bacterial luminescence system exhibit a population density–responsive induction, called **autoinduction**, in which transcription of the *luxCDABE* genes is controlled by a regulatory protein, LuxR, and an inducer molecule, acyl homoserine lactone (AHL, ◀ Section 7.7 and Figure 7.18). During growth, cells produce AHL, which can rapidly cross the cytoplasmic membrane in either direction, diffusing in and out of cells. Under conditions in which a high local population density of cells of a given species is attained, as in a test tube, a colony on a plate, or in the light organ of a fish or squid, AHL accumulates. Only when it reaches a certain concentration in the cell is AHL bound by LuxR, forming a complex that activates transcription of *luxCDABE*; cells then become luminous (Figure 23.33*b*, ◀ Figure 1.3).

This gene regulatory mechanism is called *quorum sensing* because of the population density–dependent nature of the phenomenon (◀ Section 7.7). The strategy of quorum-sensing induction of luminescence ensures that luminescence develops only when population densities are high enough to allow the light produced to be visible to animals. The bacterial light can then attract animals to feed on the luminous material, thereby bringing the bacteria into the animal's nutrient-rich gut for further growth. Alternatively, the luminous material may function as a light source in symbiotic light organ associations (Figure 23.33*c*, *d*). Quorum sensing is a form of

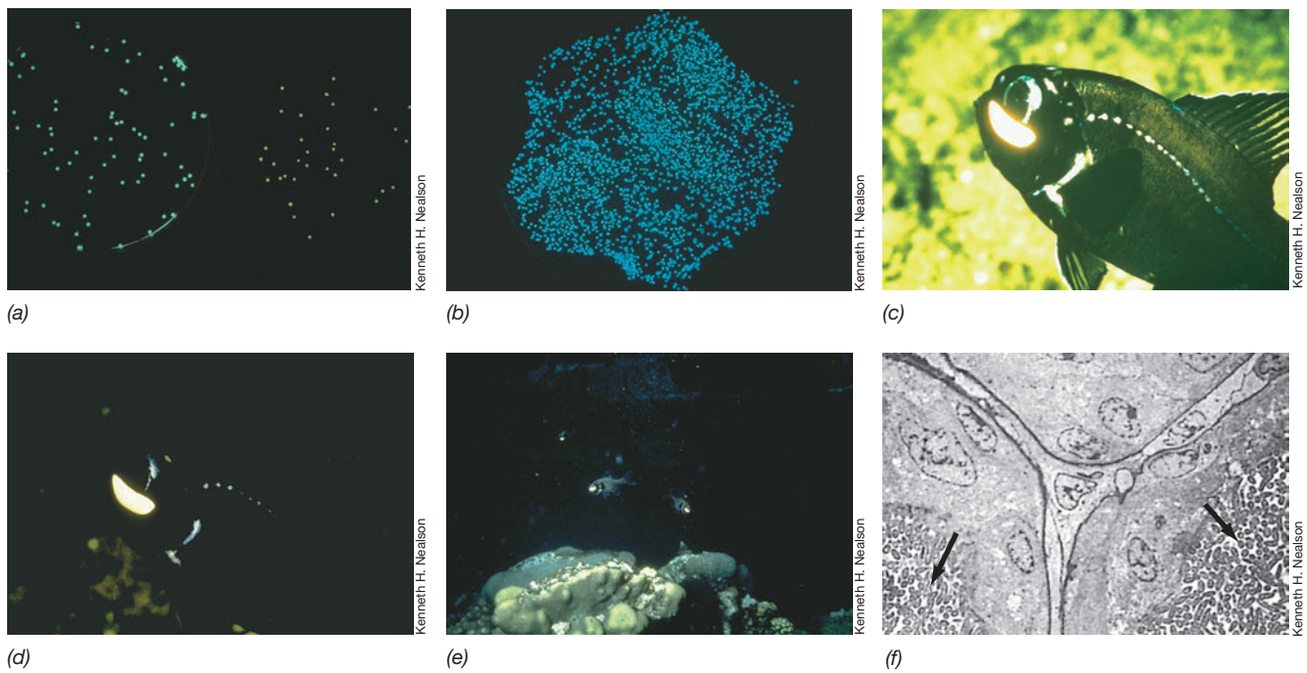


Figure 23.33 **Bioluminescent bacteria and their role as light organ symbionts in the flashlight fish.** *(a)* Two Petri plates of luminous bacteria photographed by their own light. Note the different colors. Left, *Aliivibrio fischeri* strain MJ-1, blue light, and right, strain Y-1, green light. *(b)* Colonies of *Photobacterium phosphoreum* photographed by their own light (◀ Figure 1.3). *(c)* The flashlight fish *Photoblepharon palpebratus*; the bright area is the light organ containing bioluminescent bacteria. *(d)* Same fish photographed by its own light. *(e)* Underwater photograph taken at night of *P. palpebratus*. *(f)* Electron micrograph of a thin section through the light-emitting organ of *P. palpebratus* showing the dense array of bioluminescent bacteria (arrows).

regulation that has been detected in many different bacteria, controlling activities such as the production of extracellular enzymes and expression of virulence factors for which a high population density is beneficial if the bacteria are to have a biological effect.

The Squid–*Aliivibrio* System as a Model Symbiosis

The Hawaiian bobtail squid, *Euprymna scolopes*, is a small marine invertebrate (**Figure 23.34*a***) that harbors a large population of the bioluminescent bacterium *Aliivibrio fischeri* (Figure 23.33*a*) in a light organ located on its ventral side (Figure 23.34*b* and see Figure 23.35). Squid and bacterium are partners in a mutualism. The bacteria emit light that resembles moonlight penetrating marine waters, and this is thought to camouflage the squid from predators that strike from beneath. Several other species of *Euprymna* inhabit marine waters near Japan and Australia and in the Mediterranean, and these contain *Aliivibrio* symbionts as well.

Many features of the *E. scolopes*–*A. fischeri* symbiosis have made it an important model for studies of animal–bacterial symbioses. These include the facts that the animals can be maintained in the laboratory and that only a single bacterial species is in the symbiosis in contrast to the large number of species in symbioses such as those of the termite (Figure 23.32) or the mammalian large intestine (Chapter 24). In addition, the symbiosis is not an essential one; both the squid and its bacterial partner can survive apart from each other in the laboratory. This allows juvenile squid to be grown without bacterial symbionts and then experimentally colonized. Experiments can be done to study specificity in the symbiosis, the number of bacterial cells needed to initiate an infection, the capacity of genetically defined mutants of *A. fischeri* to initiate infection of the squid, and many other aspects of the relationship. Moreover, because the genome of *A. fischeri* has been sequenced, microbial genomics may be employed to help interpret experimental results.

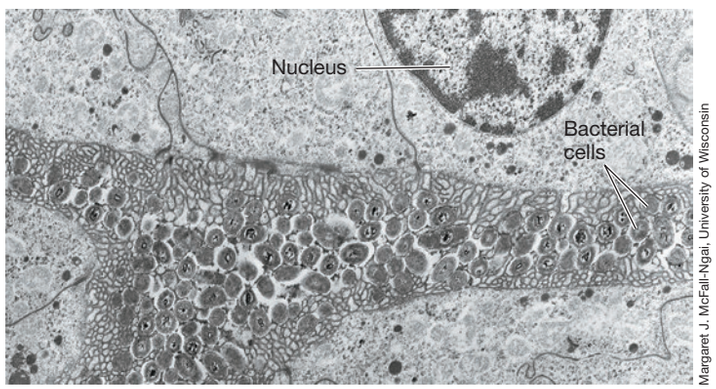
Establishing the Squid–*Aliivibrio* Symbiosis

Juvenile squid just hatched from eggs do not contain cells of *A. fischeri*. Thus, transmission of bacterial cells to juvenile squid is a horizontal (environmental) rather than a vertical (parent to offspring) event. Almost immediately after juveniles emerge from eggs, cells of *A. fischeri* in surrounding seawater begin to colonize them, entering through ciliated ducts that end in the immature light organ. Amazingly, the light organ becomes colonized *specifically* with *A. fischeri* and not with any of the many other species of gram-negative bacteria present in the seawater. Even if large numbers of other species of bioluminescent bacteria are offered to juvenile squid along with low numbers of *A. fischeri*, only *A. fischeri* establishes residence in the light organ. This implies that the animal recognizes and accepts *A. fischeri* cells and excludes those of other species.

Initial colonization of the squid light organ by *Aliivibrio* has three basic stages: (1) first contact, exclusion of unwanted microbes, and symbiont enrichment and aggregation, (2) chemotactic migration of the symbionts to the light organ colonization pore, and (3) migration of the symbionts into the crypts of the light organ interior, followed by loss of flagella and cell division (**Figure 23.35**). Contact of the squid with any bacterial cells triggers recognition in a very general way. Upon contact with peptidoglycan (a component of the cell wall of *Bacteria*, ◀ Section 2.3), the ciliated surface of the young squid's immature light organ secretes an acidic mucus, trapping the bacteria on its developing light organ. The mucus is the first layer of specificity in the symbiosis, as it makes gram-negative but not gram-positive bacteria aggregate (Figure 23.35*a*).

Within the aggregates, *A. fischeri* outcompetes the other gram-negative bacteria to form a monoculture within 2 h of a juvenile's hatching from an egg. Specific colonization is aided by the chemical environment generated by squid, including production of antimicrobial peptides and the gas nitric oxide (NO, see below), which helps the aggregating *A. fischeri* compete against attempted colonization by other gram-negative bacteria and facilitates their migration into the light organ (Figure 23.35*b*). The aggregated cells respond chemotactically to a gradient of specific sugars released from colonization pores (Figure 23.35*c*), migrating up this chemical gradient and into the pores. From there the cells continue their migration into the crypts of the light organ (Figure 23.35*d*). At this point, another critical selection process takes place. Only a single *Aliivibrio* cell enters and grows in each crypt of the light organ; when inside, it sheds its flagella and divides to form a dense population (Figures 23.35*d* and 23.34*b*). These initial colonization events trigger developmental events in the squid that lead to maturation of the light organ (Figure 23.35*e*). Each light organ in a mature *E. scolopes* contains between 10^8 and 10^9 *A. fischeri* cells.

(a)



(b)

Figure 23.34 Squid–*Aliivibrio* symbiosis. *(a)* An adult Hawaiian bobtail squid, *Euprymna scolopes*, is about 4 cm long. *(b)* Thin-sectioned transmission electron micrograph through the *E. scolopes* light organ shows a dense population of bioluminescent *Aliivibrio fischeri* cells.

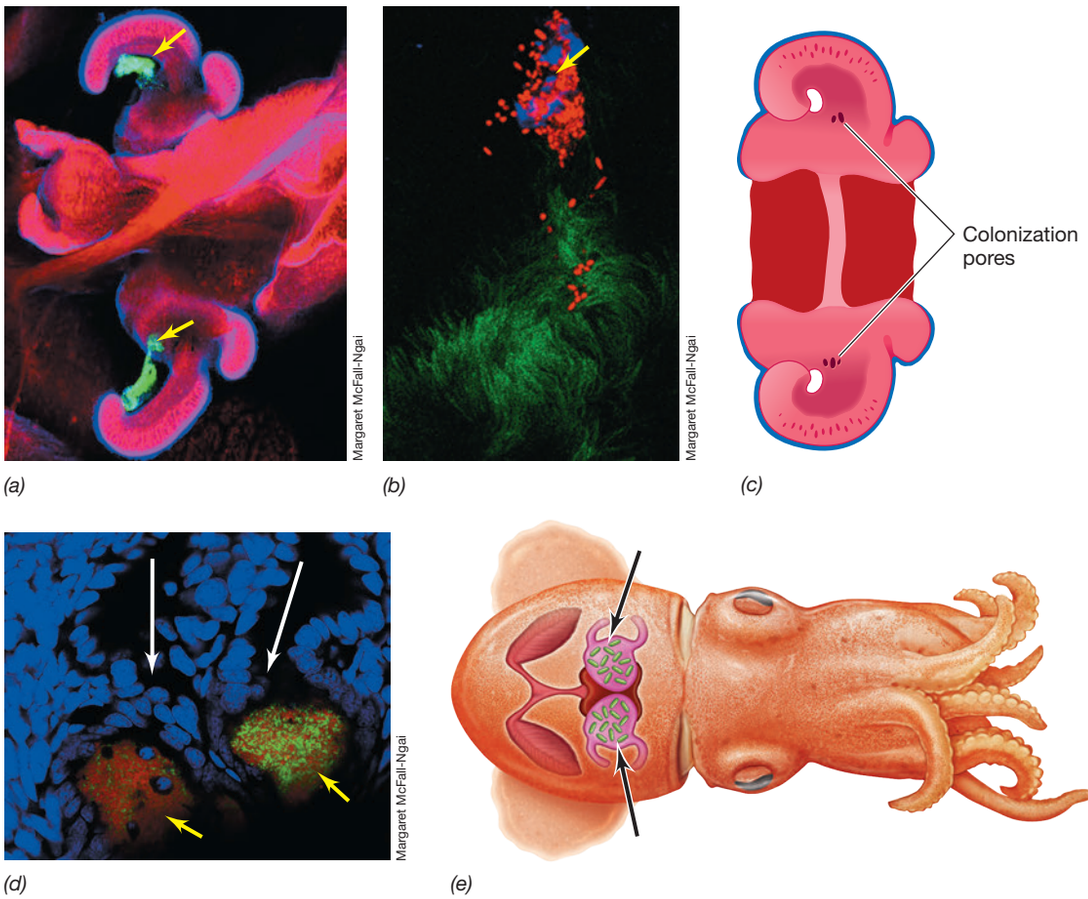


Figure 23.35 **Squid light organ symbiont colonization.** *(a) Aliivibrio fischeri* (green fluorescence) aggregates on the mucus-covered (blue) immature light organ surface of *Euprymna scolopes* (aggregates are approximately 100 μm long). *(b, c)* Chemotactic migration of *A. fischeri* cells (red fluorescence) along the ciliated surface (green) of the light organ, following a gradient of sugars released from the colonization pores. *(d) A. fischeri* cells migrate from the pore through the light organ along the direction of the white arrows to colonize the crypts (yellow arrows). Host nuclei are stained blue and *A. fischeri* cells fluoresce red or green (each cell aggregate is approximately 40 μm wide). *(e)* Following colonization of the light organs, the symbionts trigger the loss of the ciliated lobe-like appendages that are no longer needed for collection of *A. fischeri* cells from seawater. *A. fischeri* cells were genetically modified to express the green fluorescent protein or red fluorescent protein (◀ Section 19.4).

Colonization by *A. fischeri* in the squid is assisted by nitric oxide (NO). Nitric oxide is a well-known defense response of animal cells to attack by bacterial pathogens; the gas is a strong oxidant and causes sufficient oxidative damage to kill bacterial cells (▶ Section 26.7). Nitric oxide produced by the squid is incorporated into the aggregates in the mucus and is present in the light organ itself. As *A. fischeri* colonizes the light organ, NO levels diminish rapidly. It appears that cells of *A. fischeri* can tolerate exposure to NO and actually consume it through the activity of NO-inactivating enzymes. The inability of other gram-negative bacteria in the aggregates to detoxify NO helps explain the sudden enrichment of *A. fischeri* in the ducts even before the actual colonization of the light organ. Then, after establishment, continued production of NO in the light organ prevents colonization by other bacterial species.

Propagating the Symbiosis

The squid matures into an adult in about 2 months and then lives a strictly nocturnal existence in which it feeds mostly on small crustaceans. During the day, the animal buries itself and remains quiescent in the sand. Each morning at dawn the squid nearly empties its light organ of *A. fischeri* cells and begins to grow a new population of the bacterium; by midafternoon, the structure contains the dense populations of *A. fischeri* cells required for the production of visible light. The actual emission of light requires a certain density of cells and is controlled by quorum sensing (◀ Section 7.7). This daily growth and expulsion of bacterial cells is thought to be a mechanism for seeding the environment with cells of the bacterial symbionts; this increases the chances that the next generation of juvenile squid will be colonized and the symbiosis maintained.

A. fischeri clearly benefits from the squid symbiosis. The bacterium grows much faster in the light organ than in the open ocean, presumably because it is supplied with nutrients by the squid and is in a protected environment where it can avoid predation. *A. fischeri* is not a particularly abundant marine bacterium. Thus, the bacterium benefits by having an alternative habitat to seawater in which rapid growth and dense populations are possible. Daily expulsion of *A. fischeri* cells from the light organ increases the bacterium's numbers in the microbial community. Hence, the symbiotic relationship of the bacterium with the squid helps maintain larger populations of *A. fischeri* than if the organism were strictly free-living. Because the competitive success of a microbial species is to some degree a function of population size (◀ Section 20.1), this boost in cell numbers may confer an important ecological advantage on *A. fischeri* in its marine habitat.

Check Your Understanding

- What substrates and enzyme are required for an organism such as *Aliivibrio* to emit visible light?
- What is quorum sensing, and how does it control bioluminescence?
- What features of the squid–*Aliivibrio* symbiosis make it an ideal model for studying animal–bacterial symbioses?

23.11 Marine Invertebrates at Hydrothermal Vents and Cold Seeps

Diverse invertebrate communities develop near undersea hot springs called *hydrothermal vents*. We covered the geochemistry and microbiology of hydrothermal vents and cold seeps of natural gas in Sections 20.16 and 21.1. Here we focus on hydrothermal vent invertebrates and their microbial symbionts.

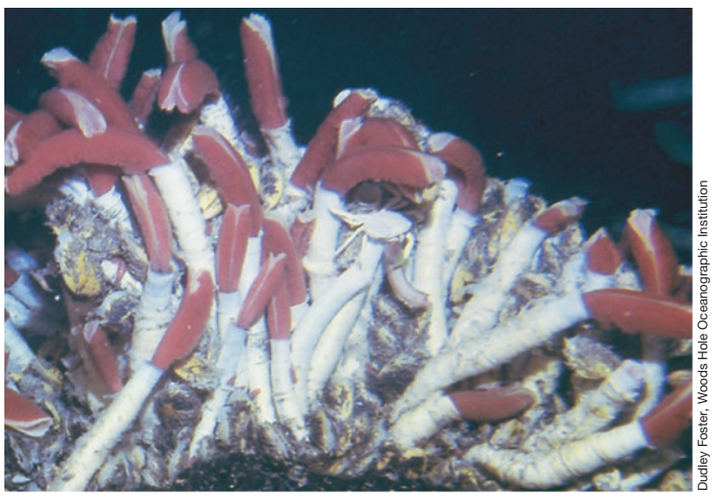
Macroinvertebrates, including tube worms over 2 m in length and large clams and mussels, are present near these vents (**Figure 23.36**). Photosynthesis cannot support these invertebrate communities because they exist below the photic zone. However, hydrothermal fluids contain large amounts of reduced inorganic materials, including H_2S, Mn^{2+}, H_2, and CO (carbon monoxide), and some vents contain high levels of ammonium (NH_4^+) instead of H_2S; all of these are good electron donors for chemolithotrophs—*Bacteria* and *Archaea* that use inorganic compounds as electron donors and fix CO_2 as their carbon source (Chapter 14). Thus, hydrothermal vent invertebrates thrive in permanent darkness because they receive nourishment through a symbiotic association with these autotrophic bacteria.

Tube Worms, Mussels, and Giant Clams

Hydrothermal vent–associated animals either feed directly on cells of free-living chemolithotrophs or have formed tight symbiotic associations with them. Mutualistic chemolithotrophs are either tightly attached to the animal surface (that is, as *epibionts*, Section 23.2 and Figures 23.5 and 23.29*b*) or actually live within the animal tissues, supplying organic compounds to the animals in exchange for a safe residence and ready access to the electron donors needed for their energy metabolism. For example, the 2-m-long tube worms (Figure 23.36*a*) lack a mouth, gut, and anus, but contain an organ consisting primarily of spongy tissue called the *trophosome*. This structure, which constitutes half the worm's weight, is filled with sulfur granules and large populations of spherical sulfur-oxidizing bacteria (**Figure 23.37**). Bacterial cells taken from trophosome tissue show activity of enzymes of the Calvin cycle, a major pathway for autotrophy (◀ Section 3.12), but interestingly, they also contain enzymes of the reverse citric acid cycle, a second autotrophic pathway (◀ Section 14.2). In addition, they contain the sulfur-oxidizing enzymes necessary to obtain energy from reduced sulfur compounds (◀ Sections 14.7 and 15.12). The tube worms are thus nourished by organic compounds produced from CO_2 and secreted by the sulfur chemolithotrophs.

Along with tube worms, giant clams and mussels (Figure 23.36*b*) are also common near hydrothermal vents, and sulfur-oxidizing bacterial symbionts have been found in the gill tissues of these animals. Phylogenetic analyses have shown that each individual animal harbors one or more different strains of bacterial symbiont and that a variety of species of bacterial symbionts inhabit different species of vent animal. With the exception of the bacterial symbiont of *Riftia* (tube worms), which also has a free-living stage (Table 23.2), none of the bacterial symbionts of hydrothermal vent animals have yet been obtained in laboratory culture, even though they are fairly closely related to free-living sulfur chemolithotrophs (◀ Sections 14.7 and 15.12).

The red plume of the tube worm (Figure 23.36*a*) is rich in blood vessels and is used to trap and transport inorganic substrates to the bacterial symbionts. Tube worms contain unusual hemoglobins that bind H_2S and O_2; these are then transported to the trophosome where they are released to the bacterial symbionts. The CO_2 content of tube-worm blood is also high, about 25 mM, and presumably this is released in the trophosome as a carbon source for the symbionts. In addition, stable isotope analyses (◀ Section 19.10) of elemental sulfur from the trophosome have shown that its $^{34}S/^{32}S$ composition is the same as that of the sulfide emitted from the vent. This ratio is distinct from that of seawater sulfate and is solid evidence that geothermal sulfide is actually entering the worm in large amounts.



Dudley Foster, Woods Hole Oceanographic Institution

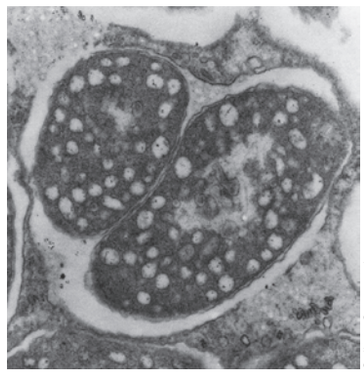
(a)

Carl Wirsen, Woods Hole Oceanographic Institution

(b)

Figure 23.36 Invertebrates living near deep-sea thermal vents. *(a) Riftia* (tube worms, phylum *Annelida*), showing the sheath (white) and plume (red) of the worm bodies. *(b)* Mussel bed in vicinity of a warm vent. Note yellow deposition of elemental sulfur from the oxidation of H_2S emitted from the vents.

(a)



(b)

Figure 23.37 Chemolithotrophic sulfur-oxidizing bacteria associated with the trophosome tissue of tube worms from hydrothermal vents. *(a)* Scanning electron micrograph of trophosome tissue showing spherical chemolithotrophic sulfur-oxidizing bacteria. Cells are 3–5 μm in diameter. *(b)* Transmission electron micrograph of bacteria in sectioned trophosome tissue. The cells are frequently enclosed in pairs by an outer membrane of unknown origin. Reprinted with permission from *Science 213*: 340–342 (1981), © AAAS.

Other Invertebrate Symbionts

Other marine invertebrates have coevolved bacterial symbioses that supply their nutrition as well (Table 23.3). For example, methanotrophic (CH_4-consuming) symbionts are present in giant clams that live near cold seeps of natural gas at relatively shallow depths in the Gulf of Mexico. Although not autotrophs (CH_4 is an organic compound), the methanotrophs do provide nutrition to the clams; the methanotrophs use CH_4 as their electron donor and carbon source and secrete organic carbon to the clams.

Molecular hydrogen (H_2) is used as an electron donor by the mussel *Bathymodiolus puteoserpentis*, the most abundant macrofauna in vent fields associated with the peridotite-hosted vent systems of the Mid-Atlantic Ridge (◀ Section 20.16 and Figure 20.45). These systems release high levels of H_2 and CH_4, with H_2 concentrations as high as 19 mM. This mussel was previously shown to live in a dual symbiosis with methane-oxidizing bacteria and chemolithotrophic sulfur-oxidizing bacteria localized to the gill tissue. Remarkably, the sulfur-oxidizing symbiont of *B. puteoserpentis* also has the capacity to use H_2 as an electron donor, making this mussel one of the most metabolically versatile of vent macrofauna.

Genomics and Hydrothermal Vent Symbioses

Genome sequencing is revealing additional features of the metabolic interaction and coevolution of marine invertebrates and their bacterial symbionts. The genome sequence of the gill endosymbiont of the giant vent clam *Calyptogena magnifica* provides direct evidence for autotrophy by way of the Calvin cycle because the genome encodes the key enzymes of the cycle, ribulose bisphosphate carboxylase and phosphoribulokinase (◀ Section 3.12). This genome also contains genes encoding key sulfur-oxidation processes and genes encoding the biosynthesis of most vitamins and cofactors and all 20 amino acids needed to support the host. However, because few substrate-specific transporters are encoded by the symbiont genome—proteins necessary to secrete organic compounds to the host—it is likely that the clam actually digests symbiont cells for nutrition, as do mussels.

TABLE 23.3 Marine animals with chemolithotrophic or methanotrophic endosymbiotic *Bacteria*

Host phylum (genus or order)	Common name	Habitat	Symbiont metabolic type
Porifera (*Demospongiae*)	Sponge	Seeps	Methanotrophs
Platyhelminthes (*Catenulida*)	Flatworm	Shallow water	Sulfur chemolithotrophs
Nematoda (*Monhysterida*)	Mouthless nematode	Shallow water	Sulfur chemolithotrophs
Mollusca (*Solemya*, *Lucina*)	Clam	Vents, seeps, shallow water	Sulfur chemolithotrophs
Mollusca (*Calyptogena*)	Clam	Vents, seeps, whale falls[a]	Sulfur chemolithotrophs
Mollusca (*Bathymodiolus*)	Mussel	Vents, seeps, whale and wood falls[a]	Sulfur and H_2 chemolithotrophs, methanotrophs
Mollusca (*Alviniconcha*)	Snail	Vents	Sulfur chemolithotrophs
Annelida (*Riftia*)	Tube worm	Vents, seeps, whale and wood falls[a]	Sulfur chemolithotrophs

[a]Whale and wood falls are sunken whale carcasses and wood, respectively.

Like the obligate symbionts of insects, most symbionts of marine invertebrates have small genomes (Table 23.2), indicating reduced function and an obligate association with their host. The bacterial symbiont of the giant tube worm *Riftia pachyptila* is an exception, having a genome larger than some free-living sulfur-oxidizing chemolithotrophs (Table 23.2). The *R. pachyptila* symbiont is acquired by uninfected juvenile animals from the environment (horizontal transmission), and its larger genome is likely important for survival in the free-living state. For more on the tube worm–microbe symbiosis, see Explore the Microbial World "Pattern Recognition Receptors of Hydrothermal Vent Tube Worms Facilitate Endosymbiosis" in Chapter 26.

Check Your Understanding

- How do giant tube worms receive their nutrition?
- What are the similarities of the obligate symbioses of insects and hydrothermal vent invertebrates?
- Why might some marine invertebrates contain large genomes and others small genomes?

23.12 Entomopathogenic Nematodes

Two families of nematodes—the *Heterorhabditidae* and *Steinernematidae*—are obligate pathogens of insects and together constitute a group of entomopathogenic (insect-killing) invertebrates that have a wide range of insect hosts. The basis for their insect lethality is a specific association between the nematode and its bacterial symbionts, which produce a variety of insecticidal compounds. However, unlike the insects making defensive use of symbiont products that we explored in Section 23.8, the nematode uses the compounds produced by its bacterial symbiont to kill insect prey.

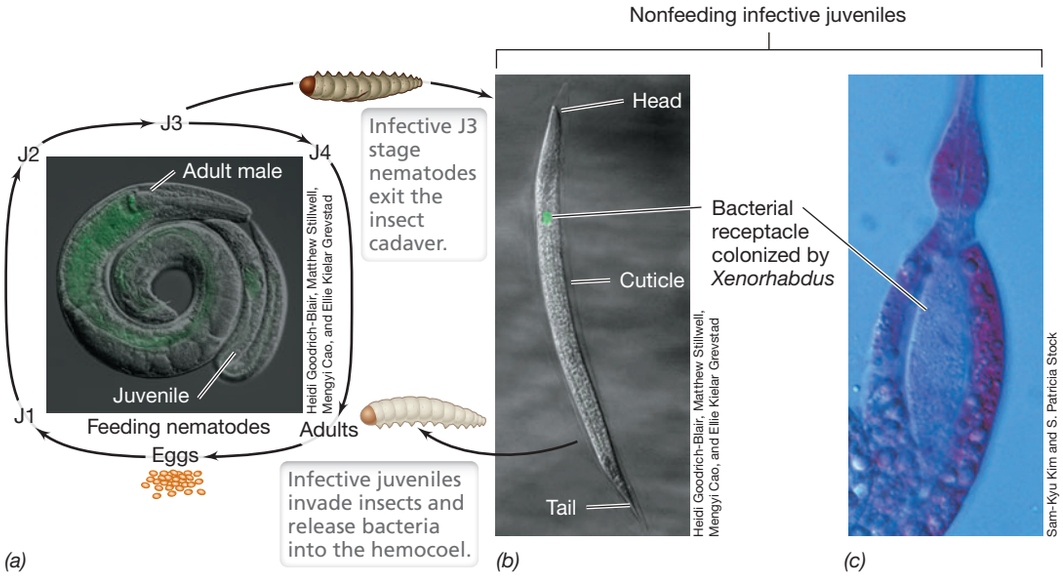


Figure 23.38 Nematode–*Xenorhabdus* life cycle. *(a)* In the presence of nutrients released in the infected insect, the nematodes develop through four juvenile stages (J1–J4), molting between stages, to egg-laying adults. When nutrients become limiting, the nematodes develop into an alternative nonfeeding J3 stage (known as the infective juvenile) that closes off most of its intestine and forms a receptacle in the gut where symbiotic bacteria localize. The infective J3 stage nematodes are then released into the environment to infect other insects. *(b) Xenorhabdus* localized to the bacterial receptacle of the infective juvenile. *(c)* Bacteria-filled receptacle imaged by differential interference contrast microscopy. *Xenorhabdus* colonizing the nematodes shown in *a* and *b* were genetically engineered to express the green fluorescent protein (◀ Sections 8.1 and 19.4) for imaging by fluorescence microscopy. Nematodes are approximately 50 μm in diameter.

Specificity of Entomopathogenic Nematodes for Their Symbionts and Insect Hosts

Species of the gram-negative bacteria *Photorhabdus* and *Xenorhabdus* are the primary bacterial symbionts of the entomopathogenic nematodes. Comparative 16S rRNA sequence analysis (◀ Section 13.11) has shown a high specificity of association between bacterial and nematode species. Species of *Photorhabdus* specifically associate with *Heterorhabditidae*, whereas *Xenorhabdus* species selectively associate with *Steinernematidae*. Each *Steinernema* species is thought to associate specifically with only one *Xenorhabdus* species. However, a single *Xenorhabdus* species may be associated with more than one nematode species. In turn, the nematodes comprising these two families can be separated into several phylogenetic groups that correspond to the phylogenetic relationships of their insect hosts. Together these data point to a history of coevolution between nematodes and their bacterial symbionts that has resulted in the various entomopathogenic nematodes having different insect host specificities.

The Nematode Life Cycle and Lethality

All entomopathogenic nematodes have a similar life cycle in which only one stage, the nonfeeding third-stage infective juvenile, survives outside the host (**Figure 23.38**). The long association between nematode and bacterial symbiont has also resulted in modification of the anterior part of the infective juvenile nematode's intestine. This region forms a discrete chamber in the infective juvenile called the *bacterial receptacle* (Figure 23.38*b*, *c*). This vessel becomes filled with bacteria by growth of the symbiont during maturation of the infective juvenile, protecting the symbiont for the next infective cycle.

When the infective juvenile encounters an appropriate insect host, it invades the insect's hemocoel (the body cavity containing the bloodlike hemolymph of the insect's circulatory system) through a natural opening such as the mouth or anus. The bacteria are then released from the bacterial receptacle into the hemolymph and grow rapidly. They are able to thwart the insect's natural immune system in part by producing a variety of hemolysins, toxins, and digestive enzymes (e.g., proteases, lipases, chitinases) that promote the release of nutrients from host tissues. The bacterial symbionts also produce antibiotics to inhibit competitive colonization by other microbes. Both the multiplying bacteria and digested host tissues nourish the multiplying nematodes, and this results in a slow insect death, taking anywhere from 1 to 22 days. As the nutrients in the host become depleted, the adult nematodes produce new infective nonfeeding juveniles that are adapted to withstand the outside environment (Figure 23.38*a*), and the life cycle repeats.

Practical Applications of Entomopathogenic Nematodes

Entomopathogenic nematodes have been extensively studied as biocontrol agents, providing an alternative to broad-spectrum chemical pesticides in the control of insect pests. In addition to alleviating possible human health effects of chemical pesticides, biologically based insect control strategies can be highly specific, targeting the pest species but not other insects. This specificity helps maintain the natural insect biodiversity in environments that require pest species management, most commonly in agricultural settings. Apart from their application in pest management, this symbiosis has also provided a powerful model system for developing a detailed understanding of the evolution, physiology, and genetics of beneficial host–microbe interactions.

Check Your Understanding

- What evidence suggest that the nematodes and their bacterial symbionts have coevolved?
- What prevents other bacteria from colonizing the dead insect and competing with the nematode and *Xenorhabdus* for nutrients?

23.13 Reef-Building Corals

Coral reef ecosystems are the products of mutualistic associations between microscopic phototrophs and simple marine animals. The

extensive ecosystems associated with the worldwide distribution of these mutualisms support thousands of species of fish and marine invertebrates.

Phototrophic Symbioses with Animals

Some animals establish mutualistic associations with algae or cyanobacteria (**Table 23.4**). The animals in most of these associations are in phyla that display very simple body plans, for example, the *Porifera* (sponges) and *Cnidaria* (corals, sea anemones, and hydroids). These mutualistic animal–bacterial associations live in clear tropical waters where nutrients for the animals are scarce, and the animal body typically has a large surface area relative to its volume and is thus well suited for capturing light.

There are only a few instances of algae forming associations with more complex animals, such as those in the phyla *Platyhelminthes* (flatworms), *Mollusca* (snails and clams), and *Urochordata* (sea squirts). In these cases, either the animal has a suitable surface-to-volume ratio or has evolved specific light-gathering surfaces. The unicellular phototrophic symbionts are phylogenetically diverse and include cyanobacteria (◀ Section 15.3), and red and green algae, diatoms, and dinoflagellates (Chapter 18). The most common symbionts are green algae (*Chlorella*, associating with sponges and freshwater hydras), cyanobacteria (associating with marine sponges), and species of the dinoflagellate genus *Symbiodinium* (◀ Section 18.4; **Figure 23.39**). We focus here on the symbiotic association between the dinoflagellate *Symbiodinium* and the stony coral cnidarians (see also MicrobiologyNow on page 780).

The Staghorn Coral Symbiosis

The staghorn coral is representative of the mutualism between the stony corals (phylum *Cnidaria*) and *Symbiodinium*. Stony corals are among the most spectacular and ecologically significant animal–phototroph associations. Each coral is a colony of genetically identical polyps (**Figure 23.40**) that together build the calcium carbonate structure. The *Symbiodinium* symbiont is distributed throughout the coral skeleton in the coral tissue (Figure 23.40*c*). Together the corals and dinoflagellates form the trophic and structural foundation of the coral reef ecosystem.

The stony corals possess a simple two-tissue-layer body plan (ectoderm and gastroderm) and harbor their dinoflagellate symbiont intracellularly in membrane-bound vesicles called *symbiosomes* within cells of the inner (gastrodermal) tissue layer (Figure 23.39*c*). The coral symbiosomes are analogous to the bacteroid-filled vesicles that develop in plant cells of the legume root nodules (Section 23.4). The coral skeleton is an extremely efficient light-gathering structure that greatly enhances light harvesting by *Symbiodinium* cells to maintain the symbiosis.

TABLE 23.4 Symbioses between animals and phototrophic symbionts

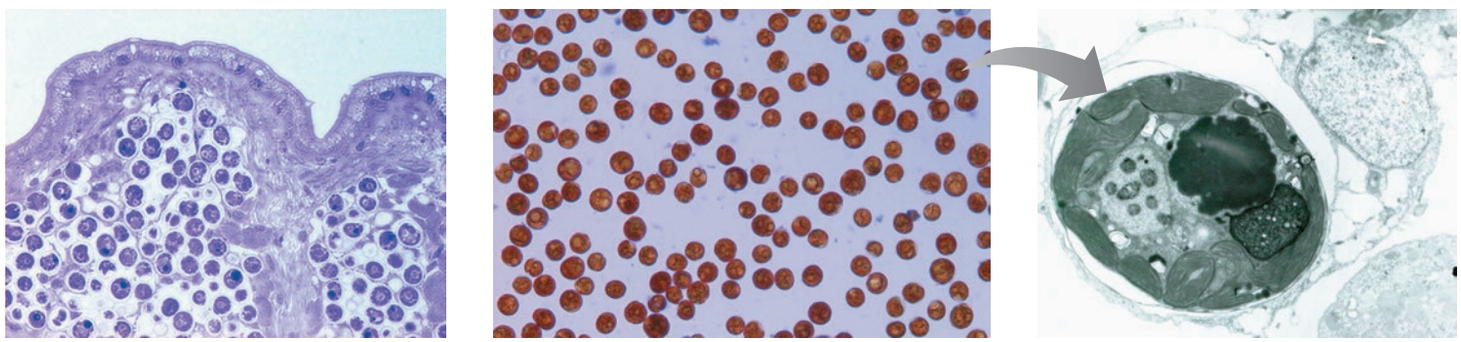
Host	Common name	Symbionts[a]
Porifera	Sponge	Cyanobacteria, *Chlorella*, *Symbiodinium*
Cnidaria	Coral, sea anemone	*Symbiodinium*, *Chlorella*
Platyhelminthes	Flatworm	Diatoms, primitive chlorophytes
Mollusca	Snail, clam	*Symbiodinium*, *Chlorella*
Ascidia	Sea squirt	Cyanobacteria

[a] Cyanobacteria are *Bacteria*; all others are eukaryotic phototrophs.

Transmission, Specificity, and Benefits of the *Symbiodinium*–Coral Association

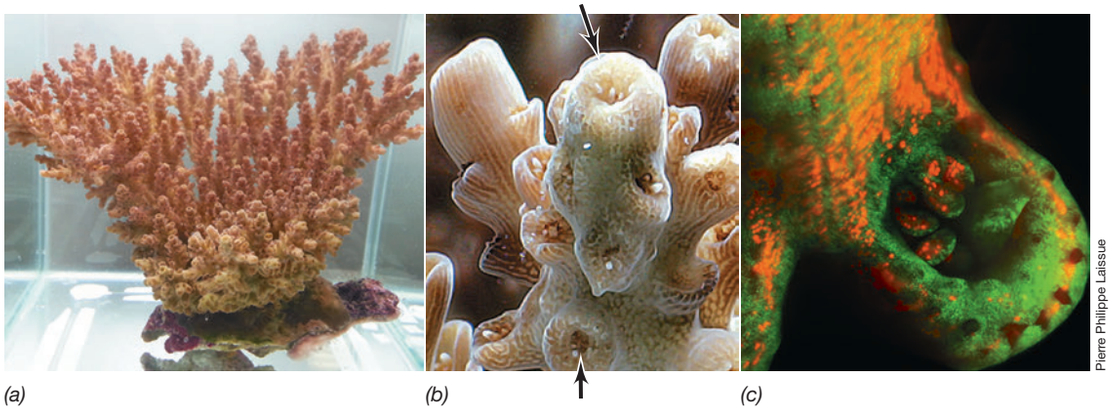
Reef-building corals reproduce sexually by releasing gametes into the seawater. A male and a female gamete fuse to form a free-swimming larva that later settles on a surface, where it may initiate a new coral colony. Algal symbionts can be present in the egg before it is released from the parent (vertical transmission), or acquired from the environment by juvenile corals (horizontal transmission). A developing coral that ingests dinoflagellates digests all of them except the particular strain of *Symbiodinium* of its mutualism. After establishing an association, the coral controls the growth of *Symbiodinium* via chemical signaling and, following each cell division, each *Symbiodinium* daughter cell is allocated to a new symbiosome.

Both partners in the stony coral–dinoflagellate mutualism have evolved adaptations for nutritional exchange. The dinoflagellates donate most of their photosynthetically fixed carbon (in the form of small molecules such as sugars, glycerol, and amino acids) to



(a) (b) (c)

Figure 23.39 *Symbiodinium* symbiont of marine invertebrates. *(a)* Thin-section micrograph of *Symbiodinium* in the mantle tissue of a giant clam. *(b) Symbiodinium* cells recovered from a soft coral. *(c)* Transmission electron micrograph of a *Symbiodinium* cell within a vacuole of a cell of the stony coral *Ctenactis echinata*. The *Symbiodinium* cell is about 10 μm in diameter.



(a) (b) (c)

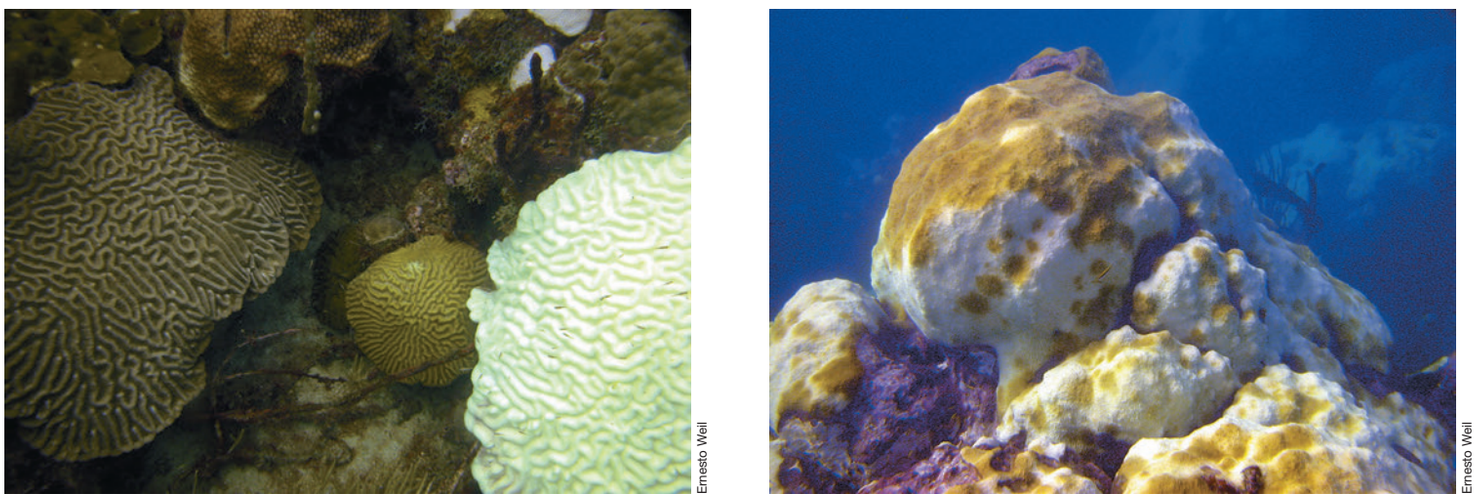
Figure 23.40 Staghorn coral. *(a)* Photograph of a large, branched colony of reef-building staghorn coral (11 cm at its widest). *(b)* Close-up of a coral branch. Each polyp (arrows) is around half a millimeter in size. The brown bands are caused by the photosynthetic symbionts living within the coral tissue. *(c)* Fluorescence microscopy of a single polyp on a branch of the colony reveals symbionts (red autofluorescence from chlorophyll) living inside the polyp's tentacles, and in bands along its sides.

the cnidarian in exchange for inorganic nitrogen, phosphorus, and inorganic carbon from the host. Moreover, in addition to providing protection and inorganic nutrients, the calcium carbonate skeleton of corals is one of the most efficient collectors of solar radiation in nature, amplifying the incident light field for the symbionts by as much as fivefold; this benefits the symbiont in carrying out photosynthesis under a light-absorbing water column. This mutualism between animal and phototroph has allowed coral reefs to thrive in large expanses of nutrient-poor ocean waters.

Coral Bleaching—The Risk of Harboring a Phototrophic Symbiont in a Changing World

Many of the extensive coral reef systems in the oceans are threatened with extinction, primarily as a consequence of human activities. Ongoing loss of these beautiful and productive ecosystems is thought to be the result of elevated atmospheric CO_2; namely, increased sea surface temperature, rising sea levels, and ocean acidification (◀ Sections 21.7 and 21.9). Coastal development also threatens reef systems, contributing to pollution from sewage discharge, eutrophication from nutrient runoff, and overfishing. These environmental changes are contributing to high mortality through disease, loss of coral structure from reduced calcification caused by acidification, and bleaching. Healthy corals harbor millions of cells of *Symbiodinium* per square centimeter of tissue. Coral bleaching is the loss of color from host tissues caused by the lysis or expulsion of these pigmented symbionts, revealing the underlying white limestone skeleton (**Figure 23.41**).

Coral reefs live close to their optimum temperature, and it is the synergistic effect of increased sea surface temperature and irradiance that causes massive bleaching. Elevated temperature and high irradiance impair the photosynthetic apparatus of the dinoflagellates, resulting in the production of reactive oxygen species (for example, singlet oxygen and superoxide, ◀ Section 4.16) that cause damage to both host and symbiont. Bleaching is thought to be caused by a protective immune response of the host that destroys compromised symbionts. Increases in sea surface temperatures as small as 0.5–1.5°C above the local maximum, if sustained for as little as several weeks, can induce rapid coral bleaching. Such thermal stress has resulted in bleaching of huge expanses of coral reefs, providing additional and highly visible evidence that climate change is upon us and that microbially based ecosystems are responding.



(a) (b)

Figure 23.41 Coral bleaching. *(a)* Two colonies of the brain coral *Colpophyllia natans*. The coral on the left is a healthy brown color, whereas the coral on the right is fully bleached. *(b)* A large colony of partially bleached mountainous star coral (*Orbicella faveolata*).

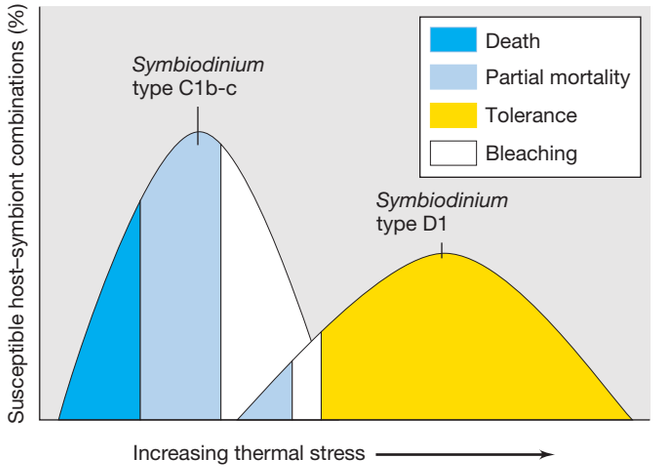


Figure 23.42 Differential stress tolerance of a coral species associated with different *Symbiodinium* phylotypes. *Pocillopora* corals symbiotically associated with *Symbiodinium* type C1b-c were much more sensitive to a thermal stress event than the same coral species associated with *Symbiodinium* type D1. The more tolerant *Symbiodinium* type D1–*Pocillopora* association suffered low mortality. The response also suggested additional genetic variation within each *Symbiodinium* type, since the two mutualisms displayed a range of sensitivity to increasing thermal stress.

Although coral reefs are clearly threatened, there is some uncertainty in projecting their future because of a lack in basic knowledge of the heat stress capacity of individual coral–symbiont mutualisms (**Figure 23.42**). For example, thermal tolerance is in part conferred by the species or strain of *Symbiodinium*, and following a bleaching event, the mutualism can shift to a more thermally tolerant symbiont (Figure 23.42). Molecular studies have indicated that there are over 150 different *Symbiodinium* phylotypes, each possibly representing a distinct species with different stress tolerance. When a coral is bleached, a more heat-tolerant symbiont can replace the bleached species, likely by symbiont swapping. In this process, the species shift results from the more robust growth of a more heat-tolerant variant already associated with the coral but in very low numbers; the variant then thrives following loss of the original symbiont and the bleaching event. Because the type of symbiont influences the ability of the coral to adapt to stresses associated with climate change, a more complete understanding of this adaptive response to thermal stress, and the limits of the response, is essential for predicting the future health of corals, their symbionts, and the spectacular and ecologically important reefs that they build.

In the final part of this chapter, we explore the symbionts of the mammalian gut of herbivorous animals as a prelude to our coverage of the human microbiome in Chapter 24.

Check Your Understanding

- Where do *Symbiodinium* cells localize within the coral skeleton and within individual coral cells?
- Describe two functions of the calcium carbonate skeleton of the stony corals.
- Besides thermal stress, list some environmental factors contributing to coral bleaching.

V • Mammalian Gut Systems as Microbial Habitats

The guts of herbivorous animals are modified in such a way as to support the growth of dense populations of anaerobic microorganisms that specialize in the digestion of plant fibers. Consumption of the products of fiber digestion and the microbes themselves allows the animal to live on a plant diet rich in carbohydrates but poor in protein.

The evolution of animals has been shaped in part by a long history of symbiotic associations with microorganisms and includes vertebrates as well as the invertebrate systems we just explored. We end this chapter by considering microbial symbioses with herbivorous animals. Microbes inhabit all sites on mammalian bodies, but the greatest diversity and density of microbes are found in the mammalian gut, and so we center our discussion there.

23.14 Alternative Mammalian Gut Systems

Some mammals are *herbivores*, consuming only plant materials, whereas others are *carnivores*, eating primarily the flesh of other animals. *Omnivores* eat both plants and animals. As **Figure 23.43** indicates, closely related mammals have evolved adaptations for differing diets. Notice that mammals of different lineages independently evolved the herbivorous lifestyle, mostly during the Jurassic period, an era in Earth's history of roughly 60 million years beginning about 200 million years ago.

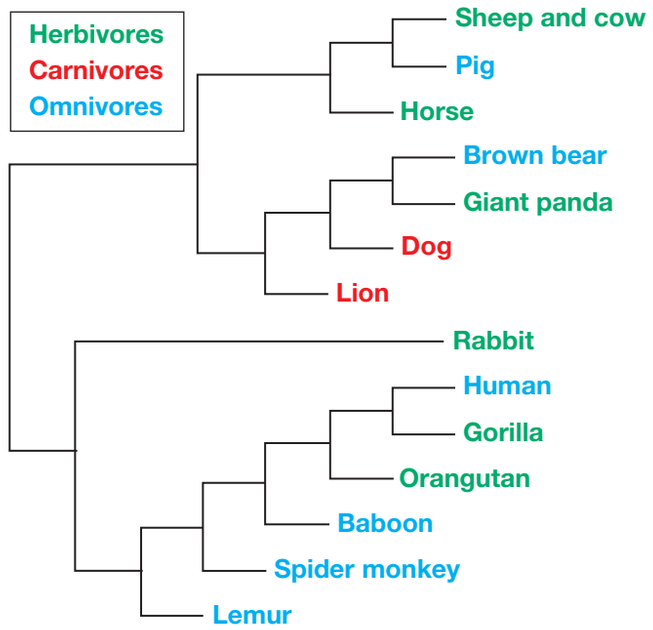


Figure 23.43 Phylogenetic tree showing multiple origins of herbivory among mammals. Some of the herbivores listed are foregut fermenters, while others are hindgut fermenters (see Figure 23.44). Instead of animal flesh, some mammalian carnivores eat only insects (the *insectivores*, such as bats), or fish (the *piscivores*, such as the river otter).

The massive evolutionary radiation of mammals during the Jurassic led to the evolution of several feeding strategies. Most mammalian species evolved gut structures that foster mutualistic associations with microorganisms. As anatomical differences evolved, microbial fermentation remained important or essential in mammalian digestion. *Monogastric* mammals, such as humans, have a single compartment, the stomach, positioned before the intestine. Such animals may get a substantial part of their energy requirement from microbial fermentation of otherwise indigestible foods in the lower intestine, while herbivores are totally dependent on such fermentations.

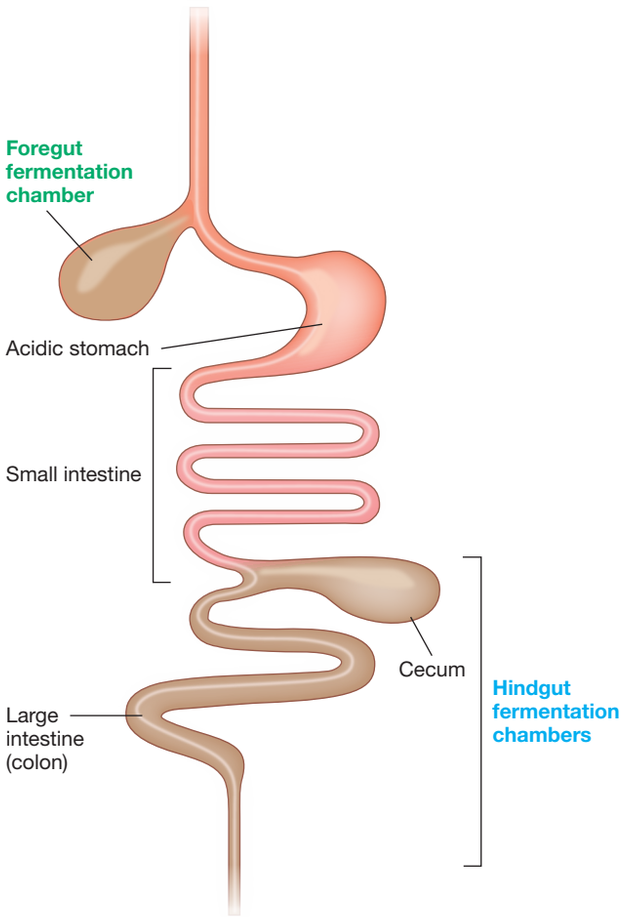
Plant Substrates

Microbial associations with various herbivorous animals led to the capacity to catabolize plant fiber, the structural component of plant cell walls. Fiber is composed primarily of insoluble polysaccharides, of which cellulose is the most abundant component. Mammals—and indeed virtually all animals—lack the enzymes necessary to digest cellulose and certain other plant polysaccharides. However, many microbes have genes encoding the hydrolases and polysaccharide lyases required to decompose these polysaccharides.

As the most abundant organic compound on Earth and one composed exclusively of glucose, *cellulose* offers a rich source of carbon and energy for animals that can digest it. The two primary traits that evolved to support herbivory are (1) an enlarged anoxic fermentation chamber for holding ingested plant material and (2) an extended retention time—the time that ingested material remains in the gut. A longer retention time allows for a longer association of microorganisms with the ingested material and thus a more complete degradation of the plant polymers.

Digestion Systems of Herbivores

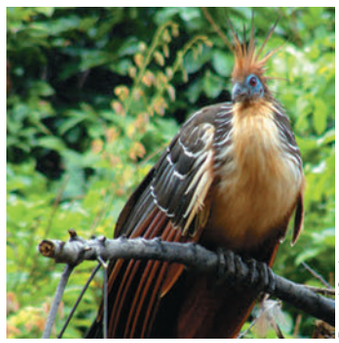
Two digestive patterns have evolved in herbivorous mammals, foregut and hindgut. In herbivores with a *foregut* fermentation, the microbial fermentation chamber *precedes* the small intestine. This gut architecture originated independently in ruminants—such as cattle and sheep—colobine monkeys, sloths, and macropod marsupials (**Figure 23.44**). These all share the common feature that ingested nutrients are degraded by the gut microbiota *before* reaching the acidic stomach and small intestine. We examine the digestive processes of ruminants, as examples of foregut fermenters, in the next section.



Foregut fermenters Examples: Ruminants (photo 1), colobine monkeys, macropod marsupials, hoatzin (photo 2)

1.

2.



Bernard Swain

Hindgut fermenters Examples: Cecal animals (photos 3 and 4), primates, some rodents, some reptiles

3.

Nancy L. Spear

4.

Figure 23.44 Variations on vertebrate gut architecture. All vertebrates have a small intestine but vary in other gut structures. Most host absorption of dietary nutrients occurs in the small intestine, whereas microbial fermentation can occur in the forestomach, cecum, or large intestine (colon). Foregut fermentation is found in four major clades of mammals and one avian species (the hoatzin). Hindgut fermentation, either in the cecum or large intestine/colon, is common to many clades of mammals (including humans), birds, and reptiles. Compare with Figure 23.43.

Horses and rabbits are herbivorous mammals, but they are not foregut fermenters. Instead, these animals are *hindgut* fermenters. They have only one stomach but use an organ called the *cecum*, a digestive organ located between the small and large intestines, as their fermentation vessel (Figure 23.44). The cecum contains fiber- and cellulose-digesting (cellulolytic) microorganisms. Mammals, such as the rabbit, that rely primarily on microbial breakdown of plant fiber in the cecum are called *cecal fermenters*. In other hindgut fermenters, both the cecum and colon are major sites of fiber breakdown by microorganisms.

Anatomical differences among monogastric mammals, foregut fermenters, and hindgut fermenters are summarized in Figure 23.44. Nutritionally, foregut fermenters have an advantage over hindgut fermenters in that the cellulolytic microbial community of the foregut eventually passes through an acidic stomach. As this occurs, most microbial cells are killed by the acidity and become a protein source for the animal. By contrast, in animals such as horses and rabbits, the remains of the cellulolytic community pass out of the animal in the feces because of its position posterior to the acidic stomach. For this reason, many hindgut fermenters, for example, rabbits, commonly eat their own feces (a phenomenon called *coprophagy*) in order to recover protein from the microbial cells.

Mastering Microbiology
Art Activity: Figure 23.38 The rumen

Check Your Understanding

- How do animals with foregut and hindgut fermentation differ in recovery of nutrients from plants?
- How does retention time affect microbial digestion of food in a gut compartment?

23.15 The Rumen and Rumen Activities

Earth's dominant herbivores are the *ruminants* (Figure 23.44), mammals that possess a special foregut digestive organ, the **rumen**, within which cellulose and other plant polysaccharides are digested by microorganisms. Some of the most important domesticated animals—cows, sheep, and goats—are ruminants. Camels, buffalo, deer, reindeer, caribou, and elk are also ruminants. Because the human food economy depends to a great extent on ruminant animals, rumen microbiology is of considerable economic significance and importance.

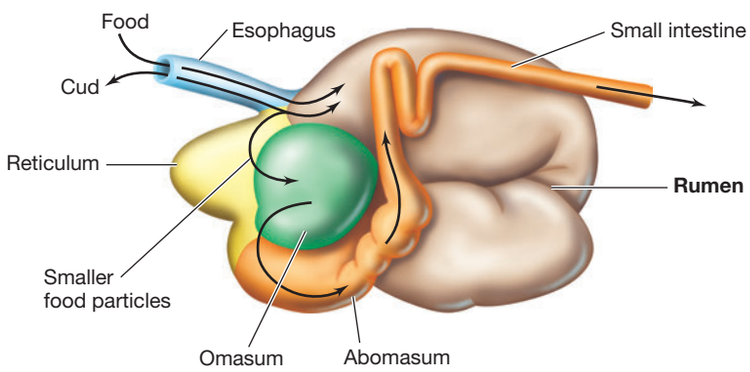
Rumen Anatomy

Unique features of the rumen as a site of cellulose digestion are its relatively large size (capable of holding 100–150 liters in a cow, 6 liters in a sheep) and its position in the gastrointestinal system *before* the acidic stomach. The rumen's warm and constant temperature (39°C), narrow pH range (5.5–7, depending on when the animal was last fed), and anoxic environment are also important factors in overall rumen function. **Figure 23.45*a*** shows the relationship of the rumen to other parts of the ruminant digestive system. The digestive processes and microbiology of the rumen have been well studied, in part because it is possible to remove samples for analysis by way of a sampling port, called a *fistula*, implanted into the rumen of a cow (Figure 23.45*b*) or a sheep.

After a cow swallows its food, the food enters the first chamber of the four-compartment stomach, the reticulum. Partially digested plant materials flow freely between the rumen and reticulum, sometimes referred to together as the *reticulo-rumen*. The main function of the reticulum is to collect smaller food particles and move them to the omasum. Larger food particles (called *cud*) are regurgitated, chewed, mixed with saliva containing bicarbonate, and returned to the reticulo-rumen where they are digested by rumen bacteria. Solids may remain in the rumen for more than a day during digestion. Eventually, small and more thoroughly digested food particles are passed to the omasum and from there to the abomasum, an organ similar to a true, acidic stomach. In the abomasum, chemical digestive processes begin that continue in the small and large intestine.

Microbial Fermentation in the Rumen

Food remains in the rumen for 20–50 h depending on the feeding schedule and other factors. During this relatively long retention



(a)

Sharisa D. Beek, Dept. Animal Science, Southern Illinois Univ.

(b)

Figure 23.45 The rumen. *(a)* Schematic diagram of the rumen and gastrointestinal system of a cow. Food travels from the esophagus into the reticulo-rumen, consisting of the reticulum and rumen. The reticulum collects smaller digesta particles and moves them into the omasum. Larger particles remain in the rumen and are regurgitated as cud (partially digested fiber). Cud is chewed until food particles are small enough to pass from the reticulum into the omasum, abomasum, and intestines, in that order. The abomasum is an acidic vessel, analogous to the stomach of monogastric animals like pigs and humans. *(b)* Photo of a fistulated Holstein cow. The fistula, shown unplugged, is a sampling port that allows access to the rumen.

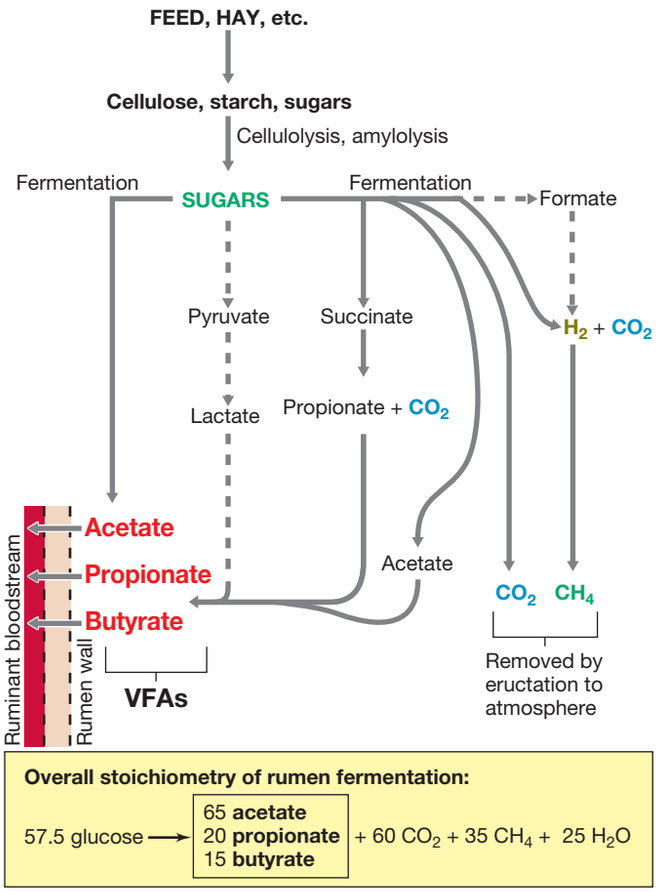


Figure 23.46 Metabolic sequences and biochemical reactions in the rumen. The major pathways are solid lines; dashed lines indicate minor pathways. Approximate steady-state rumen levels of volatile fatty acids (VFAs) are acetate, 60 mM; propionate, 20 mM; butyrate, 10 mM.

time, cellulolytic microorganisms hydrolyze cellulose, which frees glucose. The glucose then undergoes bacterial fermentation with the production of **volatile fatty acids (VFAs)**, primarily *acetic*, *propionic*, and *butyric* acids, and the gases carbon dioxide (CO_2) and methane (CH_4) (**Figure 23.46**). The VFAs pass through the rumen wall into the bloodstream and are oxidized by the animal as its main source of energy. The gaseous fermentation products CO_2 and CH_4 are released by eructation (belching).

The rumen contains enormous numbers of bacteria (10^{10}–10^{11} cells/g of rumen contents). Most of the bacteria adhere tightly to food particles. These particles proceed through the gastrointestinal tract of the animal where they undergo further digestive processes similar to those of nonruminant animals. Bacterial cells that digested plant fiber in the rumen are themselves digested in the acidic abomasum. Because bacteria living in the rumen biosynthesize amino acids and vitamins, the digested bacterial cells are a major source of protein and vitamins for the animal.

Check Your Understanding

- What physical and chemical conditions prevail in the rumen?
- What are VFAs, and of what value are they to the ruminant?

23.16 Rumen Microbes and Their Dynamic Relationships

Enormous numbers of prokaryotic and eukaryotic cells are present in the rumen, even greater than those present in fertile soils (◀ Section 20.7). These include *Bacteria* and *Archaea* as well as various fungi and nonphototrophic protists. Together these microbes constitute a highly integrated and efficient microbial community whose dynamics vary according to the types and amounts of fermentable material consumed.

Rumen *Bacteria* and *Archaea*

Anaerobic *Bacteria* and *Archaea* dominate in the rumen because it is a strictly anoxic compartment, and some anaerobic microbial eukaryotes are also present. Cellulose is converted to fatty acids, CO_2, and CH_4 (Figure 23.46) in a multistep microbial food chain, with several different anaerobes participating in the process. Analysis of 16S rRNA gene sequences from rumen samples indicate that the typical rumen contains 300–400 bacterial species (◀ Section 13.12; **Figure 23.47**), more than ten times higher than culture-dependent diversity estimates. Molecular surveys also show that species of *Firmicutes* and *Bacteroidetes* dominate bacterial diversity in the rumen, while methanogens make up virtually the entire archaeal population (Figure 23.47).

Several rumen anaerobes have been cultured and their physiology characterized (**Table 23.5**). Various rumen bacteria hydrolyze cellulose to sugars and ferment the sugars to VFAs. *Fibrobacter succinogenes* and *Ruminococcus albus* are the two most abundant cellulolytic rumen anaerobes. Although both organisms produce cellulases, *Fibrobacter*, a gram-negative bacterium, produces enzymes localized to its outer membrane, whereas *Ruminococcus*, a gram-positive bacterium (and therefore lacking an outer membrane), produces a cellulose-degrading protein complex bound to its cell wall. However, in both cases, cells of *Fibrobacter* and *Ruminococcus* need to physically bind to cellulose particles in order to degrade them. The gram-positive bacterium *Butyrivibrio* also participates in the digestion of plant cell wall polymers, specializing in the digestion of hemicellulose components such as xylan.

Rumen Protists and Fungi

In addition to huge populations of *Bacteria* and *Archaea*, the rumen has characteristic populations of ciliated protists (Chapter 18) present at a density of about 10^6 cells/ml. Many of these protists are obligate anaerobes, a physiology that is rare among eukaryotes. Although these protists are not essential for rumen fermentation, they contribute to the overall process. In fact, some protists are able to hydrolyze cellulose and starch and ferment glucose with the production of the same VFAs formed by cellulose-fermenting bacteria (Figure 23.46 and Table 23.5). Rumen protists also consume rumen bacteria and smaller rumen protists and are likely to play a role in controlling bacterial densities in the rumen. An interesting commensal interaction has been observed between rumen protists that produce VFAs and H_2 as products and methanogenic bacteria that consume the H_2, producing CH_4. Because their cells autofluoresce (◀ Section 14.15 and Figure 14.37), methanogens are easily observed in rumen fluid bound to the surface of H_2-producing protists. A similar situation exists in the hindgut of termites, but in

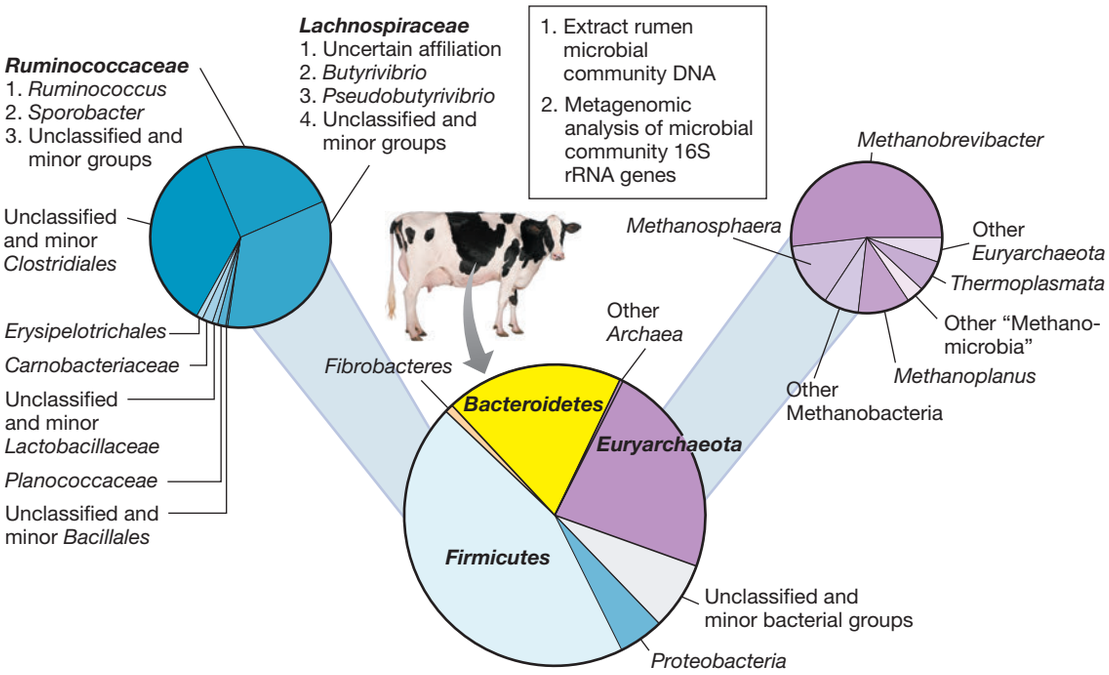


Figure 23.47 Ruminal microbial community inferred from 16S rRNA gene sequences. Pooled analyses of 14,817 sequences from several studies of ruminant animals, including cow, sheep, goat, and deer. The data reflect prokaryotic diversity and not necessarily relative abundance. Data assembled and analyzed by Nicolas Pinel.

this case, methanogens actually live *inside* anaerobic cellulolytic protozoa (◀ Figure 21.8*c*, *d*).

Anaerobic fungi also inhabit the rumen and play a role in its digestive processes. Rumen fungi are typically species that alternate between a flagellated and a thallus form, and studies with pure cultures have shown that they can ferment cellulose to VFAs. *Neocallimastix*, for example, is an obligately anaerobic fungus that ferments glucose to formate, acetate, lactate, ethanol, CO_2, and H_2. Although a eukaryote, this fungus lacks mitochondria and cytochromes and thus lives an obligately fermentative existence. However, *Neocallimastix* cells contain a redox organelle called the *hydrogenosome*; this mitochondrial analog evolves H_2 and has otherwise been found only in certain anaerobic protists (◀ Section 18.3).

Rumen fungi play an important role in the degradation of polysaccharides other than cellulose, including a partial solubilization of lignin (the strengthening agent in the cell walls of woody plants), hemicellulose (a derivative of cellulose that contains pentoses and other sugars), and pectin.

Dynamics of the Rumen Microbial Community

If a ruminant is gradually switched from cellulose to a diet high in starch (grain, for instance), the starch-digesting *Gammaproteobacteria Ruminobacter amylophilus* and *Succinomonas amylolytica* grow to high numbers in the rumen. On a low-starch diet these organisms are typically minor constituents. If an animal is fed legume hay, which is high in pectin, a complex polysaccharide containing both hexose and pentose sugars, then the pectin-digesting gram-positive bacterium *Lachnospira multipara* (Table 23.5) becomes an abundant member of the rumen microbial community. Some of the fermentation products of these rumen bacteria are used as energy sources by secondary fermenters in the rumen. For example, succinate is fermented to propionate plus CO_2 (Figure 23.46) by the bacterium *Schwartzia*, and lactate is fermented to acetate and other fatty acids by the gram-positive bacteria *Selenomonas* and *Megasphaera* (Table 23.5). Hydrogen (H_2) produced in the rumen by fermentative processes never accumulates because it is quickly consumed by methanogens for the reduction of CO_2 to CH_4. This H_2 removal facilitates greater fermentative activity because H_2 accumulation negatively affects the energetics of fermentative reactions that produce H_2 (◀ Section 14.22). Although acetate, a methanogenic substrate, is produced in large amounts in the rumen, it is absorbed by the ruminant (Figure 23.46) and thus unavailable for conversion to methane.

Dangerous and Protective Changes in the Rumen Microbial Community

Significant changes in the microbial composition of the rumen can cause illness or even death of the animal. For example, if a cow is changed abruptly from forage to a grain diet, the gram-positive bacterium *Streptococcus bovis* grows rapidly in the rumen. The normal level of *S. bovis*, about 10^7 cells/g, is an insignificant fraction of total rumen bacterial numbers. But if large amounts of grain are fed abruptly, numbers of *S. bovis* can quickly rise to over 10^{10} cells/g and dominate the rumen microbial community. This occurs because grasses contain mainly cellulose, which does not support growth of *S. bovis*, while grain contains high levels of starch, on which *S. bovis* grows rapidly.

Because *S. bovis* is a lactic acid bacterium (◀ Sections 14.18 and 16.6), large populations are capable of producing large amounts of its fermentation product, lactic acid. Lactic acid is a much stronger acid than the VFAs produced during normal rumen function (Figure 23.46). Lactate production thus acidifies the rumen below its lower functional limit of about pH 5.5, thereby disrupting the activities of normal rumen bacteria. Rumen acidification, a condition called *acidosis*, causes inflammation of the rumen epithelium, and severe acidosis can cause hemorrhaging in the rumen, acidification of the blood, and death of the animal. Despite this dangerous condition typically associated with abrupt feeding changes, ruminants such as cattle can be fed a diet exclusively of grain. However, to avoid acidosis, the animals must be switched from forage to grain *gradually* over a period of many days. The slow introduction of starch selects for VFA-producing starch-degrading bacteria (Table 23.5) instead of *S. bovis*. Under these conditions, normal rumen functions continue, and the animal remains healthy.

The overgrowth of *S. bovis* is a potent example of how a single microbial species can have a deleterious effect on animal health. In

TABLE 23.5 Characteristics of some rumen *Bacteria* and *Archaea*

Organism[a]	Morphology	Fermentation products
Cellulose decomposers		
Gram-negative		
Fibrobacter succinogenes[b]	Rod	Succinate, acetate, formate
Gram-positive		
Ruminococcus albus[c]	Coccus	Acetate, formate, H_2, CO_2
Butyrivibrio fibrisolvens[c]	Curved rod	Acetate, formate, lactate, butyrate, H_2, CO_2
Clostridium lochheadii	Rod (endospores)	Acetate, formate, butyrate, H_2, CO_2
Starch decomposers		
Gram-negative		
Prevotella ruminicola[d]	Rod	Formate, acetate, succinate
Ruminobacter amylophilus	Rod	Formate, acetate, succinate
Selenomonas ruminantium	Curved rod	Acetate, propionate, lactate
Succinomonas amylolytica	Oval	Acetate, propionate, succinate
Gram-positive		
Streptococcus bovis	Coccus	Lactate
Lactate decomposers		
Gram-negative		
Selenomonas ruminantium subsp. *lactilytica*	Curved rod	Acetate, succinate
Megasphaera elsdenii	Coccus	Acetate, propionate, butyrate, valerate, caproate, H_2, CO_2
Succinate decomposer		
Gram-negative		
Schwartzia succinovorans	Rod	Propionate, CO_2
Pectin decomposer		
Gram-positive		
Lachnospira multipara	Curved rod	Acetate, formate, lactate, H_2, CO_2
Methanogens		
Methanobrevibacter ruminantium	Rod	CH_4 (from H_2 + CO_2 or formate)
Methanomicrobium mobile	Rod	CH_4 (from H_2 + CO_2 or formate)

[a] Except for the methanogens, which are *Archaea*, all organisms listed are *Bacteria*.
[b] These species also degrade xylan, a major plant cell wall polysaccharide.
[c] Also degrades starch.
[d] Also ferments amino acids, producing NH_3. Several other rumen bacteria ferment amino acids as well, including *Peptostreptococcus anaerobius* and *Clostridium sticklandii*.

contrast, there is at least one well-studied example of how a single bacterial species can *enhance* the health of ruminant animals. This occurs in animals fed the tropical legume *Leucaena leucocephala*. This plant has very high nutritional value but contains an amino acid–like compound called *mimosine* that is converted to the toxic compounds

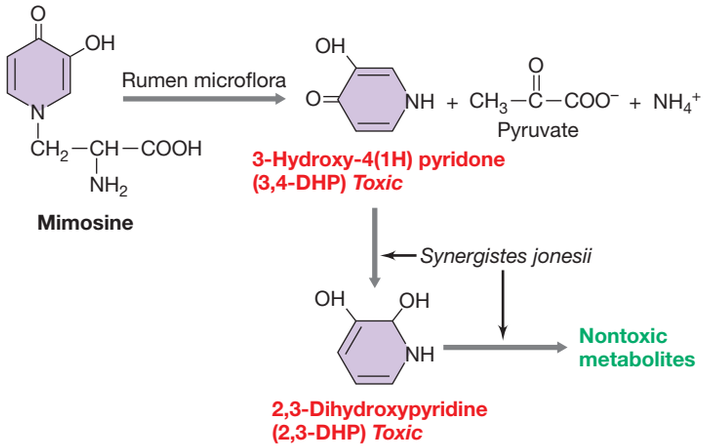


Figure 23.48 Conversion of mimosine to toxic pyridine and pyridone metabolites by ruminal microorganisms. Mimosine is converted to toxic 3,4-DHP by normal ruminal microbiota. *Synergistes jonesii* converts 3,4-DHP to nontoxic metabolites through a 2,3-DHP intermediate, preventing buildup of toxic metabolites of mimosine.

3-hydroxy-4(1H)-pyridone (3,4-DHP) and 2,3-dihydroxypyridine (2,3-DHP) by rumen microorganisms (Figure 23.48). The observation that ruminants in Hawaii, but not Australia, could feed on *Leucaena* without toxic effect led investigators to hypothesize that further metabolism of DHP by bacteria present in Hawaiian ruminants alleviated DHP toxicity. This was subsequently confirmed by the isolation of the bacterium *Synergistes jonesii*, an anaerobe related to *Deferribacter* (◀ Section 16.22) but not closely related to any other rumen bacteria. Inoculation of Australian ruminants with cells of *S. jonesii* conferred resistance to mimosine by-products, allowing the animals to feed on *Leucaena* without ill effect.

The success of this single-organism modification of the rumen microbial community has encouraged further studies of this sort, including genetic engineering of bacteria to improve their ability to utilize available nutrients or to detoxify toxic substances. A notable success has been inoculation of the rumen of sheep with genetically engineered cells of *Butyrivibrio fibrisolvens* (Table 23.5) containing a gene encoding the enzyme fluoroacetate dehalogenase. This successfully prevented fluoroacetate poisoning of sheep fed plants containing high levels of this highly toxic citric acid cycle inhibitor.

With this extensive background on the symbiotic microbial communities associated with various nonhuman animals, we now move on to Chapter 24 where the microbial world that populates and affects the health of human beings will be center stage and considered in some detail.

Check Your Understanding

- What groups of *Archaea* are present in the rumen, and how do they contribute to ruminant nutrition?
- Fungi and protists are eukaryotic microbes. How can they survive in the anoxic rumen?
- Give an example of how changes in the type of fiber given to a ruminant can result in a transition in its rumen microbes.
- Why is the metabolism of *Streptococcus bovis* of special concern for ruminant nutrition, and under what conditions is this bacterium a threat to the animal's health?

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CHAPTER REVIEW

I • Symbioses Between Microorganisms

23.1 Lichens are a mutualistic association between one or two species of fungi and an oxygenic phototroph, either an alga or a cyanobacterium.

Q How does a phototroph benefit from associating with a fungus in lichen symbiosis?

23.2 The consortium "*Chlorochromatium aggregatum*" is a mutualism between a phototrophic green sulfur bacterium and a motile heterotrophic bacterium. Mutual benefit is based on the phototroph supplying organic matter to the heterotroph in exchange for motility that permits rapid repositioning in stratified lakes to obtain optimal light and nutrients.

Q Considering its metabolic properties, what advantage to the phototroph in the "*Chlorochromatium aggregatum*" consortium might rapid repositioning confer?

23.3 Anaerobic microorganisms capable of direct interspecies electron transfer use cytochrome-rich surface structures to form direct electrical connections between cells of different species, such as between a methane-oxidizing archaeon and a sulfate-reducing bacterium. Electrons originating from the oxidation of a hydrocarbon compound (such as methane or ethane) by one species are directly transferred to the electrically connected second organism, which then uses those electrons to reduce an electron acceptor such as sulfate.

Q Assuming that methane was the only available energy source for a sulfate-reducer–ANME consortium, why can it be said that neither partner could exist without the other?

II • Plants as Microbial Habitats

23.4 One of the most agriculturally important plant–microbial symbioses is that between legumes and nitrogen-fixing bacteria. The bacteria induce the formation of root nodules within which nitrogen fixation occurs. The plant provides the energy needed by the root nodule bacteria, and the bacteria provide fixed nitrogen for the plant.

Q Describe the steps in the development of root nodules on a leguminous plant. What is the nature of the recognition between plant and bacterium, and how do Nod factors help control this? How does this compare with recognition in the *Agrobacterium*–plant system (Section 23.6)?

23.5 Mycorrhizae are mutualistic associations between fungi and the roots of plants that allow the plant to extend its root system via intimate interaction with an extensive network of fungal mycelia. Both ectomycorrhizae and endomycorrhizae are known. The mycelial network provides the plant with essential inorganic nutrients, and the plant, in turn, supplies organic compounds to the fungus.

Q How do mycorrhizae improve the growth of trees? In what way(s) are the root nodule and mycorrhizal symbioses similar? In what major way do they differ?

23.6 The crown gall bacterium *Agrobacterium* enters into a unique relationship with plants. Part of the Ti plasmid in the bacterium can be transferred into the genome of the plant, initiating crown gall disease. The Ti plasmid has also been used for the genetic engineering of crop plants.

Q Compare and contrast the production of a plant tumor by *Agrobacterium tumefaciens* and a root nodule by a *Rhizobium* species. In what ways are these structures similar? In what ways are they different? Of what importance are plasmids to the development of both structures?

III • Insects as Microbial Habitats

23.7 A large proportion of insects have established obligate mutualisms with bacteria, the basis of the mutualism often being bacterial biosynthesis of nutrients such as amino acids that are absent from the food the insect feeds on. Long-established obligate mutualisms are marked by extreme genome reduction of the symbiont, with retention of only those genes essential for the mutualism.

Q How is it possible for aphids to feed only on the carbohydrate-rich but otherwise nutrient-poor sap of phloem vessels in plants? Why do symbionts that are transmitted horizontally show less genome reduction, as opposed to the significant genome reduction observed in heritable symbionts?

23.8 The symbionts of many insects produce defensive chemicals that are toxic to specific pathogens and predators of the insect. The biosynthetic capacity to make defensive chemicals often resides on mobile genetic elements such as plasmids and transposons, allowing insects and their protective symbionts to adapt to changing predator threats by horizontal gene transfer.

Q How would horizontal versus vertical transmission of symbionts influence transfer of genes for defensive chemicals between different strains or species of symbionts?

23.9 Termites associate symbiotically with bacteria and protists capable of digesting plant cell walls. The unique termite gut configuration and the hindgut microbial community composed largely of cellulolytic bacteria and protists and acetogenic bacteria result in high levels of acetate, the primary source of carbon and energy for the termite.

Q How do the microbial communities of higher and lower termite guts differ in composition and degradation of cellulose?

IV • Other Invertebrates as Microbial Habitats

23.10 Several genera of primarily marine microorganisms have the capacity to generate light through a biochemical process known as bioluminescence, sometimes forming symbiotic associations with marine animals. A light-emitting organ on the underside of the Hawaiian bobtail squid provides a habitat for bioluminescent cells of the bacterium *Aliivibrio fischeri*. From the mutualism in the light organ, the squid gains protection from predators while the bacterium benefits from a habitat in which it grows quickly and contributes cells to its free-living population.

Q How is the correct bacterial symbiont selected in the squid–*Aliivibrio* symbiosis?

23.11 Most invertebrates living on the seafloor near regions receiving hydrothermal fluids have established obligate mutualisms with chemolithotrophic bacteria. These mutualisms are nutritional, allowing the invertebrates to thrive in an environment enriched in reduced inorganic materials, such as H_2S, that are abundant in vent fluids. The invertebrates provide the symbionts an ideal nutritional environment in exchange for organic nutrients.

Q Why is the genome of the tube-worm symbiont thought to be so much larger than the genomes of insect symbionts?

23.12 Entomopathogenic nematodes have established symbiotic associations with species of *Photorhabdus* and *Xenorhabdus* bacteria. Following invasion of an insect by the nematode, bacterial symbionts are released into the insect's hemolymph and multiply rapidly by thwarting the insect's immune system, killing the insect by the release of toxins and digestive enzymes. When nutrients are depleted, the nematodes then transition to a nonfeeding juvenile form that harbors the symbiont in a specialized receptacle and goes on to infect another insect.

Q Why are entomopathogenic nematodes so attractive for the biocontrol of insect pest species?

23.13 The mutualism between the dinoflagellate *Symbiodinium* and the stony corals produces the extensive worldwide coral reef ecosystems that sustain a tremendous diversity of marine life. Coral bleaching caused by climate change threatens these ecosystems.

Q How have both partners in the cnidarian–dinoflagellate mutualism evolved adaptations for nutritional exchange?

V • Mammalian Gut Systems as Microbial Habitats

23.14 Microbial fermentation is important for digestion in all mammals. Several microbial mutualisms have evolved in different mammals that allow for the digestion of different types of food. Herbivores derive almost all of their carbon and energy from plant fiber.

Q From a gastrointestinal standpoint, how do a sheep and a rabbit differ and how are they similar?

23.15 The rumen, the foregut digestive organ of ruminant animals, specializes in cellulose digestion, which is carried out by various microbes. The position of the rumen at the beginning of the animal's digestive tract allows for protein recovery when rumen microbes are killed in the animal's acidic stomach.

Q How can the rumen be studied and sampled in a living animal?

23.16 *Bacteria*, protists, and fungi in the rumen produce volatile fatty acids that provide energy for the ruminant while *Archaea* convert CO_2 and H_2 into CH_4 that is released to the atmosphere. Rumen microorganisms synthesize vitamins and amino acids and are also a major source of protein—all used by the ruminant.

Q Give an example of two rumen microbes and describe how they contribute to herbivore nutrition.

APPLICATION QUESTIONS

1. Choose one invertebrate discussed in this chapter and compare its microbial symbionts with those in the rumen. Why is the microbial community of these two animals considerably different? In your comparison, consider major aspects of the biology of the host (body temperature, organ systems need to resist predators, and the like), its habitat, and its food source(s), as well as the biology of the symbionts, including their habitat, metabolism, and genome characteristics.
2. Imagine that you have discovered a new animal that consumes only grass in its diet. You suspect it to be a ruminant and have available a specimen for anatomical inspection. If this animal is a ruminant, describe the position and basic components of the digestive tract you would expect to find and any key microorganisms and substances you might look for. What metabolic types of microorganisms or specific genes would you predict would be present?
3. Why would you be very surprised to find the exact same microbial symbionts inhabiting a lichen and the rumen of a cow? Consider both the physical and chemical conditions of the habitat and the requirement for specific microbes to be present in order for the symbiosis to be successful.

CHAPTER GLOSSARY

Arbuscule a branched or coiled hyphal structure within cells of the inner cortex of plants with a mycorrhizal infection

Autoinduction the induction of transcription of a set of genes, such as those responsible for bioluminescence, that occurs only when population density is great enough

Bacteriocyte a specialized insect cell in which bacterial symbionts reside

Bacteriome a specialized region in several insect groups that contains insect bacteriocyte cells packed with bacterial symbionts

Bacteroid a misshapen rhizobial cell inside a leguminous plant root nodule; can fix N_2

Bioluminescence the enzymatic production of visible light by living organisms

Coevolution evolution that proceeds jointly in a pair of intimately associated species owing to the effects each has on the other

Commensalism a symbiosis in which one symbiotic partner benefits and the other is unaffected

Consortium a two- or more-membered association of bacteria, usually living in an intimate symbiotic fashion

Infection thread in the formation of root nodules, a cellulosic tube through which *Rhizobium* cells can travel to reach and infect root cells

Leghemoglobin an O_2-binding protein found in root nodules

Lichen one or two species of fungi and an alga (or cyanobacterium) living in symbiotic association

Mutualism a symbiosis in which both partners benefit

Myc factors lipochitin oligosaccharides produced by mycorrhizal fungi to initiate symbiosis with a plant

Mycorrhizae a symbiotic association between a fungus and the roots of a plant in which nutrients are transferred in both directions

Nod factors lipochitin oligosaccharides produced by root nodule bacteria that help initiate the plant–bacterial symbiosis

Parasitism a symbiosis in which one symbiotic partner benefits and the other is harmed

Root nodule a tumorlike growth on plant roots that contains symbiotic nitrogen-fixing bacteria

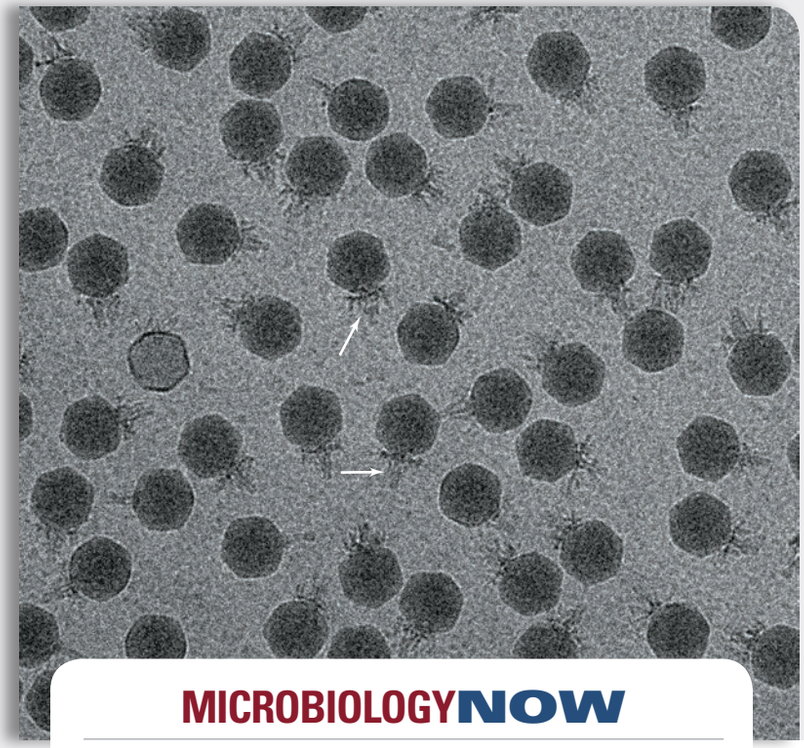
Rumen the major fermentation vessel in the multichambered gut of ruminant animals, where most cellulose digestion occurs

Symbiosis an intimate relationship between organisms, often developed through prolonged association and coevolution

Ti plasmid a conjugative plasmid in the bacterium *Agrobacterium tumefaciens* that can transfer genes into plants

Volatile fatty acids (VFAs) also called short-chain fatty acids (SCFAs), these are the major fatty acids (acetate, propionate, and butyrate) produced during fermentation in the rumen

Microbial Symbioses with Humans 24



I Structure and Function of the Healthy Adult Gastrointestinal and Oral Microbiomes 820

II Urogenital Tract and Skin Microbiomes and the Human Viral Microbiome 830

III From Birth to Death: Development of the Human Microbiome 836

IV Disorders Attributed to the Human Microbiome 839

V Modulation of the Human Microbiome 845

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One of the Most Abundant Viruses on Earth Discovered First in the Human Viral Microbiome

Past discoveries of novel viruses have required that the host they naturally infect first be identified and isolated. However, since most bacterial and archaeal diversity is still uncultured, new viral discoveries were few until next-generation sequencing allowed virus-like particles collected from the environment to be characterized directly. Today, viral metagenomics is the primary method of viral discovery and has revealed a vast reservoir of novel genetic diversity, most of which has no precedent in known viruses.

One of the most remarkable findings from metagenomic viral studies thus far has been the discovery of a human gut–associated bacterial virus (bacteriophage) that comprises up to 90% of viral genomic sequences found there. The virus was named *crAssphage* for the *cr*oss-*ass*embly method used to reconstruct its roughly 100-kbp circular DNA genome from metagenomic sequences obtained from different subjects. Genomic sequences from crAssphage were found to match more than one-fifth of all viral genomic sequences recovered from marine, terrestrial, and animal sources. Thus, crAssphage-like viruses are probably one of the most abundant biological entities on Earth! But a remaining mystery was "Whom do crAssphage-like viruses infect?"

The hunt was on using the standard method of screening cultured bacteria for infection by crAssphage. By screening 54 gut species for infection by phage-enriched fecal filtrates, the first crAssphage virus was isolated on the gut bacterium *Bacteroides intestinalis*; the phage has an icosahedral capsid about 80 nm in diameter (photo) and a short tail (arrows).The crAssphage-like viruses are now known to comprise a large viral family predicted to infect many species of *Bacteroidetes* in diverse environments.

Since changes in the gut virome are associated with human gastrointestinal abnormalities such as inflammatory bowel disease and malnutrition, we can expect that the isolation of new bacteriophages discovered through metagenomics will reap important rewards for human health.

Source: Shkoporov, A.N., Khokhlova, E.V., Fitzgerald, C.B., et al. 2018. ϕCrAss001 represents the most abundant bacteriophage family in the human gut and infects *Bacteroides intestinalis*. *Nat. Commun. 9*: 4781.

I • Structure and Function of the Healthy Adult Gastrointestinal and Oral Microbiomes

Our bodies host tremendous numbers of microorganisms distributed among different body sites, together comprising the human microbiome. Colonization begins at birth, and the assembly, composition, and activities of our gastrointestinal and oral microbiomes in particular greatly influence our health and predisposition to disease.

The human body contains huge numbers of microbes—our **microbiome**—that our very existence depends on. We begin our consideration of this microbial menagerie with an overview of the human microbiome and then consider the **microbiota**—the types of organisms present in specific regions of the body.

The clinical benefits of knowing a person's microbiome seem promising and include the development of biomarkers for predicting predisposition to specific diseases, the design of therapies increasing or decreasing the activities of selected microbial species in particular body sites, personalized drug therapies, and tailor-made probiotics (Section 24.12). However, there is a caveat. Not only is a person affected by his or her microbiota, but a person's microbiota also responds to his or her activities, health, and diet. Thus, cause and effect relationships are often not immediately obvious and can sometimes be difficult to sort out.

Microbiome studies to date have revealed an incredible diversity of microbial life in and on the human body and have signaled several likely connections between the microbial composition of a body site and the health status of the person. However, at this point most microbial associations with health or disease states are only correlations or are based on animal models; a firm causal relationship between a person's microbiome and health status has been well established in only a few instances.

24.1 Overview of the Human Microbiome

The sites of the human body inhabited by microorganisms include the mouth, nasal cavities, throat, stomach, intestines, urogenital tracts, and skin (Figure 24.1). It is estimated that the microbes in the **human microbiome** number around 10^{13} cells, which is about the same as the number of human cells in a single person. Together, the human body as the **host** and its associated microbes constitute a *host–microbiome supraorganism*. For example, the gut microbial community in the healthy human was once considered to consist of merely commensal microbes, but we now know that gut microbes are critical to development of the immune system, overall health later in life, and predisposition to disease. This recognition has stimulated the formation of several national and international research programs devoted to examining the human microbiome in great detail (Table 24.1). Collectively, these studies have shown that on the one hand the microbial diversity between individuals is so great that no one microbial species is present in all individuals, but on the other hand, particular microbial groups typically dominate.

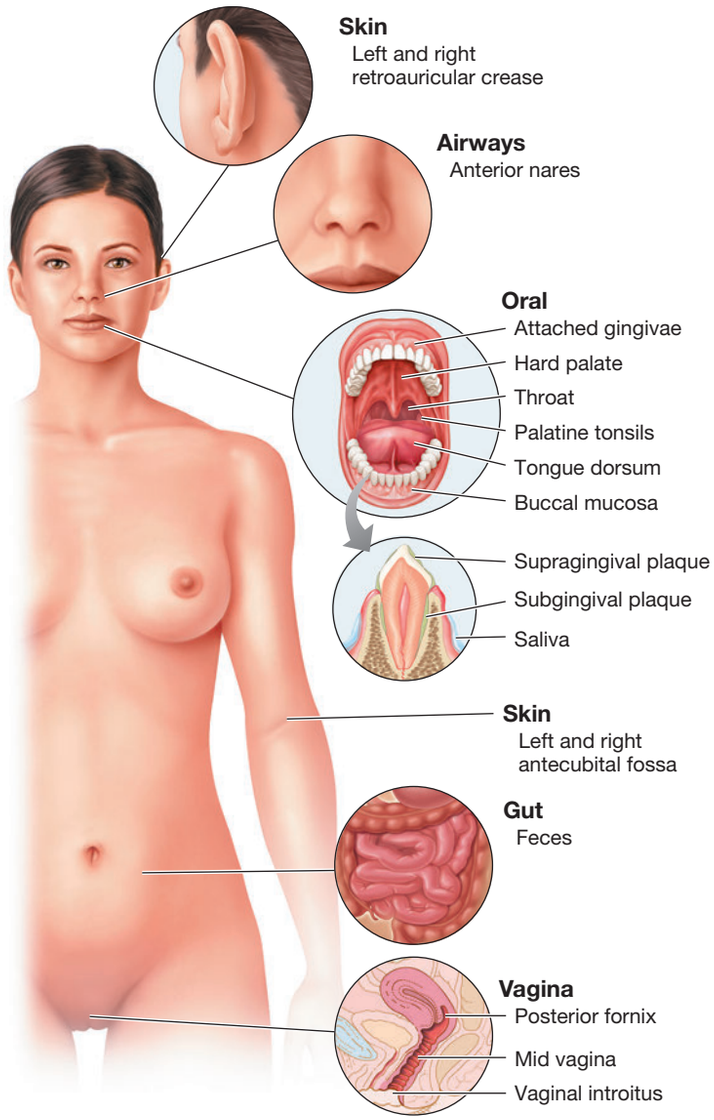


Figure 24.1 Microbial habitats of the human body. Primary body sites characterized in ongoing studies of the human microbiome (see Table 24.1).

Methodologies for Probing the Human Microbiome

Because the vast majority of microorganisms cannot yet be readily cultured or enumerated using growth-dependent approaches, it was the development of advanced nucleic acid sequencing methods (Chapters 10 and 19) that first offered the means to initiate a robust census of microbial diversity and abundance patterns in humans. The current "megasequencing era" has allowed microbiologists to quickly compare the microbiomes of different individuals and to begin to resolve the dynamic relationship between the microbiome and host history, genetics, health status, and diet.

This focus on molecular sequences in microbiome studies does not diminish the importance of cultivation in the study of the human microbiome. Indeed, since sequence-based surveys have shown that many human-associated microorganisms have not yet been isolated, there has been a renaissance in efforts to bring those microorganisms

TABLE 24.1 Major human microbiome research programs

Research program	Participating countries	Programmatic objectives
MetaGenoPolis	France	Demonstrate the impact of the human gut microbiota on health and disease using metagenomics technology
International Human Microbiome Standards	European Commission	Optimize methods for the assessment of the effects of the gut microbiome on human health through the standardization of procedures and protocols
Korean Twin Cohort Project	Korea	Characterize microbiota associated with epithelial tissue in a twin cohort study group, with the goal of identifying targets for early disease diagnosis and prevention
NIH Human Microbiome Project (HMP)	USA	Characterize the microbes that live in and on the human body, and assess the ability to demonstrate correlations of changes of the human microbiome with health
Canadian Human Microbiome Initiative	Canada	Characterize the microorganisms colonizing the human body. Evaluate their relationship to health and examine compositional changes associated with chronic disease
NIH Jumpstart Program	USA	Generate the complete genome sequences of 200 bacterial strains isolated from the human body; recruit donors for securing samples from five body regions, and perform 16S rRNA and metagenomic sequence analysis of the sampled body regions
American Gut: An Open Platform for Citizen Science Microbiome Research	USA	Crowdsourcing model to secure fecal samples for comparative 16S rRNA, metagenomic, and metabolomic analyses

into culture, and the development of successful culture conditions has been guided by the results of metagenomic sequencing and the other omics tools of the microbial ecologist (◀ Section 19.8). Using the omics toolbox, longitudinal studies of "at risk" human study groups have explored how the composition of the human microbiome, often determined early in life, affects such major diseases as obesity, diabetes, and inflammatory bowel disease. The results clearly show microbial links to these conditions.

A Snapshot of the Human Microbiome

An overview of the bacterial composition of the four heavily colonized human body sites (skin, oral, urogenital, and gastrointestinal), as defined by higher taxonomic levels, is shown in **Figure 24.2**. In each site, one group of bacteria tends to dominate, and at each site, the dominant group is different. For example, the diversity of gram-positive bacteria is greatest on the surface of the skin and the urogenital tract while the diversity of gram-negative bacteria is greatest in the gastrointestinal tract. The following sections in this chapter will examine each of these sites at a higher resolution to more fully describe the diversity of microbes inhabiting the human body. Later sections will address the functional significance of the microbiome and how it might be modified for health benefit.

A remarkable feature that emerged early in human microbiome studies is the individuality and resilience of the different microbial communities. For example, an adultlike gut microbiome develops in an individual within three years of birth and shows a species composition unique to that individual that is retained throughout life. An individual's microbiome also has tremendous plasticity, responding quickly to disturbances such as changes in diet, antibiotic therapy, or cohabitation with people and pets. However, following the removal of the disturbance, an individual's unique microbiome tends to reemerge.

As we will explore in later sections, differences in the strains or even species of microbes in people's microbiomes likely influence their response to therapies, such as fecal transplants designed to alter their microbiota for health benefit (Section 24.11). Similarities in the microbiomes of different individuals are more evident at higher bacterial taxonomic levels (such as phyla, families, and to some extent genera) rather than at the species and strain levels.

We start our tour of the human microbiome with a focus on the microbial communities that inhabit the gut, the human body site most heavily colonized by microbes and a site where the absence or presence of specific microbes may play a major role in an individual's overall health or lack thereof.

Check Your Understanding

- Which major body sites do microbes most heavily colonize?
- What methods have been used to assess the human microbiome?
- Why might knowing our microbiome and how it functions be useful?

24.2 Gastrointestinal Microbiota

The human gastrointestinal tract consists of the stomach, small intestine, and large intestine (**Figure 24.3**), and is responsible for the digestion of food and the absorption of nutrients; many important nutrients are also produced by the indigenous microbiota. Starting with the stomach, the human digestive tract is a long, folded tube of nutrients mixed with microbes, primarily species of *Bacteria*. The host and its gut microorganisms share the easily digestible nutrients. The nutrients are moved through this tube, encountering ever-changing microbial communities and abundance (Figure 24.3).

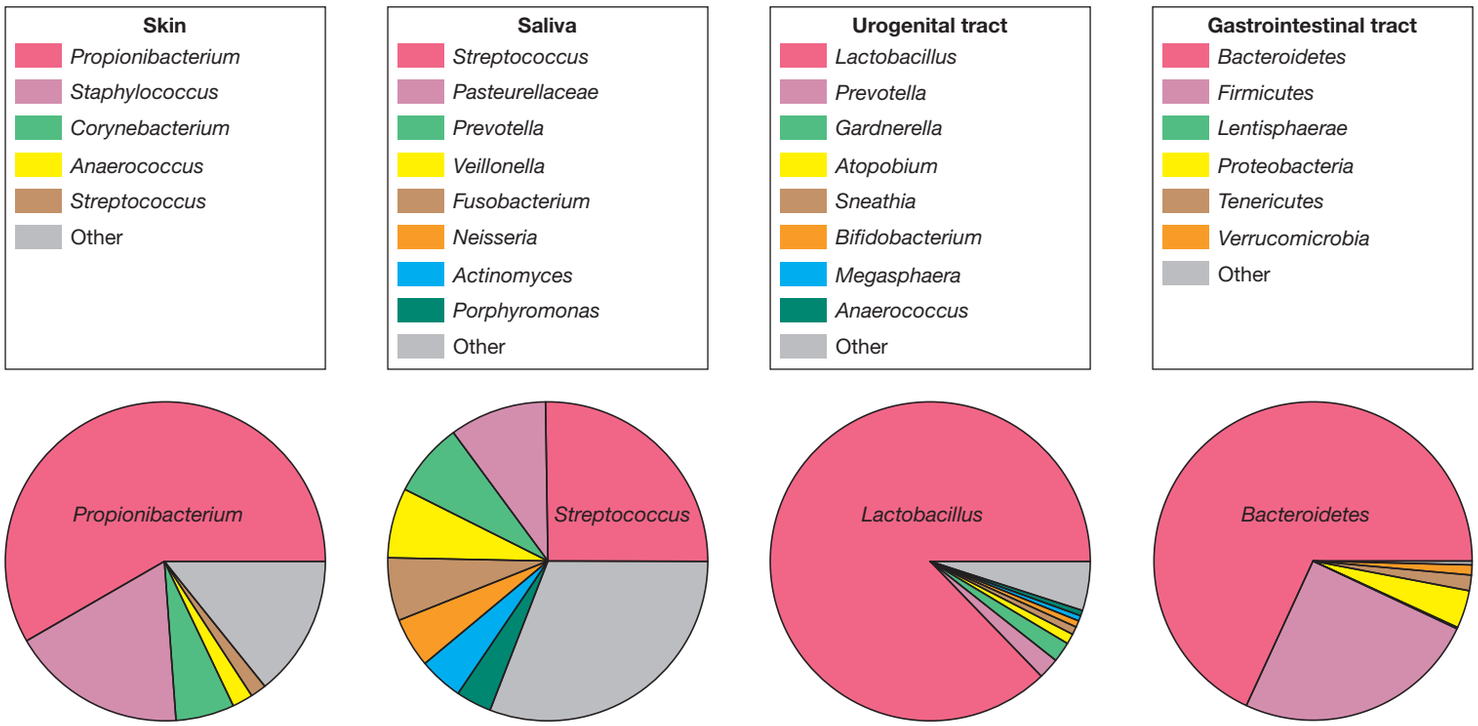


Figure 24.2 Overview of major microbial populations in the body sites sampled by human microbiome projects. Diversity among body sites was evaluated by 16S rRNA gene sequence analysis (◀ Section 13.11). Note that each body site tends to be dominated by one population type: skin by *Propionibacterium* species; oral by *Streptococcus* species; urogenital by *Lactobacillus* species; and gastrointestinal by *Bacteroides* species.

The human gastrointestinal tract has about 400 m^2 of surface area and is home to a total of about 10^{13} microbial cells. In the human duodenum, ingested food passed down from the stomach is blended with bile, bicarbonate, and digestive enzymes. About 1–4 h after ingestion, food reaches the gut (the large intestine, with near-neutral pH) and total bacterial numbers have increased from about 10^4/g (in the stomach) to 10^8/g (in the small intestine) to 10^{11}–10^{12} per g (in the large intestine) (Figure 24.3).

The Stomach and Small Intestine

The stomach was long thought to be either sterile or only minimally populated until the discovery in 1983 that the highly acid-tolerant bacterium *Helicobacter pylori* colonizes the stomachs of about 50% of all humans. This discovery prompted a closer inspection of the microbiology of the human stomach through both cultivation and 16S rRNA sequence analyses. The stomach is now recognized as the home of a vibrant bacterial community with hundreds of phylotypes distributed between the gastric lumen (pH 1–2) and the mucus layer of the wall (pH 6–7). Although low pH prevents bacterial overgrowth, the healthy human stomach holds a core microbiome that is distinct from the transient passage of ingested oral populations and is dominated by *Bacteroidetes* (*Prevotella*), *Firmicutes* (*Streptococcus, Veillonella, Lactobacillus*), *Actinobacteria* (*Rothia, Propionibacterium*), *Fusobacteria*, and *Proteobacteria* (*Haemophilus, Methylobacterium*).

Firmicutes, Bacteroidetes, and *Actinobacteria* dominate gastric fluid samples, whereas *Firmicutes* and *Proteobacteria* are more abundant in gastric mucosal samples. When present, *H. pylori* accounts for the vast majority of stomach microbial biomass and can cause upper gastrointestinal tract disease (▶ Section 31.10). Distal to the stomach, the intestinal tract consists of the small and large intestines, each of which is divided into different anatomical segments and supports a characteristic microbiota (Figure 24.3). The small intestine has three distinct chambers, the *duodenum* and the *ileum* connected by the *jejunum*. The duodenum, adjacent to the stomach, is fairly acidic and its microbiota typically resembles that of the stomach. From the duodenum to the ileum, the pH gradually becomes less acidic and bacterial numbers increase. In the lower ileum, cell numbers of 10^5–10^7/gram of intestinal contents are common, even though the environment becomes progressively more anoxic. Fusiform (spindle-shaped) anaerobic bacteria are typically present, with one end attached to the intestinal wall (**Figure 24.4**). Whereas the colonic microbiota (next subsection) is largely supported by the degradation of complex indigestible carbohydrates, microbial residents of the small intestine must compete with the host for the rapid uptake of small carbohydrates and therefore consist of bacteria well-adapted to "feast or famine" conditions.

Structure, Cell Numbers, and Retention Times in the Large Intestine

The ileum empties into the *cecum*, the beginning of the large intestine. The *colon* makes up the rest of the large intestine. In the colon, *Bacteria* are present in enormous numbers and *Archaea* (primarily

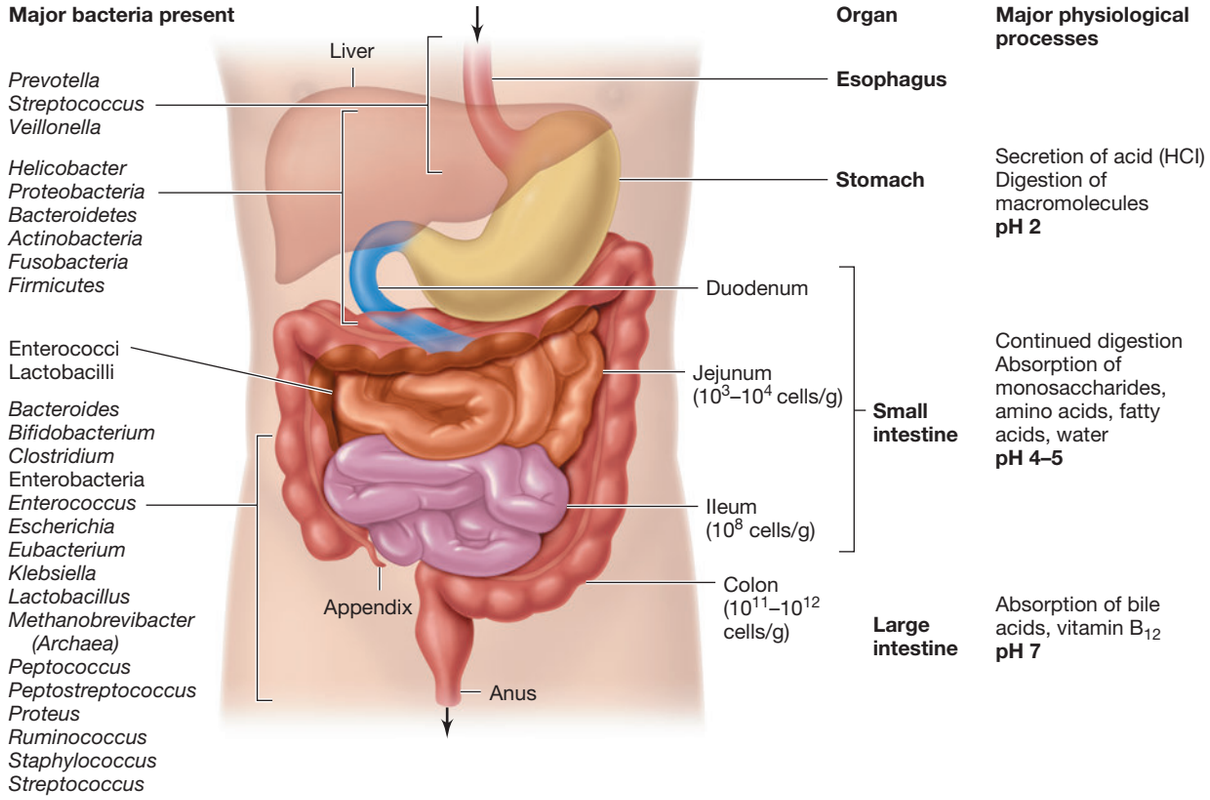
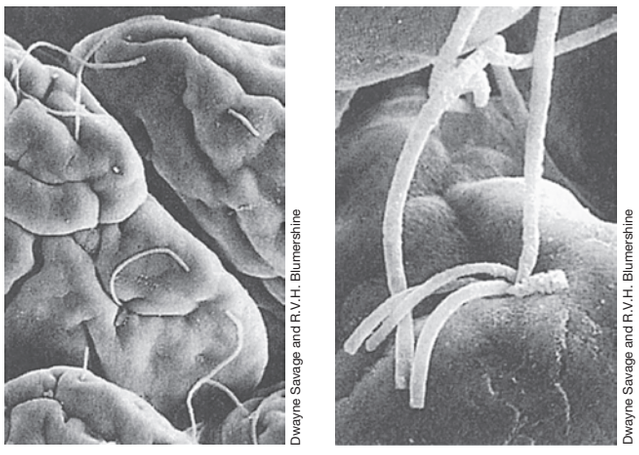


Figure 24.3 The human gastrointestinal tract and major members of its microbiota. These taxa are representative of microorganisms found in healthy adults. Not every individual harbors all of these microorganisms.

methanogens) can be present, too, depending on the individual. The colon is essentially a fermentation vessel, with the microbiota using nutrients derived from the digestion of food (Figure 24.3). Facultative aerobes such as *Escherichia coli* are present but in much smaller numbers than other bacteria; instead, obligate anaerobes predominate. The facultative aerobes consume traces of oxygen that make it to the colon, rendering the large intestine strictly anoxic. Anoxia promotes growth of obligate anaerobes such as *Clostridium* and *Bacteroides* species; indeed, *Bacteroidetes* and gram-positive *Bacteria* account for more than 99% of all prokaryotic cells.

The total number of obligate anaerobes in the colon is enormous. *Bacteria* are by far the dominant population—bacterial cell counts of 10^{10} to 10^{11}/gram of fecal contents are typical. During the passage of food through the gastrointestinal tract, water is absorbed from the digested material, which gradually becomes more concentrated and is converted to feces (**Figure 24.5**). Bacteria compose about one-third of the weight of fecal matter. Organisms inhabiting the lumen of the large intestine are continuously displaced downward by the flow of material, and bacteria that are lost are continuously replaced by new growth, similar to a continuous culture system (◀ Section 4.8). The time needed for passage of material through the human gastrointestinal tract is about 24 h, and the growth rate of bacteria in the lumen averages one to two doublings per day.

A person sheds a total of about 10^{13} bacterial cells each day in feces. Most of those organisms are restricted to the lumen of the large intestine. Production of **mucin** (a thick liquid secretion containing water-soluble proteins and glycoproteins) by *goblet cells* (a specialized class of epithelial cells) in the intestinal epithelium forms a protective layer (inner mucus layer) immediately adjacent to the intestinal epithelium (Figure 24.5). This inner mucus layer, unlike the outer mucus layer, is rarely colonized by bacteria. Goblet cells also produce various antimicrobial peptides that help prevent microbial contact with the underlying epithelium.



(a) *(b)*

Figure 24.4 Microbiota in the small intestine. Scanning electron micrographs of the microbial community on epithelial cells in the mouse ileum. *(a)* An overview at low magnification shows long, filamentous fusiform bacteria on epithelial cells in the mouse ileum (part of the small intestine, see Figure 24.19). *(b)* Higher magnification shows several filaments attached at a single depression. The attachment is at the end of the filaments. Individual cells are 10–15 μm long.

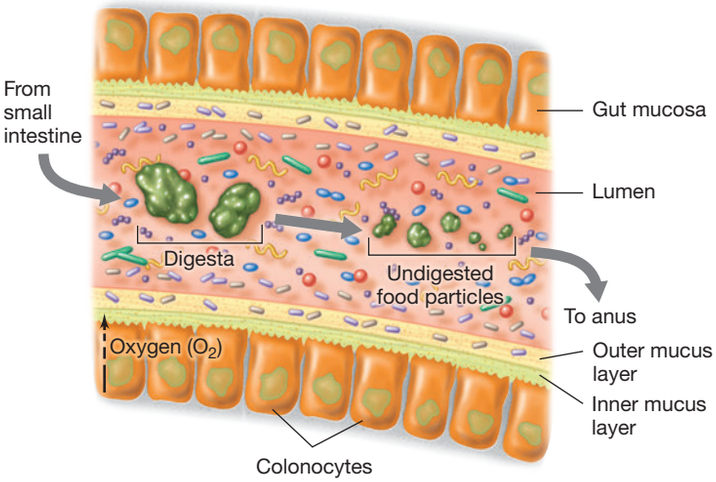


Figure 24.5 Different microenvironments in the large intestine. The inner mucin layer, produced by and contacting the gut mucosa, is partly oxygenated but generally free of bacteria. The sparsely populated outer mucus layer is adjacent to the heavily colonized anoxic lumen, which receives undigested food particles from the small intestine.

Bacterial Diversity of the Large Intestine

Figure 24.6 is a molecular snapshot of bacterial diversity in the human colon as determined by 16S rRNA gene sequence analysis of feces. Two major families of *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*) dominate gram-positive bacterial diversity in the gut and are important in the digestion of polysaccharide polymers in plant fiber, such as cellulose and pectin; these are depolymerized and the sugars fermented in the large intestine. *Ruminococcus* species play an important role in the digestion of starch and resistant starch, a form of starch common in seeds and whole grains that is not digested in the small intestine. Since fermentation of resistant starch in the colon generates beneficial short-chain fatty acids (see Section 24.9), there is increasing interest in using resistant starch as a food additive, called a *prebiotic* (see Section 24.12), to encourage the growth of ruminococci and other fermenting species.

Gut *Bacteroidetes* largely consist of different species of the genus *Bacteroides*, organisms that also specialize in the degradation of polysaccharides, although some species are proteolytic. When dietary polysaccharides are not available, or in low abundance, glycolytic *Bacteroides* use host glycans and glycoproteins derived from the mucin layer (Figure 24.5).

In contrast to the rather limited *phylum-level* gut bacterial diversity, gut *species* diversity is enormous. For instance, one study of diversity in human fecal samples (based on the analysis of millions of 16S rRNA sequences from a large sample of people) identified between 3500 and 35,000 bacterial "species," depending on exactly how one defines a bacterial species (◀ Section 13.12). A relatively small number of these, several hundred or so, are shared by most individuals. *Archaea* (represented by a phylotype closely related to the methanogen *Methanobrevibacter smithii*), yeasts, fungi, and protists are either absent or compose only a minor part of the human gut community (compare this with the rumen, ◀ Section 23.16). When eukaryotic microbes are present, they are usually yeasts of the genus *Candida* (▶ Sections 34.1 and 34.2).

Although there is high variability from person to person in gut community composition at the species level, any particular individual's community is relatively stable over long periods. In a longitudinal study of healthy adults, about 65% of strains persisted for at least 5 years and most strains likely remain residents for decades, perhaps even for the lifetime of an individual. Three broad gut communities (called *enterotypes)* have been described, differing primarily by the enrichment of one microbial group in each enterotype. Enterotype 1 is enriched in *Bacteroides*, enterotype 2 is enriched in *Prevotella*, and enterotype 3 is enriched in *Ruminococcus* (Figure 24.6). The enterotypes are functionally distinct, differing in capacity for producing vitamins and energy-rich metabolites that can be used by the host. Evidence also exists that a person's enterotype influences the response to diet and drug therapy and may contribute to health or disease status in yet unknown ways.

Products of Intestinal Microbiota

Some products of gut microbial metabolism are relatively simple compounds (**Table 24.2**), such as the volatile fatty acids generated by microbial fermentation of plant material, as also occurs in the rumen of herbivores (◀ Figure 23.46). Other products generated by

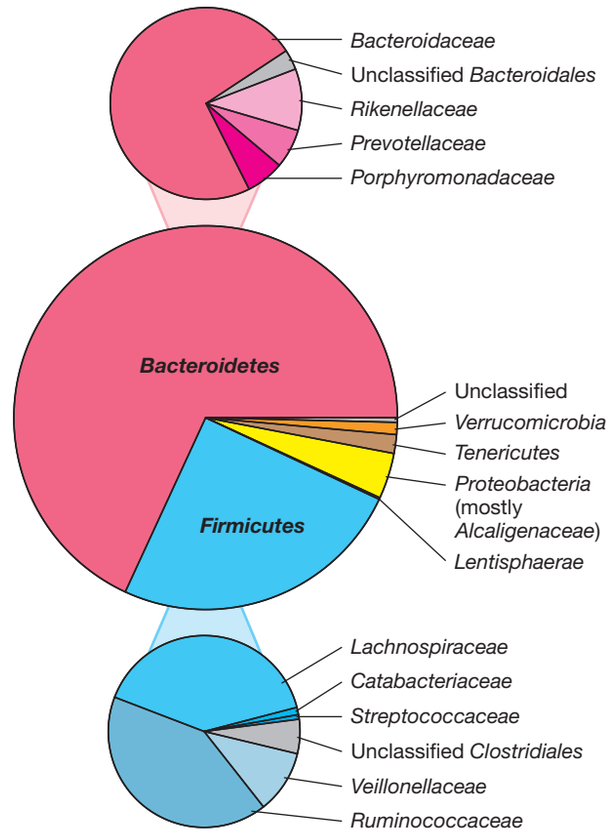


Figure 24.6 Bacterial diversity of feces. The results are pooled analyses of approximately 1 million sequences (from 184 samples) of the V1–V3 region of the 16S rRNA gene (◀ Figure 13.24). Members of the *Bacteroidetes* and *Firmicutes* dominate the normal microbiota of the large intestine. Many of these groups are discussed in Chapters 15 and 16. Data assembled and analyzed by Nicolas Pinel.

TABLE 24.2 Biochemical/metabolic contributions of intestinal microorganisms

Process	Product or enzyme
Vitamin synthesis	Thiamine, riboflavin, pyridoxine, B_{12}, K
Amino acid synthesis[a]	Asparagine, glutamate, methionine, tryptophan, lysine, and others
Gas production	CO_2, CH_4, H_2
Odor production	H_2S, NH_3, amines, indole, skatole, butyric acid
Organic acid production	Acetic, propionic, butyric acids
Glycosidase reactions	β-Glucuronidase, β-galactosidase, β-glucosidase, α-glucosidase, α-galactosidase
Steroid metabolism (bile acids)	Esterified, dehydroxylated, oxidized, or reduced steroids

[a]Capacity for amino acid biosynthesis inferred from the identification of biochemical pathways encoded in gut metagenomic sequences (◀ Sections 10.7 and 19.8).

the activities of fermentative bacteria and methanogens include H_2, CO_2, and CH_4 (similar to fermentation products of the rumen) and several other substances and nutrients (Table 24.2).

Gut microbes also produce vitamins B_{12} and K and some amino acids humans are unable to make (so-called essential amino acids, such as lysine). These essential compounds are not synthesized by humans (and vitamin B_{12} is not present in plants) but are made by the gut microbiota and absorbed from the colon. In addition, steroids, produced in the liver and released into the intestine from the gallbladder as bile acids, are modified in the intestine by the microbiota; the modified bioactive steroid compounds are then absorbed from the gut.

Many other microbial metabolites or transformation products that can be generated in the gut have significant influence on host physiology. These include post-translationally modified peptides such as the lantibiotics and bacteriocins (**Table 24.3**), substances that function to help secure colonization of the producing organism by inhibiting organisms closely related to the producer. Gut bacteria can also synthesize high levels (>100 mg/day in some cases) of metabolites derived from the reduction of amino acids. These include substances such as *tryptamine*, a tryptophan metabolite thought to function as a neurotransmitter that signals the enteric nervous system, and *4-ethylphenylsulfate*, shown to affect behavior in mice (Table 24.3). These examples suggest that an animal's microbiome and its nervous system—called the *gut–brain axis*—are likely connected (see Explore the Microbial World, "The Gut–Brain Axis").

The Gut Microbiome and "Educating" the Immune System

The gut microbiome has far-reaching effects on the long-term health of a human. For example, the immune system does not properly develop in the absence of microbial stimulation, and exposure to a variety of microorganisms soon after birth is essential for developing immune tolerance of beneficial microorganisms (our **normal microbiota**) and recognizing pathogens as foreign. Indeed, the result of excessive hygiene in an infant's development may be a poorly trained immune system that is more likely to attack beneficial organisms with an inflammatory response. Such an immune status is thought to promote autoimmune conditions such as allergies, asthma, and inflammatory bowel diseases later in life.

Much of our understanding of how the immune system is trained to accept the normal microbiota comes from the study of mice (Section 24.7). For example, a key factor contributing to the successful colonization of *Bacteroides fragilis*, part of the normal microbiota of the mouse gut, is production of a "symbiosis factor" called *polysaccharide A* (Table 24.3). Polysaccharide A is a diffusible oligosaccharide derived from the *B. fragilis* outer membrane (◀ Section 2.4) that signals the host's immune system to promote the tolerance needed for successful colonization by this bacterium. Tolerance is associated with the capacity of polysaccharide A to induce the production of cytokines (▶ Section 26.5) in the host that suppress inflammation or activate specific immune cells. *Clostridium* species also suppress inflammation and promote tolerance using a similar mechanism. Besides promoting its own colonization, *B. fragilis* has also been shown to protect mice from colitis caused by a pathogenic bacterium, *Helicobacter hepaticus*. In experimental animals colonized with *B. fragilis* mutants unable to express polysaccharide A, *H. hepaticus* colonizes the gut and elicits inflammatory bowel disease, whereas control animals colonized by wild-type *B. fragilis* do not.

The gut is a complex structure that hosts a diversity of microbes, and these examples of the "dialogue" between gut microbiota and host indicate the importance of exposing infants to a variety of microbes early in life in order to develop a healthy immune system that can readily distinguish "friend from foe." The identification of common and variably distributed biologically active compounds

TABLE 24.3 Small bioactive molecules produced by bacteria in the large intestine

Class	Compound	Example producer	Activity
RiPP[a] (lantibiotic)	Ruminococcin A	*Ruminococcus gnavus*	Antibiotic
RiPP[a] (bacteriocin)	Ruminococcin C	*Ruminococcus gnavus*	Antibiotic
Amino acid metabolite	Indolepropionic acid	*Clostridium sporogenes*	Protective antioxidant
Amino acid metabolite	4-Ethylphenylsulfate	Undefined	Neuromodulatory
Amino acid metabolite	Tryptamine	*Ruminococcus gnavus*	Neurotransmitter
Volatile fatty acid	Propionic acid	*Bacteroides* spp.	Immunomodulatory[b]
Oligosaccharide	Polysaccharide A	*Bacteroides fragilis*	Immunomodulatory[b]

[a]Ribosomally synthesized and post-translationally modified peptides.
[b]These small molecules promote colonization by normal microbiota.

The Gut–Brain Axis

Interactions between the gut microbiota and the host brain and general nervous system (called the *gut–brain axis*) have been gaining attention because of possible contributions to behavioral disorders. We know that there is a clear relationship between gut dysbiosis and pathologies such as inflammatory bowel disease and obesity (Section 24.9). However, gut dysbiosis is also associated with autism, a general term for a spectrum of behavioral disorders (together called *autism spectrum disorder*) that can emerge in the first 36 months of life, causing substantial impairments in social interaction and communication and marked by repetitive behaviors and unusual interests.

Human autism has been attributed to a combination of genetic and environmental factors. An environmental risk factor for a child developing autism and other behavioral disorders is maternal immune activation (MIA), which involves elevated levels of inflammatory factors in the blood, placenta, and amniotic fluid during pregnancy and can be caused, for example, by viral infection. Although the relationship between MIA during pregnancy and impaired development of the fetal central nervous system is complex, mouse models suggest that some features of autism can be alleviated by correcting associated defects in the gut microbial community.

The offspring of mice in which MIA has been induced display autism-like behaviors, such as communication defects and anxiety (**Figure 1*a, b***). Those behavioral changes are associated with a loss of intestinal integrity, a shift in the composition of gut clostridial and bacteroidal populations reminiscent of changes observed in autistic humans, and a 46-fold increase in serum levels of the chemical *4-ethylphenylsulfate* (Figure 1*d*). This compound is made from the amino acid tyrosine by certain gut microbiota. When administered alone, it induces anxiety-like behavior in normal mice.[1] However, a remarkable finding is the ability to ameliorate defects in communication, anxiety, 4-ethylphenylsulfate levels, and gut permeability by feeding the affected mice a human gut commensal bacterium, either *Bacteroides fragilis* or *Bacteroides thetaiotaomicron* (Figure 1*c*). It is thought that these organisms displace the 4-ethylphenylsulfate producers and return gut chemistry to normal.

These studies offer an example of the importance of the gut microbiota–brain connection in behavior and point to the possibility of modifying the gut community, such as might be possible with targeted probiotic therapy (Section 24.12), to alleviate symptoms in human neurodevelopmental disorders such as autism. Furthermore, amelioration of aberrant behavior by reducing serum levels of the gut microbiota–derived metabolite 4-ethylphenylsulfate to normal levels suggests that other neurodevelopmental illnesses may also be linked to the accumulation of microbial metabolites in serum by an unbalanced gut microbiota. However, one must be cautious about overinterpreting this study because the science thus far is only at the early stages of associating the gut microbiota with behavioral disorders.

[1]Hsiao, E.Y., S.W. McBride, S. Hsien, et al. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell 155*: 1451.

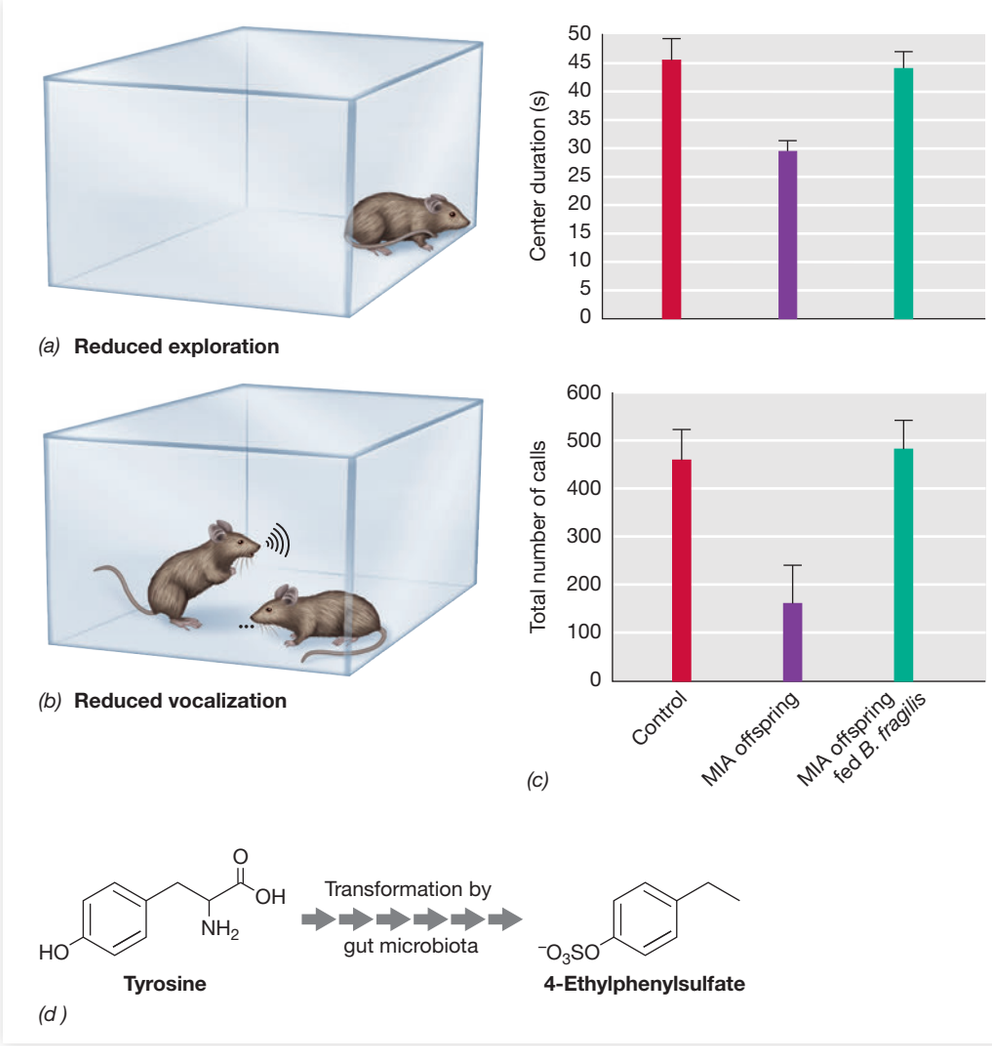


Figure 1 **Influence of gut microbiota on behavior.** Mice offspring of mothers with maternal immune activation (MIA) show autistic-like behavior, marked by *(a)* a fear of exploring the center of the test field and *(b)* reduced vocalization. *(c)* Feeding the affected offspring the human commensal bacterium *Bacteroides fragilis* ameliorates these behavioral abnormalities. *(d)* Certain gut microbiota can convert the amino acid tryptophan into 4-ethylphenylsulfate, the neuroactive compound thought to trigger the mouse autistic-like behaviors shown in *a* and *b*. Modified from Hsiao, E.Y., et al. 2013. *Cell 155*: 1451.

produced by the human microbiota is a rapidly emerging research area of great significance and will be critical to predicting future health outcomes and developing therapies for human pathologies linked to the gastrointestinal microbiome.

Check Your Understanding

- How does the general metabolism of microorganisms colonizing the small and large intestines differ and why?
- What is an enterotype, and what data indicate that it a stable feature of an individual?

24.3 Oral Cavity and Airways

Mucous membranes throughout the body support the growth of a normal microbiota that helps prevent infection by pathogenic microorganisms. Mucous membranes consist of epithelial cells, tightly packed cells that interface with the external environment, and are found throughout the body, lining the urogenital, respiratory, and gastrointestinal tracts (Figure 24.5). Mucous membranes secrete *mucin*, forming the mucus layer that retains moisture and inhibits microbial attachment; invaders are usually swept away by physical processes like swallowing or sneezing, but some microorganisms adhere to the epithelial surface and colonize it. Here we discuss two mucosal environments and their resident microbes. In Chapter 25 we explore the mechanism of specific attachment of microbes to mucous membranes leading to growth and development of the normal microbiota or, alternatively, to microbial disease.

Oral Microbial Diversity

The complex mixture of nutrients present in the mouth is supplied by saliva (containing proteins, glycoproteins, amino acids, peptides, and vitamins) and the ingestion of food. This nourishes a diverse community comprised of both aerobes and anaerobes. However, saliva itself is not a good microbial growth medium because saliva contains antibacterial substances. These include *lysozyme*, an enzyme that cleaves glycosidic linkages in peptidoglycan of the bacterial cell wall, weakening the wall and causing cell lysis. Another enzyme, *lactoperoxidase*, found in both milk and saliva, kills bacteria by a reaction that generates singlet oxygen (a toxic form of oxygen, ◀ Section 4.16). Despite the activity of these antibacterial substances, food particles and cell debris provide high concentrations of nutrients near surfaces such as the teeth and gums, creating favorable conditions for extensive local microbial growth that can contribute to tissue damage and disease.

The oral cavity provides a variety of microbial habitats, each colonized by species that grow primarily as biofilms (◀ Sections 4.9, 8.10, and 20.4). The microbiota in saliva consists of microorganisms shedding from multiple sites within the oral cavity and provides an overview of oral microbial diversity (**Figure 24.7**). The oral microbiome is essentially as diverse as that of the gut, but human populations share greater proportions of common taxa for the mouth than for the gut. Almost all of the microorganisms inhabiting the healthy oral cavity can be assigned to seven bacterial phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Saccharibacteria*, and *Spirochaetes*.

At least 750 species of aerobic and anaerobic microbes, including a minor representation of methanogenic *Archaea* and yeast, reside in the human oral cavity, distributed among teeth, tissue surfaces, and saliva. Most of these microorganisms have facultatively aerobic metabolisms, but some, such as *Bacteroidetes* and *Spirochaetes*, are obligately anaerobic. A few have strictly aerobic metabolisms, such as species of *Neisseria*, *Campylobacter*, and *Haemophilus* (a member of the *Pasteurellaceae*) in the *Proteobacteria* phylum. The most abundant genera are *Firmicutes*, major degraders of polysaccharides derived from the host or diet. Among *Firmicutes*, *Streptococcus* (~20%) and the obligately anaerobic *Veillonella* (~9%) are most abundant, although their relative numbers vary greatly as a function of oral cleanliness. Acid-producing, carbohydrate-fermenting genera physiologically and morphologically similar to the streptococci are also common *Firmicutes*, including species of *Abiotrophia* (*Aerococcaceae*), *Gemella* (*Gemellaceae*), and *Granulicatella* (*Carnobacteriaceae*).

Nanosynbacter lyticus is the first described species of the phylum *Saccharibacteria* (Figure 24.7) and is unique among oral bacteria (**Figure 24.8**). *N. lyticus* is a member of the ultrasmall (200–300 nm) bacterial radiation first observed in the terrestrial subsurface (◀ Section 20.8 and Explore the Microbial World, "Tiny Cells" in Chapter 1). This

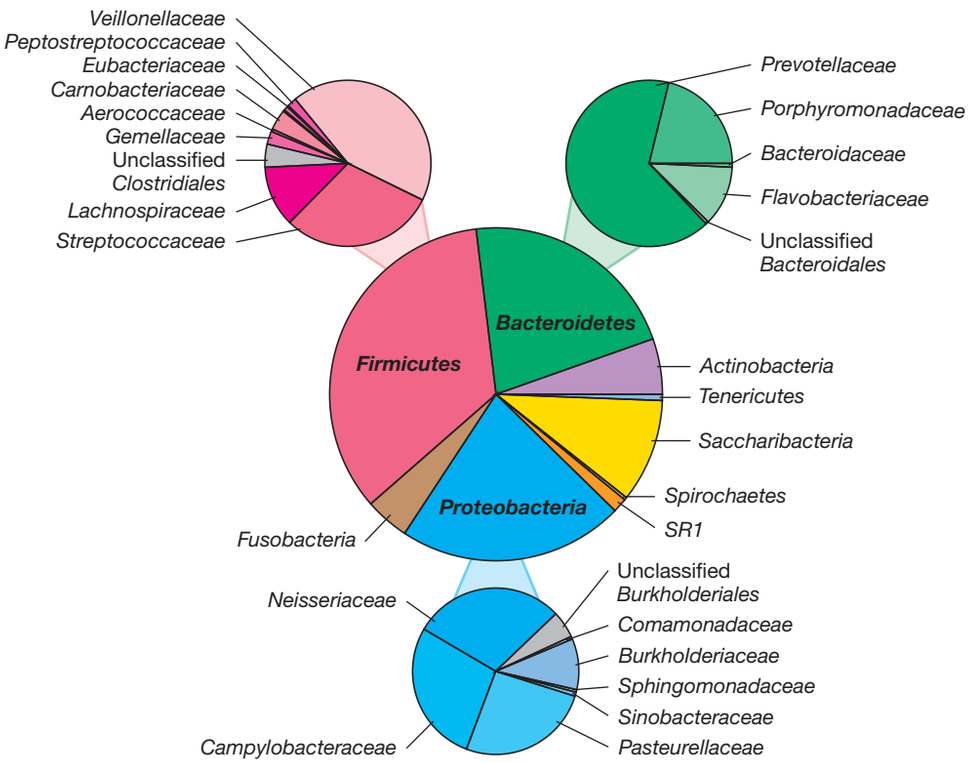
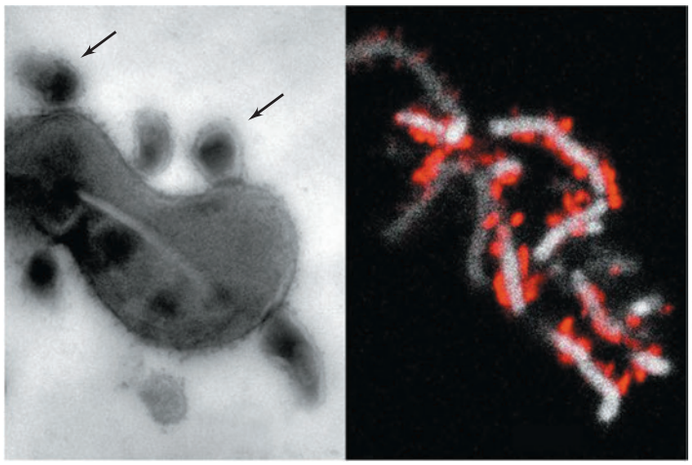


Figure 24.7 Bacterial diversity of saliva. The results are pooled analyses of approximately 750,000 sequences (from 159 samples) of the V1–V3 region of the 16S rRNA gene (◀ Figure 13.24). Note the lower fraction of anaerobes affiliated with *Fusobacteria* and *Spirochaetes* relative to the distribution of taxa observed in subgingival plaque (Figure 24.11). Many of these groups are discussed in Chapters 15 and 16. Data assembled and analyzed by Nícolas Pinel.

Mastering Microbiology
Art Activity: Figure 24.8 Section through a tooth



Ryan Hunter, Batbileg Bor, Xuesong He, and Jeffrey McLean

Figure 24.8 ***Nanosynbacter lyticus.*** The epiparasite *N. lyticus* is a species of the phylum *Saccharibacteria*. The tight physical association between *N. lyticus* cells (arrows) and its host *Actinomyces odontolyticus* is shown in *(a)* a transmission electron micrograph of a thin section, and *(b)* fluorescence in situ hybridization (FISH, ◀ Section 19.5) imaging of intact cells (red *N. lyticus* cells attached to white *Actinomyces* filaments). An *N. lyticus* cell is about 250 nm in diameter.

diminutive bacterium has a highly reduced genome and lacks the ability to synthesize any amino acid, vitamin, or cell wall precursor. It exists as an obligate epiparasite, drawing nutrients from other oral bacteria by physical attaching to them. Lactate, a major metabolic product of oral streptococci, seems to be an important carbon and energy source for *N. lyticus*, as assessed from genomic analysis of cultured *N. lyticus* cells grown with oral actinobacteria as host (Figure 24.8). A similar symbiotic relationship occurs in the *Archaea* where the tiny parasitic and hyperthermophilic *Nanoarchaeum equitans* grows attached to the surface of cells of *Ignicoccus* (◀ Section 17.6 and Figure 17.17).

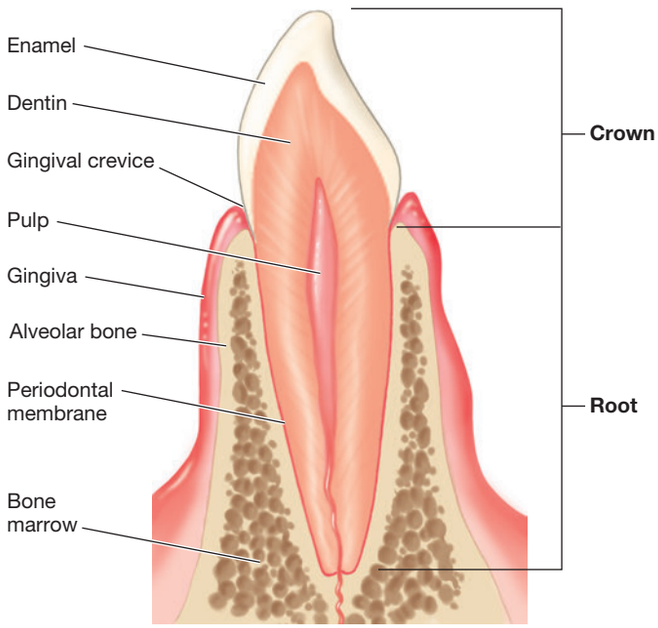
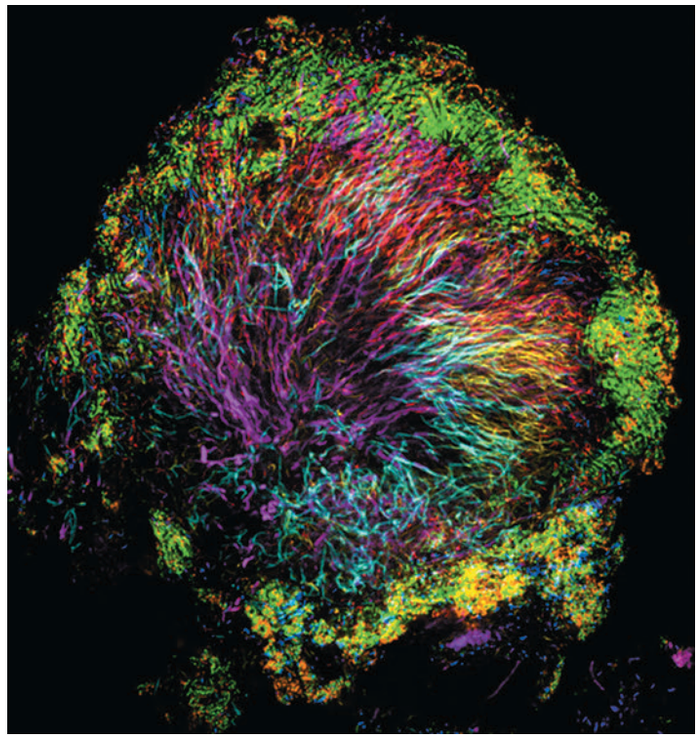


Figure 24.9 **Section through a tooth.** The diagram shows the tooth architecture and the surrounding tissues that anchor the tooth in the gum.

Oral Microenvironments and Their Microbiota

Bacteria found in the mouth during the first year of human life (when teeth are absent) are predominantly aerotolerant anaerobes such as streptococci and lactobacilli, and a few aerobes. When the teeth appear, the newly created surfaces are rapidly colonized by aerotolerant and obligate anaerobes that are specifically adapted to growth in biofilms on the surfaces of the teeth and in the gingival crevices (**Figure 24.9**). The formation of biofilms on teeth (*dental plaque*) is an ordered process of microbial assembly, where order is determined by highly specific binding between cell surface adhesins of one species and specific receptors on another species. *Streptococcus* and *Actinomyces* are the pioneer colonizers of a freshly cleaned tooth surface, followed by successive adherence by species of *Veillonella*, *Prevotella*, *Fusobacterium*, and other genera. The highly ordered structure can be revealed using fluorescence microscopy to visualize the layers of different genera in plaque (**Figure 24.10**). Close physical proximity may serve the function of metabolic cooperation. For example, *Veillonella* and *Streptococcus* establish a close physical and



Jessica Mark-Welch and Gary Borisy

Figure 24.10 **Structured microbial community of dental plaque.** Organization of different bacterial genera in a thin section through human dental plaque visualized by fluorescence in situ hybridization (FISH, ◀ Section 19.5). The micrograph shows streptococci (stained green) located primarily at the periphery of the plaque beyond several other genera that combine to form a scaffold emerging from the tooth surface. These include early-colonizing *Corynebacterium* spp. (purple) near the tooth surface, covered by later-colonizing fusobacteria (*Fusobacterium*, yellow; *Leptotrichia*, blue-green), and capped by species of *Capnocytophaga* (red), *Streptococcus* (green), and *Haemophilus* (orange) in this highly organized assembly.

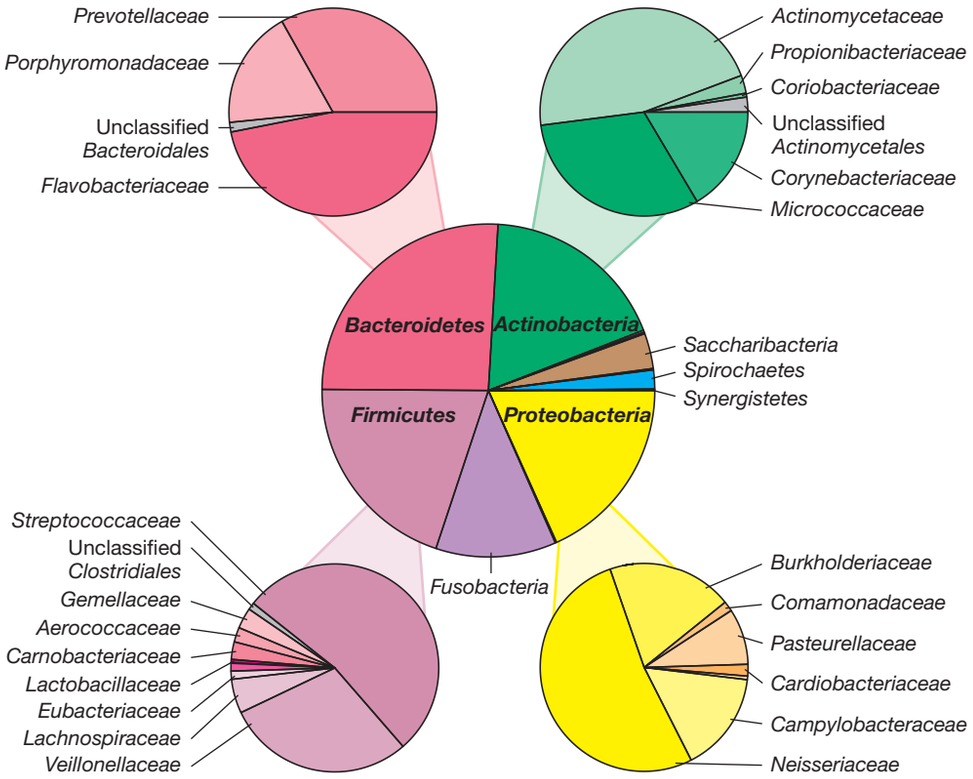


Figure 24.11 Bacterial diversity of subgingival plaque. The results are pooled analyses of approximately one million sequences (from 183 samples) of the V1–V3 region of the 16S rRNA gene (◀ Figure 13.24). Compare the fractional distribution of different bacterial taxa with that observed in saliva (Figure 24.7), noting the higher representation of anaerobic fusobacteria and spirochete populations in the oxygen-limited gingival crevice. Many of these groups are discussed in Chapters 15 and 16. Data assembled and analyzed by Nícolas Pinel.

metabolic association in which the *Veillonella* consumes the lactate produced by streptococcal fermentation of carbohydrates.

Most of these bacteria contribute to the health of the host by keeping pathogenic bacteria in check and preventing them from gaining access to mucosal surfaces. Tooth decay, gum inflammation, and periodontal disease are among the most visible manifestations of a breakdown in these generally stable mutualisms. We discuss the microorganisms associated with the hard tooth surface and their contribution to the formation of dental plaque and dental caries in Section 25.2.

There is significant site variation in the diversity and specificity of colonization by different oral bacterial species. For example, there are differences in abundant microbial taxa associated with subgingival plaque (plaque that forms under the gums) (**Figure 24.11**) compared with that found in saliva (Figure 24.7). Compare Figure 24.7 and Figure 24.11 and note the greater representation by *Actinobacteria* and *Spirochaetes* in the subgingival plaque. Different species of *Corynebacterium* (a genus of the *Actinobacteria*, ◀ Section 16.10) demonstrate significant site specificity. For instance, *Corynebacterium matruchotii* is almost exclusively found in the supragingival plaque, whereas *Corynebacterium argentoratense* occurs mostly in the saliva. *Lautropia mirabilis* (*Proteobacteria*) selectively colonizes the supragingival plaque, whereas the spirochete *Treponema socranskii* is found mostly in subgingival plaque, presumably because this site provides the low-oxygen environment needed for microaerobic growth of this bacterium. The distribution of *Firmicutes, Proteobacteria*, and *Bacteroidetes* is similar between the oropharynx (see Figure 24.12) and saliva. The hard palate (roof of the mouth) harbors a much lower diversity of microbiota than does the gingival plaque, possibly as a result of constant shedding of epithelial cells and the shear forces associated with chewing and swallowing.

Microenvironments of the Respiratory Tract

The anatomy of the respiratory tract is shown in **Figure 24.12** and Figure 24.1. In the **upper respiratory tract** (including the sinuses, nasal and oral cavities, and throat—pharynx, tonsils, and larynx), microbes live bathed in secretions from the mucous membranes. Bacteria continually enter the upper respiratory tract from the air during breathing, but most are trapped in the mucus of the nasal and oral passages and expelled with nasal secretions or swallowed and then killed by the acid in the stomach. However, a few microbes colonize respiratory mucosal surfaces in all individuals; those most commonly present are species of staphylococci, streptococci, diphtheroid bacilli, and gram-negative cocci.

Mastering Microbiology
Art Activity: Figure 24.10 The respiratory tract

Occasionally, potential pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* are part of the normal microbiota in

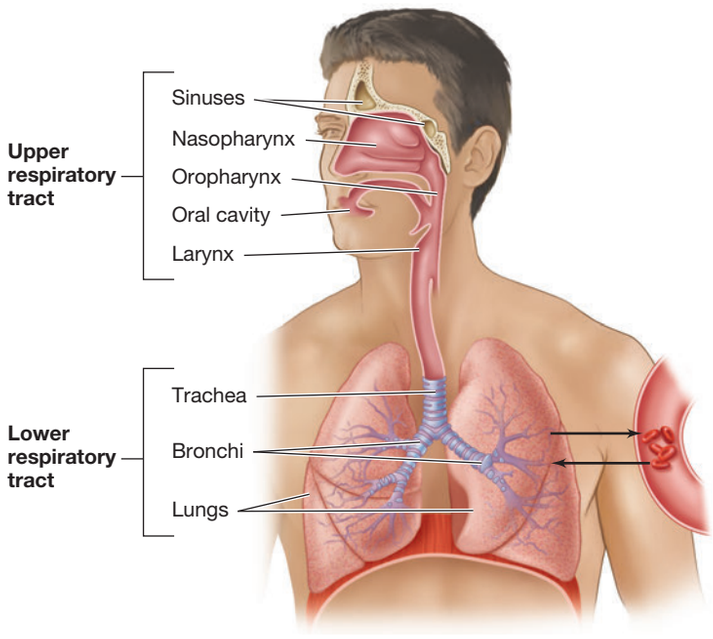


Figure 24.12 The respiratory tract. In healthy individuals the upper respiratory tract has a large variety and number of microorganisms. By contrast, the lower respiratory tract in a healthy person has few microorganisms.

the nasopharynx of healthy individuals. These individuals are *carriers* of the pathogens but do not normally develop disease, presumably because the other resident microorganisms compete successfully for nutritional and metabolic resources and limit pathogen attachment, colonization, or activities. The innate immune system (Chapter 26) and components of the adaptive immune system such as secreted antibodies (Chapter 27) are particularly active at mucosal surfaces and inhibit growth and invasion by potential pathogens.

The **lower respiratory tract** (trachea, bronchi, and lungs, Figure 24.12) has no naturally resident microbiota in healthy adults, but does harbor very low numbers of bacteria (primarily *Prevotella*, *Veillonella*, and *Streptococcus*) that are introduced from the upper respiratory tract during normal breathing. Dust particles, which are fairly large, settle in the upper respiratory tract. As the air passes into the lower respiratory tract, the flow rate decreases, and organisms settle onto the walls of the respiratory passages. The walls of the entire respiratory tract are lined with ciliated epithelial cells, and the cilia, beating upward, push bacteria and other particulate matter toward the upper respiratory tract where they are then expelled in saliva and nasal secretions or are swallowed. Only particles smaller than about 10 μm in diameter reach the lungs. Nevertheless, these include some pathogenic microbes, most notably certain bacteria and viruses that cause pneumonia (inflammation of the lungs, ▶ Sections 31.1, 31.2, and 31.8).

We now leave the oral cavity and explore the microbial diversity of other potentially heavily colonized body sites, the urogenital system and the surface of the skin.

Check Your Understanding

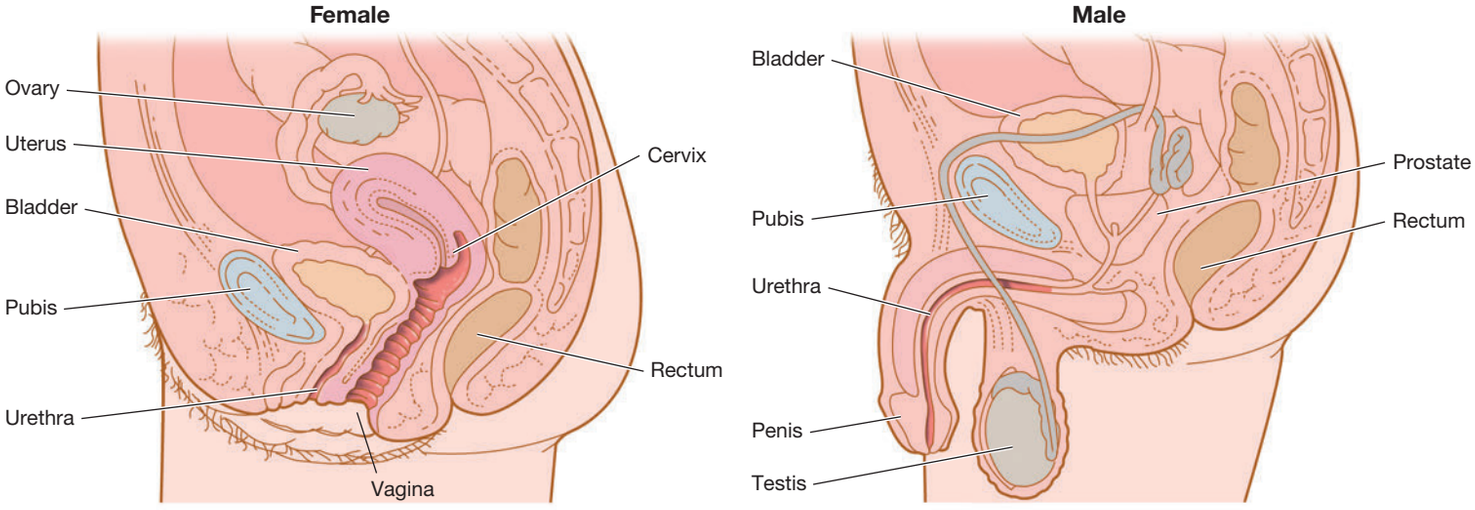
- Compare the microbial microenvironments in the oral cavity in newborns and adults.
- Identify the major microbes that predominate in the adult oral cavity by taxa and metabolic requirements.
- Why is the lower respiratory tract typically microbe-free?

II • Urogenital Tract and Skin Microbiomes and the Human Viral Microbiome

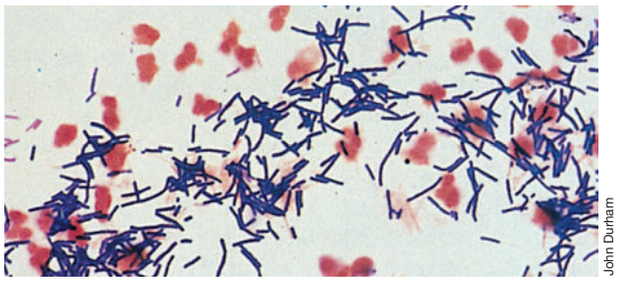
A resilient lactobacilli community colonizes the healthy vagina and maintains a weakly acidic environment. Different oily, moist, and dry microenvironments of healthy skin select for different distributions of primarily gram-positive bacteria. The human viral microbiome (virome) consists of both animal viruses and bacteriophages.

24.4 Urogenital Tracts and Their Microbes

In the urogenital tracts of healthy adults (Figure 24.13), the kidneys and bladder are believed to be sterile; however, facultatively aerobic gram-negative bacteria colonize epithelial cells lining the distal



(a)



(b)

Figure 24.13 Microbial growth in the urogenital tracts. *(a)* The urogenital tracts of the human female and male, showing regions (red) where microorganisms often grow. The upper regions of the urogenital tracts of both males and females are sterile in healthy individuals. *(b)* Gram stain of *Lactobacillus acidophilus*, the predominant organism in the vagina of women between the onset of puberty and the end of menopause. The individual gram-positive rods are 3–4 μm long. Species of *Lactobacillus* are anaerobic bacteria that ferment glucose and other sugars primarily to lactic acid as a fermentation product (◀ Section 16.6).

urethra, and the bladder of even healthy individuals may harbor a resident microbial community similar in composition to that of the urethra. Potential pathogens such as *Escherichia coli* and *Proteus mirabilis*, normally present in small numbers in the body or in the local environment, can multiply in the urethra and cause disease if conditions such as changes in pH occur. Such organisms are a frequent cause of urinary tract infections, especially in females. *Proteus* is especially notorious as a urinary tract pathogen. This bacterium is a strong urease producer; it generates ammonia from urea and uses the ammonia as a nitrogen source. Ammonia also causes urine pH to become quite alkaline, and this can trigger other urinary tract conditions such as the formation of kidney stones.

The vagina of the adult female is weakly acidic (pH ~5) and contains significant amounts of glycogen. *Lactobacillus acidophilus*, a resident organism in the vagina, ferments the polysaccharide glycogen, producing lactic acid that maintains a local acidic environment (Figure 24.13*b*). Other organisms, such as species of the yeasts *Torulopsis* and *Candida*, various streptococci, and *E. coli*, may also be present. Before puberty, *L. acidophilus* is absent, the female vagina is neutral in pH and does not produce glycogen, and the microbiota consists predominantly of staphylococci, streptococci, corynebacteria, and *E. coli*. After menopause, glycogen production ceases, the pH rises, and the microbiota again resembles that found before puberty.

Metagenomic 16S rRNA sequence analyses have confirmed earlier culture-based observations that the vaginal microbial community is less complex at the genus level than the oral or gut communities. A healthy vaginal microenvironment is dominated by lactobacilli (Figures 24.1 and 24.13*b*) and *vaginosis* (major changes in the balance of microbes in the vagina) is characterized by increased bacterial diversity, elevated pH, and a vaginal discharge. But even in the healthy adult female, vaginal microbiota consists of over 100 bacterial genera that group into several primarily gram-positive bacterial families (**Figure 24.14**).

There are at least five types of "normal vaginal communities" containing different compositions of *Lactobacillus* spp., the dominant gram-positive organisms in the vagina. Four types are defined by dominance by one of *L. crispatus, L. iners, L. reuteri,* or *L. jensenii* (see also Figure 24.27), while the fifth is a more heterogeneous type characterized by higher overall diversity and a greater proportion of other strict anaerobes relative to the lactobacilli. Although all vaginal community types are associated with an acidic pH, pH varies with community type. The *L. crispatus* type shows the lowest average pH (~4.0), whereas the heterogeneous type shows the highest average pH (~5.3).

Thus, unlike the picture of microbial diversity we have seen in the gut or the oral cavity, the microbiota in the vagina of the healthy female is overwhelmingly dominated by lactobacilli (Figure 24.14). In contrast to the vagina, studies of the penis microbiota are fewer, but the general picture shows that the bacterial diversity of the penis is similar to that in the vagina, the patterns being especially so in sexual partners. Species of *Lactobacillus, Streptococcus*, and *Sneathia* are commonly found in both the male and female urethra. However, the microbiota on the circumcised versus the uncircumcised penis can be quite different, and bacterial abundance on the uncircumcised penis is typically much greater as well.

Check Your Understanding

- What is the importance of vaginal *Lactobacillus* in healthy adult women?
- What variable feature of the vagina is most closely associated with different community compositions of *Lactobacillus* species?

24.5 The Skin and Its Microbes

The skin is a complex human organ functioning primarily to prevent loss of moisture and restrict the entry of pathogens. An average adult human has about two square meters (2 m^2) of skin surface that varies greatly in chemical composition and moisture content. Skin also provides an environment for part of the human microbiome. The skin microbiota consists of a rich community of microorganisms that associates intimately with the host's hormonal, nervous, and immunological systems.

There are approximately 1 million resident bacteria per square centimeter of skin, for a total of about 10^{10} skin microorganisms covering the average adult. Although these numbers are much lower than the oral and gut communities, molecular analyses have shown that the skin harbors a diverse microbial community of bacteria and fungi (primarily yeast) that varies significantly with location on the body as a function of the diversity of habitats. These habitats consist of microenvironments of varying temperature, pH, moisture, sebum content (sebum is the oily secretions of the sebaceous glands), and surface characteristics. One distinct set of microenvironments includes moist skin areas such as the inside of the nostril, the armpit, and

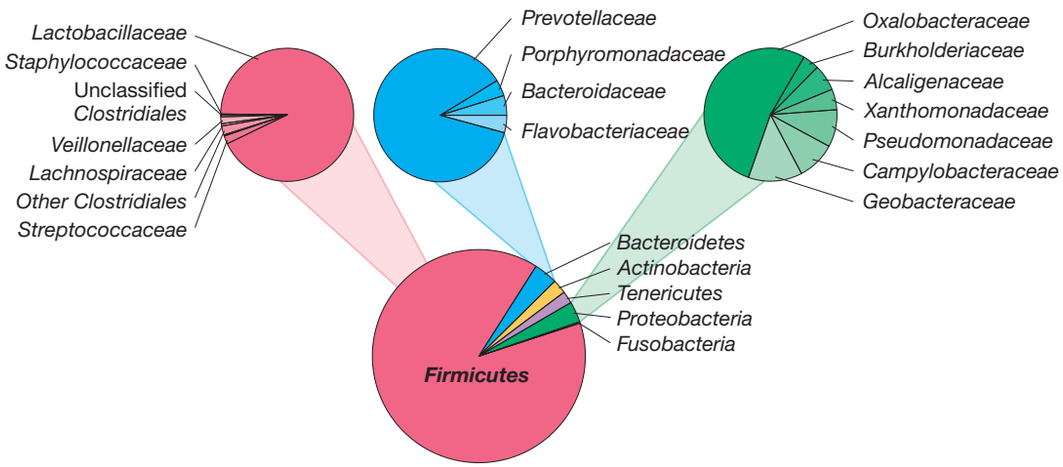


Figure 24.14 Vaginal bacterial diversity. The results are pooled analyses of approximately 600,000 sequences (from 86 samples) of the V1–V3 region of the 16S rRNA gene (◀ Figure 13.24). Note the dominant fractional representation by *Lactobacillus* species and related *Firmicutes*, as compared to the diversity found at other body sites. Many of these groups are covered in Chapters 15 and 16. Data assembled and analyzed by Nícolas Pinel.

Mastering Microbiology
Art Activity: Figure 24.13*a* Normal skin microbiota

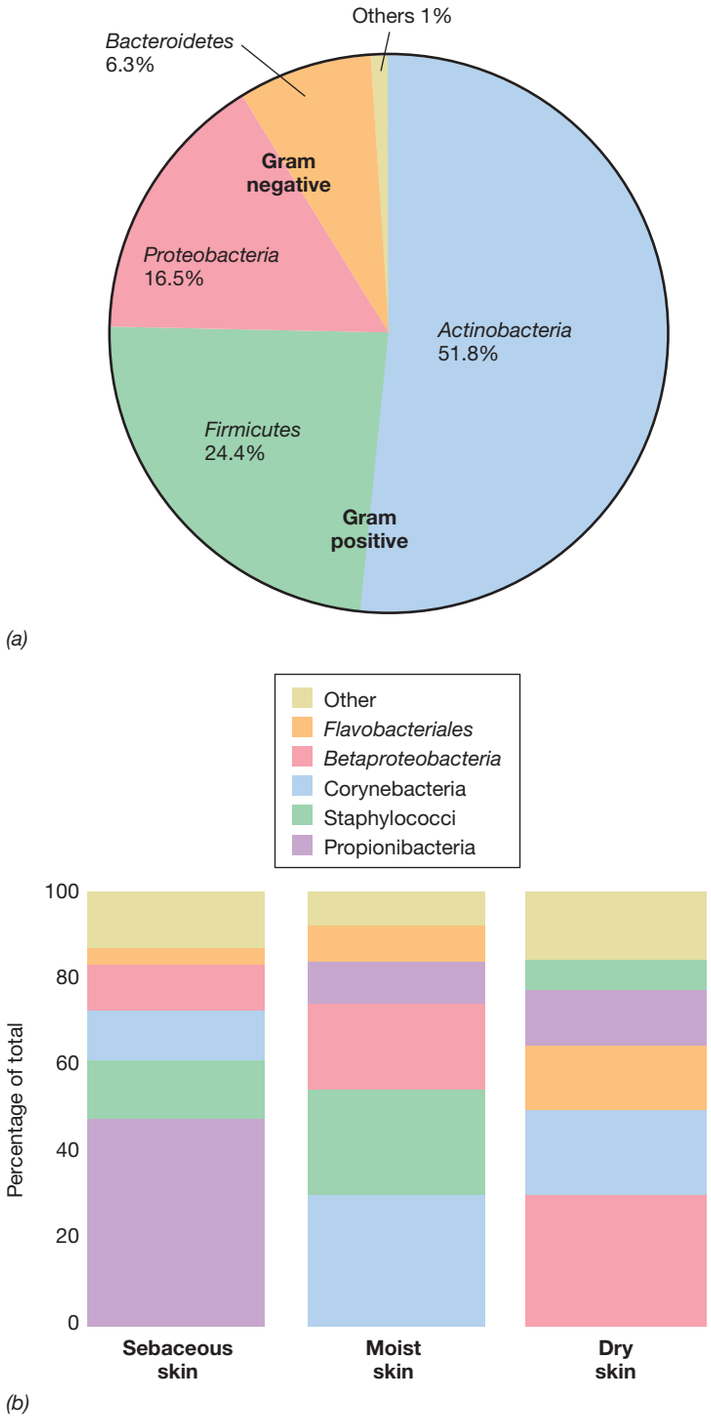


Figure 24.15 Overview of the skin microbiota. *(a)* Analysis of the skin microbiome from 10 healthy human volunteers detected 19 bacterial phyla. Four phyla were predominant. *(b)* Composite populations of *Bacteria* from the same volunteers, divided according to sebaceous, moist, and dry skin microenvironments. Data adapted from Grice, E.A., et al. 2009. *Science 324*: 1190.

the umbilicus. Moist skin is separated by only a few centimeters from dry microenvironments such as the forearms and the palms of the hands. A third microenvironment consists of areas with high concentrations of sebaceous glands such as those by the side of the nose, the back of the scalp, and the upper chest and back. In addition to these site-specific differences, sweat is high in salt and other antimicrobial substances such as free fatty acids and antimicrobial peptides and thus plays a role in controlling diversity.

Microbial Diversity of Skin Microenvironments

A 16S rRNA sequencing comparison of 20 diverse skin sites categorized as moist, dry, or oily revealed tremendous diversity and variation among sites and individuals and also showed some common patterns (**Figure 24.15**). Collectively, nearly 20 bacterial phyla were detected, but most phylotypes affiliated with one of four groups: *Actinobacteria* (~52%), *Firmicutes* (~24%), *Proteobacteria* (~16%), and *Bacteroidetes* (~6%). Over 200 different genera were identified, but species of three genera, *Corynebacterium* and *Propionibacterium* (both *Actinobacteria*) and *Staphylococcus* (*Firmicutes*) typically dominated the observed phylotypes (Figure 24.15*b*).

Each skin microenvironment showed a unique microbiota. Moist sites are dominated by corynebacteria and staphylococci, while drier sites support a mixed population dominated by *Betaproteobacteria*, corynebacteria, and *Flavobacteriales*. Species of *Propionibacterium* predominate in sebaceous areas (Figure 24.15*b*). For example, colonization of the follicular sebaceous gland system by *Propionibacterium acnes* is promoted by its ability to hydrolyze triglycerides present in sebum, resulting in release of free fatty acids that promote adherence of this bacterium, which sometimes causes disease (acne, Section 24.10).

A higher-resolution molecular diversity study of 400 different body sites of one male and one female subject revealed 850 distinct species, as bacterial species are currently defined (◀ Section 13.12). The results of a high-resolution analysis of a single dry skin site (the inside of the elbow) are shown in **Figure 24.16**. As shown by the more general studies (Figure 24.15*a*), the most common microbial phyla were *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, but in this work, the breakdown of each phylum into family-level taxa shows a significant hidden diversity within each major group. Although staphylococci, propionibacteria, and *Betaproteobacteria* dominate, many other groups are also present (Figure 24.16).

The abundance of specific bacterial taxa can also be visualized in a "heat map" diagram (◀ Section 10.10 and Figure 10.28) showing the major locations of different taxa on the skin. An example is shown in **Figure 24.17** where it can be seen that *Propionibacterium* tends to localize on sebaceous regions (head, face, upper back, and upper chest), whereas species of *Staphylococcus* and *Corynebacterium* are more prevalent on less exposed regions, such as the groin, armpit, and toe web—areas higher in temperature and moisture content (Figure 24.17).

Other Aspects of the Skin Microbiome

Eukaryotic microbes and *Archaea* are also present on the skin. The yeast *Malassezia* is the most common fungus found on the skin, and at least five different species of this yeast are typically present on the skin of healthy individuals. In an individual with a weakened immune system, for example, someone who has HIV/AIDS or whose normal microbiota has been compromised, *Candida* and other potentially pathogenic fungi can also colonize the skin and cause serious (even fatal) infections (fungal pathogens are discussed in Chapter 34). A remarkable finding emerging from 16S rRNA gene surveys of skin is that ammonia-oxidizing *Archaea* (◀ Section 17.5)

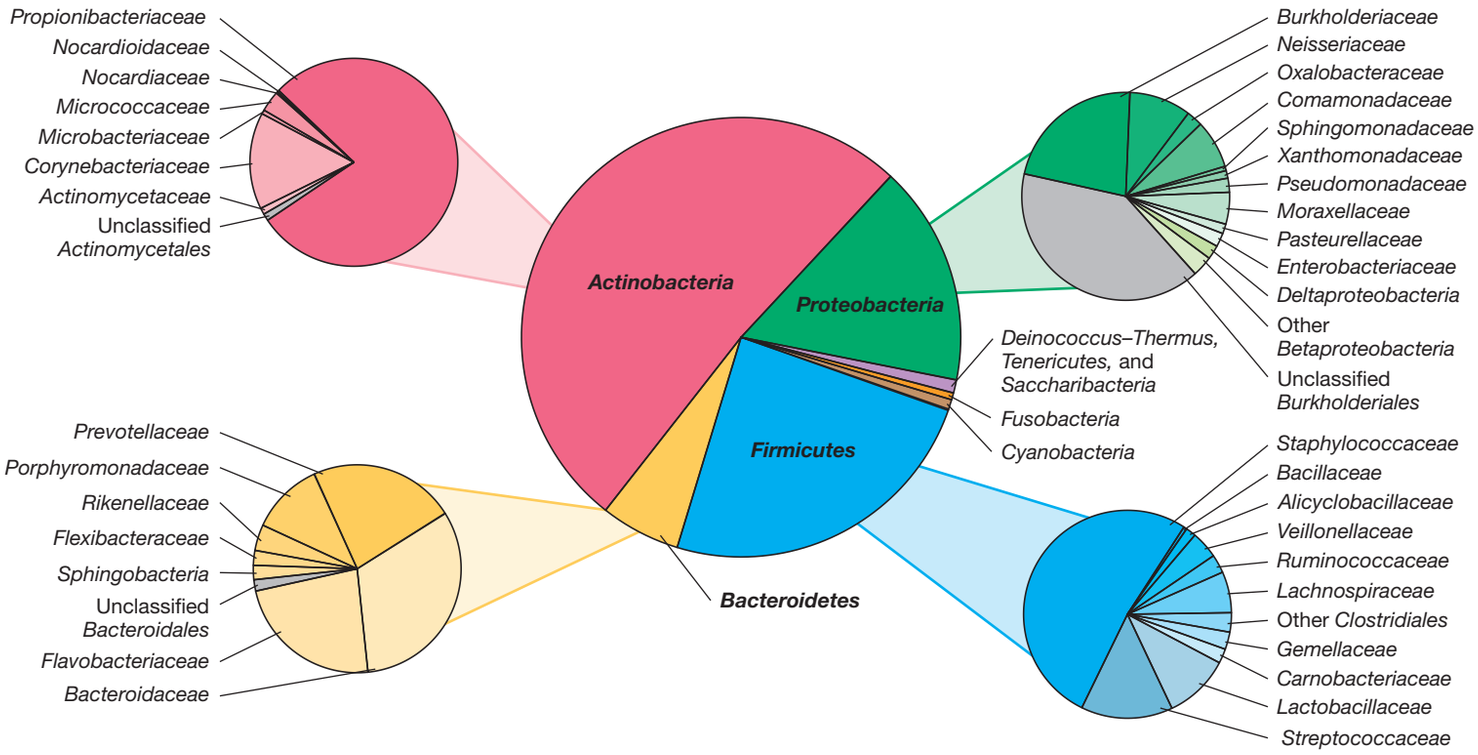


Figure 24.16 Skin bacterial diversity at the inside of the elbow. Note that *Propionibacterium* species comprise the vast majority of the *Actinobacteria*, and that *Staphyloccocus* species dominate skin-associated *Firmicutes*. The results are pooled analyses of approximately 80,000 sequences (from 123 samples) of the V1–V3 region of the 16S rRNA gene (◀ Figure 13.24). Many of these groups are discussed in Chapters 15 and 16. Data assembled and analyzed by Nícolas Pinel.

can comprise as much as 4% of the skin microbiota in some individuals, presumably sustained by ammonia present in the sweat of more physically active individuals.

Environmental and host factors influence the composition of the normal skin microbiota. For example, the *weather* may cause an increase in skin temperature and moisture, which increases the abundance of the skin microbiota. The *age* of the host also has an effect; young children have a more varied skin microbiota and carry more potentially pathogenic gram-negative *Bacteria* than do adults. *Personal hygiene* also greatly influences the resident skin microbiota; individuals with poor hygiene typically have higher microbial population densities on their skin. And finally, many microorganisms that would otherwise colonize skin cannot survive there simply because of its low moisture content and presence of antimicrobial fatty acids. Thus, the skin is a natural barrier to microbial colonization (▶ Figure 26.2) while at the same time supporting a diverse array of normal microbiota.

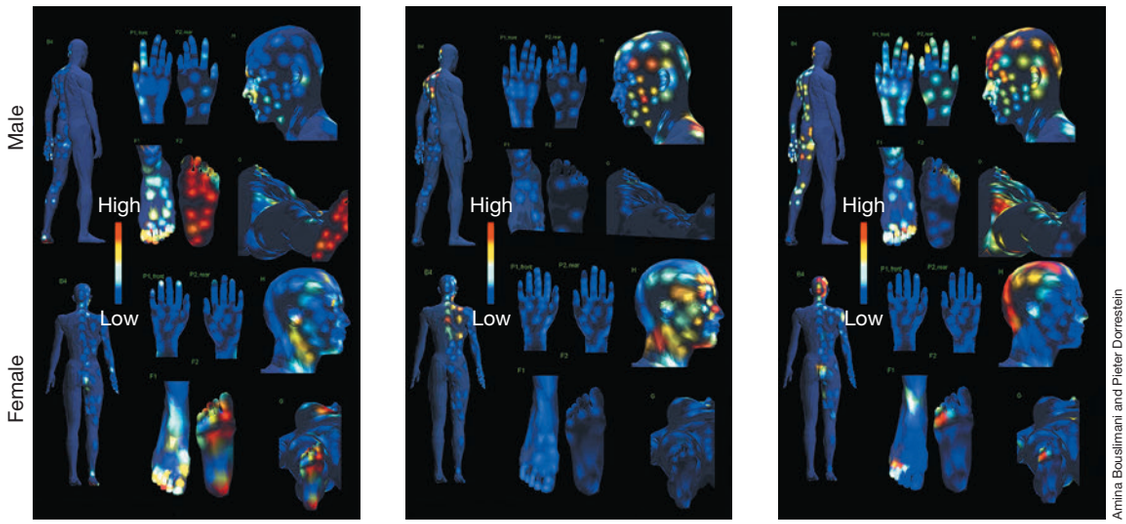
As yet, there have been no significant longitudinal studies of early colonization and succession of the skin microbiome as have been conducted for the gut microbiome (Section 24.8). However, it is well recognized that the skin microbiome undergoes a major transition associated with sexual maturation. The skin of young children is dominated by *Streptococcus* spp., *Betaproteobacteria*, and *Gammaproteobacteria*. By contrast, these taxa are largely absent from the skin of postadolescent young adults; in this group the skin is characteristically dominated by species of *Propionibacterium* and *Corynebacterium*, as we have seen (Figures 24.15 and 24.17). And finally, encounters with man's best friend can contribute to the human skin microbiota. Studies have shown that adult dog owners have more skin microbes in common with their own dogs than with other dogs. This demonstrates that close and regular contact between two distinctly different species of animal can result in a major sharing of their microbiomes. In contrast to the skin microbiota, microbes in the mouths and guts of canines differ quite distinctly from those of their owners.

Check Your Understanding

- Compare the populations of microorganisms in the three major skin microenvironments.
- Describe the properties of microorganisms that grow well on the skin.

24.6 The Human Virome

In Chapter 5 we highlighted the infection process of animal viruses, and we described the general morphology and genomic structure of common animal viruses in Chapter 11 (◀ Figures 11.2 and 11.3). Animal viruses differ from bacteriophages and archaeal viruses in that an animal cell takes up the *entire virion* instead of just the viral genome. There are also more than two lifestyles for animal viruses, including virulent infection, latent infection, persistent infection, and cellular transformation (◀ Figure 5.22). A healthy human is teeming with viruses—not just animal viruses but also bacteriophages and possibly even some plant viruses—and we examine this suite of viruses now.



(a) ***Staphylococcus*** (b) ***Propionibacterium*** (c) ***Corynebacterium***

Figure 24.17 Distributions of *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* on the human skin. Heat maps of microbial species distributions at 400 different body sites inferred from 16S rRNA gene sequences from the skin of male (top) and female (bottom) subjects (in scale bar, red indicates higher relative abundance and blue lower abundance). *Propionibacterium* species tend to localize on sebaceous regions (head, face, upper back, and upper chest); *Corynebacterium* species are most common on the head, groin, and toes; and *Staphylococcus* species are most abundant on the foot.

The Human Body and the Virome

While profiling the viruses of the human body has been hampered by limitations in cell culturing, the power of metagenomics has not only allowed for the human *microbiome* to be characterized (Section 24.1), but also the human **virome**. The human virome encompasses the entire population of viruses present in and on the human body (**Figure 24.18**). And, as for the human microbiome, the human virome is both unique to an individual and relatively stable over long periods.

Depending on the individual, animal viruses of the human virome include those that cause severe diseases such as hepatitis and severe acute respiratory syndrome, those that cause milder acute infections such as influenza and viruses of the common cold, and those that are completely benign. Other common animal viruses of the human virome cause latent infections, such as the herpesviruses, in particular human cytomegalovirus, present in most human adults (the biology of these viruses is covered in Chapter 11 and the disease syndromes themselves in Chapters 31–33).

Viruses that contain DNA genomes dominate the viromes of healthy humans. Areas of the human body in which the virome has been assessed are the nose, skin, mouth, and gastrointestinal tract (from fecal samples). Animal viruses commonly detected in these areas include the single-stranded DNA anelloviruses and circoviruses and the double-stranded DNA adenoviruses, polyomaviruses, and papillomaviruses (Figure 24.18*a*). Anelloviruses are nonenveloped viruses that establish persistent infections in the body early in life with no known connection to disease. Circoviruses possess some of the smallest of all viral genomes and are commonly found in poultry and pigs, suggesting that their presence in the human gastrointestinal tract may originate from these food sources.

Adenoviruses are common respiratory viruses that have been detected in children with fevers but are also found in the nose and upper respiratory tract of healthy humans. Similarly, polyomaviruses are commonly found in healthy individuals, but in immunocompromised individuals they can also cause urinary tract infections and a type of brain disorder called *leukoencephalopathy*. Several different papillomaviruses are also common in the human virome, especially in the skin and saliva. *Papillomaviruses* are nonenveloped double-stranded DNA viruses (Baltimore class I, ◀ Figure 11.2) that replicate on skin and in mucosal epithelia of virtually all mammals. Most papillomavirus infections are asymptomatic; however, the human papillomavirus (HPV) causes persistent infections that can develop into skin warts, and certain strains of HPV can cause premalignant lesions in the female reproductive tract that can lead to cervical cancer later in life. Nearly all cervical cancers are attributed to HPV, and this virus also causes many cancers of the vagina, vulva, penis, anus, and oropharynx.

Besides these common animal viruses, most human viromes also contain viruses that infect plants, such as the pepper mild mottle virus (Figure 24.18*a*). These viruses are undoubtedly transmitted to humans from foods and pass through the intestines. While the host specificity of these viruses is for plants, it is thought that their presence in humans may trigger inflammation, which may lead to some of the symptoms susceptible individuals have to spicy foods or certain plant products. Another possible interaction between the human virome and the immune system is the prevalence of *human endogenous retrovirus* (HERV) elements in human chromosomes; HERVs are remnants of retroviral genes that have been incorporated into and constitute 5–8% of the human genome. Although most HERVs are thought to be harmless, connections have been proposed between certain HERVs and the autoimmune disorders rheumatoid arthritis and systemic lupus erythematosus (▶ Section 28.1), and with other afflictions such as inflammatory bowel (Crohn's) disease and multiple sclerosis.

Bacteriophages and the Human Virome

While every human body contains a unique mixture of different animal viruses, the most abundant viruses in all body sites are not animal viruses but instead are bacteriophages. The large intestine is a hotbed for such viruses, as this organ contains an abundance of prokaryotic cells and virions (numbering about 10^{11} and 10^{9}, respectively, per gram of feces). However, unlike the marine virosphere (◀ Section 20.13), for example, in which bacteriophage virions greatly outnumber bacteria, the virion to microbe ratio (VMR)

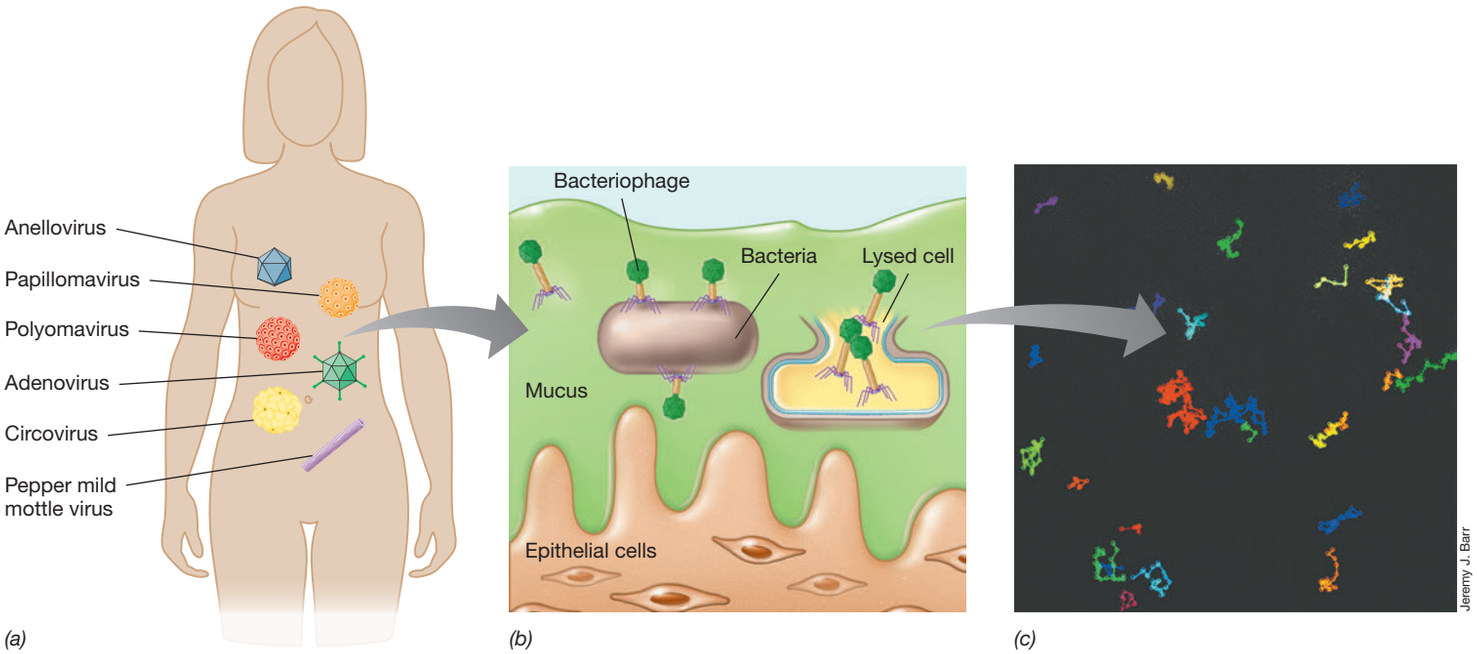


Figure 24.18 The human virome. *(a)* Common viruses of eukaryotes found in the virome of a healthy human. *(b)* Bacteriophages within the mucosa of the human respiratory and gastrointestinal tracts protect epithelial cells from the invasion of bacterial pathogens. *(c)* Movement of T4 bacteriophages in mucus. Tracks represent movement of fluorescently labeled individual bacteriophage virions within a microfluidic chip containing 1% mucin to simulate the mucus environment shown in part *b*.

in the gut is much lower and could be as low as one virus for every 100 bacteria. As with the animal viruses of the human virome, DNA bacteriophages dominate, and many of these viruses are thought to benefit bacterial cells by transferring genes encoding antibiotic resistance or enzymes for specialized metabolisms through the genetic transfer processes of transduction (◀ Section 9.7) and lysogeny (◀ Section 5.6).

A unique feature of the gut virome is that most bacteriophages are in a lysogenic state, reproducing in concert with their host bacterium and therefore persisting for prolonged periods. Observations of bacteriophage diversity within an individual have shown it to be remarkably stable, with 80–95% of bacteriophages retained for over 2 years, and likely for much longer. Persistence and stability likely derive from the stability of the bacterial community (see Section 24.8) and a preference for the bacteriophage to enter into a stable lysogenic state. Thus, although genetic transfers from gut bacteriophages to their hosts likely help the gut microbiota adapt to changing nutrient conditions and stabilize it from the stresses of antibiotic treatment, the mechanisms and frequency of exchange may be quite different in the gut than in open environmental systems.

Metagenomic surveys of DNA recovered from virions separated from feces have shown that between 75% and 99% of bacteriophages in the gut microbiome are not closely related to known viral genomes. Thus, matching bacteriophage genomes with the bacterial hosts they infect is a daunting challenge. Even so, important progress has been made in identifying novel bacteriophages by matching CRISPR sequences (◀ Section 9.12) between phage and bacterial genomes to search for links in a virus–host association. Using this strategy, the isolation of a major group of novel tailed bacteriophages (called *crAssphage*) present in at least half of the human population was achieved by recovering the bacteriophage on cultures of the *Bacteroides* species predicted by metagenomic sequencing to serve as its host (see MicrobiologyNow on page 819).

Function of the Human Virome

Why are there so many bacteriophages in and on us? Probably because our bodies are home to such enormous levels of bacteria. To our benefit, phages likely play a protective role in human health. Bacteriophages of the human virome can be a first line of defense against certain pathogens, especially within mucosal surfaces where bacteriophages accumulate. It has been estimated that 20 times more bacteriophages than bacteria exist in the mucosa of our lungs and intestines. The bacteriophages present in mucous linings are anchored to sugar residues produced by mucosal cells (Figure 24.18*b*). Here bacteriophages presumably scavenge invading pathogens and kill them before they can cross the mucous barrier. Thus, phages within the mucus layer can be considered to have a symbiotic relationship with the human host and provide a form of host-independent immunity. Using fluorescently labeled T4 phages, movement of the phages through a model mucus layer can actually be tracked microscopically in the laboratory (Figure 24.18*c*).

Besides killing or conferring antibiotic resistance to bacteria within the microbiome, the bacteriophage component of the virome can also *enhance* the pathogenicity of certain bacteria. An example of this is the lysogenic bacteriophage CTXϕ and its host, the bacterium *Vibrio cholerae* (the bacterium that causes cholera). It is the phage genome rather than the host genome that actually encodes the cholera toxin that induces disease symptoms (▶ Section 33.3), and cells of *V. cholerae* that are not CTXϕ lysogens are nonpathogenic. This

phage–bacterium link is also seen in the *toxin-coregulated pilus*, a structure essential for *V. cholerae* cells to attach to intestinal cells. Genes encoding the pilus are part of a viral genome that is integrated into the host's genome (a prophage, ◀ Section 5.6) and is only present in pathogenic strains of *V. cholerae*.

With recognition of the potentially huge impact on human health by the virome coupled with the continually improving field of metagenomics, we can expect to have a much clearer understanding of the role bacteriophages play in health and disease in the near future. Indeed, a handful of therapeutic bacteriophage treatments have already been developed for humans and shown to be effective (see MicrobiologyNow at the beginning of Chapter 5 and the MicrobiologyNow on page 819).

We now turn our attention to how the human microbiome first develops and the importance to human health of early exposure to the microbial world.

Check Your Understanding

- How does the virome differ from the microbiome?
- What accounts for the remarkable stability of the gut virome?
- How do bacteriophages benefit and harm human hosts?

III • From Birth to Death: Development of the Human Microbiome

Different body sites of the newborn are rapidly colonized, receiving microbes from the mother and the local environment. A mature gut community takes about three years to develop and is greatly influenced by early life events. Each individual harbors a unique microbiome but one that can be altered by diet and aging.

At birth, a baby is exposed to both maternal and environmental microorganisms. These early encounters determine the composition of microorganisms that first colonize different body sites. In this part of the chapter we focus primarily on colonization of the gut, the body site harboring the largest part of the human microbiome, and consider factors that govern early colonization and subsequent successional events of a community now recognized to be critical to general health and the education of the immune system.

24.7 Human Study Groups and Animal Models

Establishing relationships between the composition of a person's microbiome and its contribution to health and disease has been complicated by problems surrounding sampling, the uncertainty about the contribution of the host's genetic background, and the limitations of controlling diet and other contributing lifestyle factors. Nevertheless, several human study groups and animal model experiments have revealed some of the basic principles and given microbiome studies a starting point.

Human Microbiome Study Groups

Most functional understanding of the human microbiome has been based on surveys of selected study groups (Table 24.1). For example, one of the most ambitious early studies was the Human Microbiome Project (HMP) funded by the U.S. National Institutes of Health. This study collected samples from 242 individuals (all American medical students in good health), sampling different body sites (15 to 18 sites depending on gender) at one to three time points, and then evaluated bacterial diversity based on 16S rRNA gene sequencing and limited metagenomic analyses (Figures 24.1 and 24.2).

The objective of the HMP was to develop baseline information about what constituted a "healthy" microbiome. Although the HMP generated a huge amount of data, the study group represented only a small fraction of human diversity. This limitation was clearly revealed in the Global Gut Project, which examined three distinct populations (U.S. citizens, Malawians, and a group of indigenous peoples from Venezuela) and different age groups within those populations. The gut microbiomes of the two non-U.S. populations were distinct from those of the U.S. individuals, showing that the HMP greatly underestimated the potential for variation in gut microbiomes across nationalities. These studies also suffered from a lack of appropriate metadata; for example, detailed information about dietary habits (vegetarian, vegan, omnivorous) and how much fiber or protein was ingested daily. Because there is still very limited understanding of the influence of specific environmental variables such as lifestyle, diet, gender, and genetics on microbiome structure and function, new and ongoing studies (e.g., the American Gut Project) are obtaining these valuable metadata at the time of sampling. The overarching goal is to develop robust correlations between the microbiome, host genetics, diet, lifestyle, health, and pathologies thought to have a microbial connection, in particular, heart disease, cancer, stroke, diabetes, and obesity.

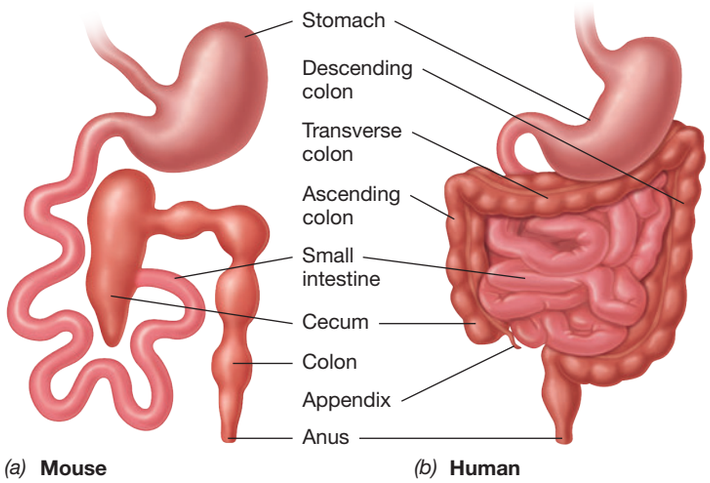


Figure 24.19 Anatomy of the mouse and human intestinal tracts. The mouse *(a)* and human *(b)* intestinal tracts have significant structural differences that are associated with differences in the composition of the microbiota of mice and humans despite similarities in their physiologies.

The Mouse Model

Even if appropriate metadata are available, a major limitation of the human microbiome studies to date is establishing *causality*, something that is only possible with highly controlled animal studies. Hence the mouse has become the major model animal system for linking cause and effect in the gut microbiome.

Although the mouse and human digestive systems have some significant differences (**Figure 24.19**), the mouse (and to a more limited extent, the rat) has been the workhorse of experimental microbiome studies. Compared to humans, mice have a relatively larger colon and cecum, which are needed to extract nutrients derived primarily from the fermentation of plant materials. For example, the average ratio of the length of the murine small intestine to its colon is about 2.5 whereas in humans it is about 7. In mice, fermentation of plant material is compartmentalized in the cecum (◀ Section 23.14), while in humans this fermentation takes place in the colon. Nevertheless, mice have several experimental advantages, including the availability of well-defined genetic lines, low maintenance costs, and short life cycle. This has allowed researchers to explore the importance of the host genetic background through selective gene knockouts, the manipulation of gut microbiota composition using germ-free mice (raised free of all microbes), tests of the influence of a strictly controlled diet, assessing the consequences of antibiotic treatment, and exploring the transfer of physiological traits through fecal transplantation. These studies have clearly associated the composition of the mouse microbiome to different pathologies, including obesity and inflammatory bowel disease, as we will discuss in Section 24.9.

Unfortunately, because of anatomical differences (Figure 24.19), mouse studies are not directly applicable to humans, and these anatomical differences may in part account for observed differences in the relative abundance of dominant bacterial genera in mouse and human guts. For example, the genera *Prevotella, Faecalibacterium* (*Firmicutes*), and *Ruminococcus* are in high abundance in the human gut (Figure 24.6), whereas the genera *Lactobacillus, Alistipes*, and *Turicibacter* are more abundant in the mouse gut. Thus, although the mouse model gives us much experimental latitude not available in gut microbiome studies of humans, actual results from the two systems are not directly comparable. However, despite these differences in bacterial composition, experimenters have glimpsed much useful information from the study of animal models that has accelerated our understanding of the human gut microbiome (for example, see Section 24.9 and Figure 24.25).

We now follow development of the human gut microbiota from birth through adulthood, comparing and contrasting the major organisms seen in healthy humans. An overview of the mature and highly stable gut microbiota was presented in Figures 24.2, 24.3, and 24.6.

Check Your Understanding

- In hindsight, which aspects of the HMP were well controlled for and which were not?
- Why has it been so difficult to associate human disease, or health, with changes in the gut microbial community?
- What are some of the major differences between the mouse and human gastrointestinal systems?

24.8 Colonization, Succession, and Stability of the Gut Microbiota

Colonization of an initially sterile gut begins immediately after birth; a succession of microbial populations replace each other in turn until a relatively stable, adult microbial community is established. Colonization begins when the infant first encounters microbes by passage through the mother's vagina. The newborn is quickly inoculated by bacteria present in breast milk and skin, and later through contact with visitors and household pets.

The structure of the infant's gut community in the first year of life is highly dynamic and greatly influenced by early life events. In particular, birth by vaginal delivery versus cesarean section has a profound influence on early community structure. It is also now known that these early colonization events are critical to the microbe–immune system "cross talk" essential to the development of a well-functioning immune system. Disruption of a normal colonization sequence, such as may result from early-life treatment with antibiotics, is correlated with the development in later life of allergy and asthma, as well as type 1 diabetes and obesity. As we will review in Section 24.12, modulation of the developing gut community by early feeding of beneficial probiotic microorganisms is increasingly viewed as a means to avoid diseases associated with the early disturbance of the normal gut colonization sequence.

Microbial Community Structure and Function in the First Three Years of Life

The young infant gut community is dominated by bifidobacteria—fermentative anaerobes of the bacterial class *Actinobacteria* (◀ Section 16.10)—and does not reach an adultlike composition

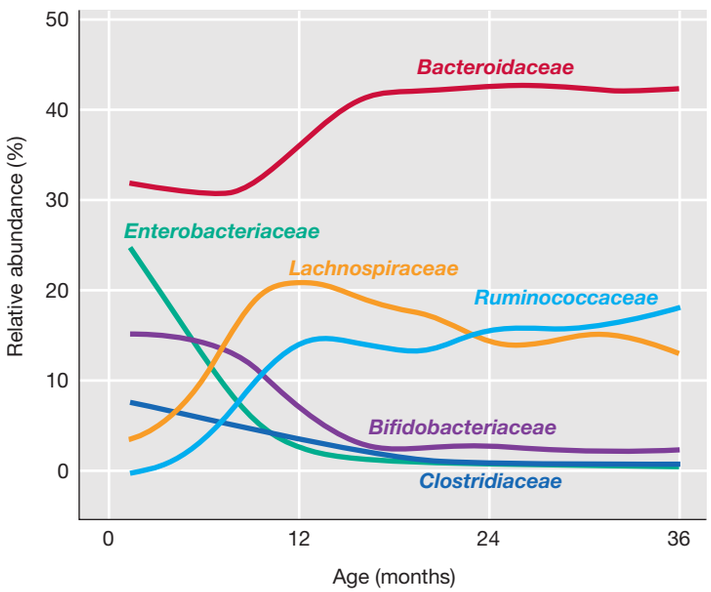


Figure 24.20 Maturation of the infant gut community. Normal maturation of the breastfed infant's gut bacterial community during the first three years of life. *Bifidobacterium* and *Bacteroides* species are important early colonizers. As the gut community matures with termination of breastfeeding, species of *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*) and *Bacteroidaceae* increase in relative abundance while *Enterobacteriaceae* decrease to very low levels.

until about age 3. The newborn's relatively simple community progressively evolves into a more complex and adultlike composition in these first three years. The first year is a period of tremendous fluctuation in community composition; the second year is a transitional phase and is followed by maturation to a stable, adultlike composition during year three.

Early microbial colonizers are an important source of amino acids and vitamins. Microbial genes encoding the synthesis of vitamins K_2 (menaquinone), B_6 (pyridoxal), and B_7 (biotin) are elevated in the newborn's microbiota. As the gut microbiome matures, there is a greater prevalence of microbial genes encoding synthesis of the vitamins B_1 (thiamine), B_5 (pantothenate), and B_{12} (cobalamin). The immature gut community contains a large number of species affiliated with facultatively aerobic *Enterobacteriaceae* (**Figure 24.20**). The presence of bacterial genera such as *Escherichia* and *Enterococcus*, both facultative aerobes, in the newborn gut is also indicative of a more aerobic state of the early gut system and a greater role for the citric acid cycle and respiration in microbial energy production in the neonate.

Major factors controlling the early assembly of the gut microbiome following birth are whether birth was vaginal or by cesarean section (C-section) (**Figure 24.21**) and whether initial nutrition came from breast milk or from formula. A vaginally delivered infant is colonized by a gut microbiota similar to that of the mother, suggesting direct transfer from mother to neonate during passage through the birth canal and subsequent intimate contact with the mother. In contrast, the gut microbiota of a child delivered by C-section is significantly different from that of the mother. Approximately 70% of babies delivered vaginally develop a gut microbiome similar to their mothers, including species of *Escherichia* and *Bilophila* (*Proteobacteria*), *Bifidobacterium* (*Actinobacteria*), *Enterococcus* (*Firmicutes*), and *Bacteroides*. By contrast, only about 40% of the species match those of the mother in the gut of a C-section neonate (Figure 24.21). The latter tends to be enriched in *Staphylococcus* and *Streptococcus* species (*Firmicutes*), and *Veillonella* species, indicating that skin and oral microbes, respectively, as well as environmental populations, were early colonizers. *Bacteroides* species are either less prevalent or totally missing in the infants delivered by C-section (Figure 24.21*b*). The difference in the gut microbiomes of babies born vaginally or by C-section is significantly less by 12 months of age, but the C-section gut microbial community at 8 months remains distinctive and more variable in composition than that of infants born vaginally (Figure 24.21).

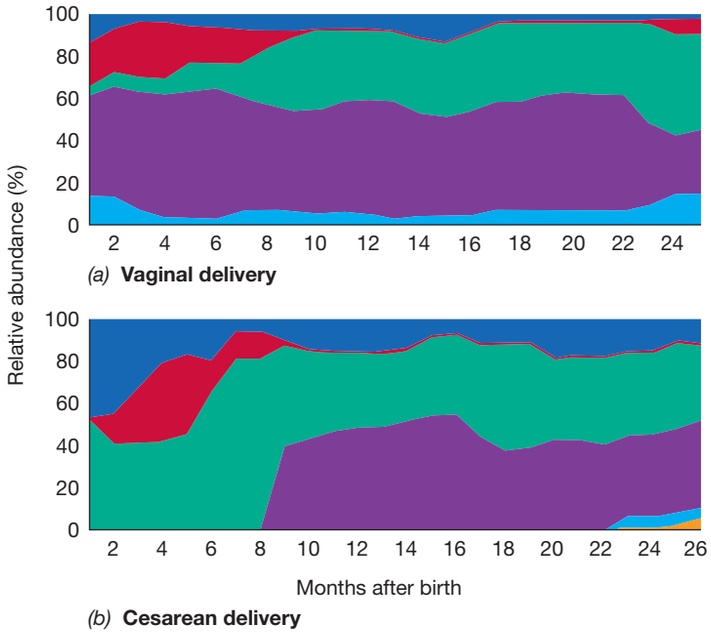


Figure 24.21 Impact of birth mode on the young infant's gut community. Early colonization and temporal changes in the relative abundance of major microbial groups in children delivered *(a)* vaginally or *(b)* by cesarean section. With vaginal delivery, species of *Bacteroides* are present from an early age and *Proteobacteria* only poorly colonize. *Bacteroides* species are undetectable during the first 8–9 months of life following delivery by cesarean section, and a higher initial colonization by *Proteobacteria* persists throughout early life.

The Microbiology of Breastfeeding versus Formula

Infants that have been breastfed develop a different gut microbiome than those who have not. Breastfeeding is associated with elevated numbers of fermentative *Bifidobacterium*, *Lactobacillus,* and *Staphylococcus* species. Viable populations of *Bifidobacterium* and *Lactobacillus* species are present in breast milk, and *Staphylococcus* species colonize the areolar skin. Thus, these species are transferred directly from mother to infant. *Staphylococcus epidermidis* is an opportunistic colonizer, essentially absent in formula-fed infants and an organism whose numbers decrease over time following cessation of breastfeeding.

The enrichment of *Bifidobacterium* species, in particular the species *B. longum*, is related to the composition of human milk. Breast milk contains a complex mixture of unusual oligosaccharides that most gut microbes (other than *Bifidobacterium* species) and humans are unable to digest; for example, the sugar monosialyl-lacto-*N*-neohexaose I (**Figure 24.22**). *B. longum* subspecies *infantis* is unique in having an exceptionally large repertoire of glycosidases and oligosaccharide transporters and grows well on this and other human milk oligosaccharides. These characteristics have also made this strain an important probiotic for treating enterocolitis in premature infants (see Section 24.12). With cessation of breast milk feeding and maturation of the infant gut microbiome, *B. longum* subspecies *infantis* is displaced by other strains of *B. longum* that can use both mammalian and plant-derived oligosaccharides. However, even with the introduction of solid food, the gut communities of infants that continue to receive some breast milk are less diverse than in those no longer getting breast milk. Thus, maturation of the gut microbiome is largely driven by the removal of breast milk from the diet—which generally occurs between 9 and 12 months—rather than by the introduction of solid foods.

At 4 months there remains a clear difference between children who have received exclusively breast milk compared with those given formula milk. Formula-fed infants tend to have elevated numbers of *Clostridioides difficile*—a potentially serious pathogen (▶ Section 30.7 and Figure 30.12)—as well as other potential pathogens such as *Citrobacter* and *Enterobacter*, whereas the guts of children that remain on breast milk at 12 months continue to be

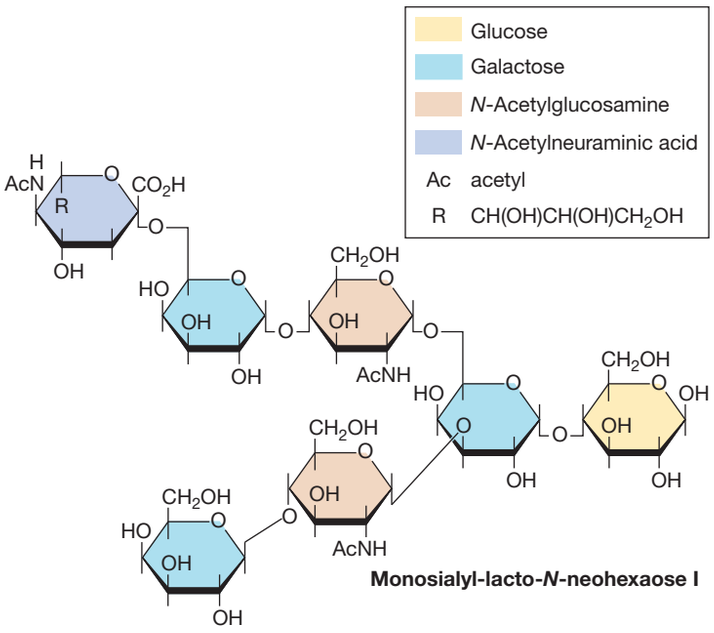


Figure 24.22 Example of an oligosaccharide found in human breast milk. This and other short polysaccharides selectively nourish and enrich for desirable microbial populations, such as *Bifidobacterium* and *Lactobacillus* species, in the infant's developing gut microbial community.

dominated by *Bifidobacterium* and *Lactobacillus*. The abundance of *B. longum* in breastfed infants also leads to the production of short-chain fatty acids; this creates an environment favoring the growth of the normal microbiota important for "educating" the immune system. In addition, since the structures of human milk oligosaccharides mimic carbohydrates lining the infant gut, they function to suppress pathogenic bacterial infection by blocking the pathogens' receptors for attachment. Therefore, in addition to providing nutrition to the newborn, breast milk selects for a specific group of earlier colonizing normal microbiota important to the overall health of the child and proper development of its immune system.

Termination of breastfeeding results in a shift of the community toward a more adultlike composition, as reflected by enrichment in *Bacteroides*, *Bilophila*, *Roseburia*, *Clostridium*, *Anaerostipes*, and *Eikenella*. Many of these later-arriving genera are efficient degraders of dietary fibers and complex carbohydrates, consistent with a transition to more solid foods following the cessation of breastfeeding; they also produce short-chain fatty acids. The increased abundance of *Bacteroides thetaiotaomicron*, a bacterium encoding a diverse set of glycan-degrading enzymes, is specifically associated with the increase in pectin in solid foods. Although the development of a fully adultlike gut microbial community varies a bit from child to child, the average is three years.

Stability of the Adult Microbiome and Transitions with Age

Although metagenomic diversity studies have identified several thousand gut bacterial species, the number of species in any given individual is closer to 200, a number that remains stable for long periods. The most stable of these species are typically affiliated with the *Bacteroidetes* and *Actinobacteria*. Although *Actinobacteria*, such as *Bifidobacterium* spp., comprise a rather small part of the adult gut microbiota compared to the *Bacteroidetes*, specific species tend to associate with individuals over long periods of time. By contrast, species of *Firmicutes* and *Proteobacteria* appear to be less stable members of the gut community. The species composition of the gut of an individual tends to reflect that of family members, and likely was established early in life. Thus, the early colonizers—including microbes acquired from parents or siblings—in large part determine the metabolic and immunological properties of the adult microbiome and may be an important factor in adult health and predisposition to disease.

As we age, so does our microbiome. The most well-established change is the age-related alteration in the relative proportions of *Firmicutes* and *Bacteroidetes*, with an increasing proportion of *Bacteroidetes* seen in the elderly as compared with the higher proportions of *Firmicutes* in young adults. Also observed with age are significant decreases in bifidobacteria and certain clostridial species. It also appears that the old age–related inability to perform routine daily activities, a measure of frailty, is correlated with decreased diversity in the gut microbiota. Consistent with this observation, elderly individuals living at home or in supportive communities have a more diverse gut community compared with those living in residential care facilities, such as nursing homes. Thus, maintaining a diverse gut microbiota is likely one of many links to health for the aged and is possibly even a positive effector of longevity.

With this overview of changes in the human microbiome with time, we move on to consider some startling discoveries about human diseases linked to unfavorable changes in the human gut microbiota. We then end this chapter by looking at some therapies for dealing with these problems.

Check Your Understanding

- What factors contribute to early colonization of the newborn's gut community?
- How do microorganisms in the infant and adult gut community contribute differently to vitamin and amino acid requirements?
- What factor(s) are most important in the transition from an immature to a mature gut microbial community?

UNIT 6

IV • Disorders Attributed to the Human Microbiome

Many major human health issues including obesity and inflammatory bowel disease correlate with disturbances of the microbiome. Maintenance of gut and general health depends on the continuous production of short-chain fatty acids by microbial fermentation of dietary fiber.

Alteration of the structure and activity of the human microbiome is associated with a variety of pathologies, including obesity, type 2 (non-insulin-dependent) diabetes, asthma, atopic dermatitis, liver disease, colorectal cancer, kidney stones, psoriasis, tooth decay

(dental caries), and periodontitis. As we learn more about the relationship between the human microbiome and health and disease, therapeutic intervention may well be possible. This might include promoting the growth of protective beneficial bacteria, inhibiting the growth of specific microbes (or specific assemblages of microbes) that compromise health, fecal transplants to introduce a desirable gut microbiota, and developing appropriate behavioral interventions, such as diet modification.

24.9 Syndromes Linked to the Gut Microbiota

A healthy gut community is loosely defined as constituting a relatively stable and resilient community associated with health of the human host. A gut microbial community demonstrating stability and resilience is said to be in a state of *homeostasis*. Variations from the homeostatic state may or may not trigger disease. We will first consider the healthy state and then explore some cases where alterations in the gut microbiome are strongly correlated with specific disease syndromes.

Diet and Gut Health

A specific definition of healthy homeostasis based on species composition has not been possible because individuals differ significantly in their specific gut community makeup, and community makeup shifts with diet. Thus, a healthy gut microbiota is increasingly being defined in dietary, metabolic, and immunological terms. Diet is a critical determinant of homeostasis. In particular, the **short-chain fatty acids** (SCFAs; primarily butyrate, propionate, and acetate) produced by fermentation of fiber by the gut microbiota serve a central role in the development and maintenance of a healthy gut. Not only are SCFAs an important energy source for intestinal cells, but they also function as signaling molecules to regulate cellular metabolism and immune surveillance (**Figure 24.23**). Binding of SCFAs to specific intestinal cell surface receptors regulates cellular metabolism, increases the secretion of antimicrobial peptides known as defensins (▶ Section 26.2), elevates production of hormones affecting energy recovery and sensations of hunger, and has a dampening effect on the inflammatory response through the regulatory T cell (Treg) signaling system (Figure 24.23 and ▶ Section 27.8).

Among SCFAs, butyrate in particular serves a critical function in the control of immune surveillance and the metabolism of colonocytes. Colonocytes are the absorptive epithelial cells of the large intestine (Figure 24.5). Butyrate activates a specific regulatory protein of colonocytes called PPAR-γ that accelerates mitochondrial beta-oxidation of fatty acids (◀ Section 14.23). This oxygen-consuming process helps to maintain an anoxic environment near the epithelial surface that is favorable to the obligately anaerobic bacteria that produce butyrate (Figure 24.23). If butyrate-producing microbes are depressed by, for example, a dramatic diet change or as a result of antibiotic treatment, colonocyte metabolism is shifted toward glucose fermentation and lower oxygen consumption. This results in greater passage of oxygen through the intestinal mucosal surface and a proliferation of facultatively aerobic bacteria (such as species of *Enterobacteriaceae*) that can invoke an inflammatory response. Since SCFA signaling also promotes tight junction formation (Figure 24.23), it is thought that a depression of PPAR-γ activity contributes to the development of *leaky gut syndrome* in humans. Disruption of tight junctions leads to a greater exposure to gut microbiota, which in turn elicits an inflammatory response. Such a disruption of healthy gut function is called **dysbiosis**, typified by increased numbers of facultative aerobes, such as species of *Enterobacteriaceae*, and a state of chronic inflammation. These physiological changes are also thought to play important roles in the development of inflammatory bowel disease and obesity, and we turn our attention to these now.

Inflammatory Bowel Disease

The gut microbiota plays important roles in shaping the immune system in infancy; during this time, the normal microbiota and their products interact with immune cells to initiate and maintain host tolerance. The failure to develop tolerance to the normal microbiota early in life is associated with different immune-mediated diseases, including allergies and chronic inflammation of the gut such as *inflammatory bowel disease* (*IBD*).

It is widely accepted that IBD is not caused by a specific pathogenic microbe but rather an imbalance, or dysbiosis, between the immune system and the normal gut microbiota. The observation that antibiotic use in early life increases the risk of IBD points to the importance of the development of a normal gut microbiota in "educating" the immune system to differentiate between the normal

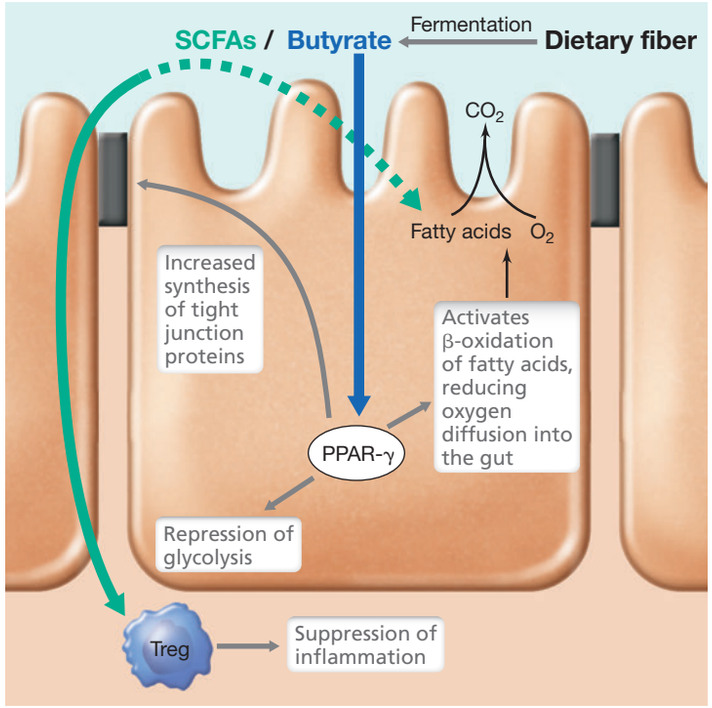


Figure 24.23 Role of short-chain fatty acids in intestinal health. Short-chain fatty acids (SCFAs) produced by microbial fermentation of fiber and dietary polysaccharides modulate the metabolic activity of colonocytes (see Figure 24.5) lining the intestinal epithelium. Butyrate activates the PPAR-γ regulatory protein, which then accelerates beta (β)-oxidation of fatty acids and the synthesis of tight junction proteins between cells and concurrently depresses colonocyte glycolysis. SCFAs also signal to regulatory T cells (Treg), suppressing the potential inflammatory response to normal gut microbiota. Together these shifts in metabolic activities reduce oxygen leakage into the lumen and maintain the integrity of the epithelial barrier.

microbiota and invading pathogens. For example, relative to healthy children, children with IBD have higher levels of species of *Veillonella*, *Prevotella*, *Lactobacillus*, and *Parasporobacterium* and lower levels of species of *Bifidobacterium* and *Verrucomicrobium*.

There is also evidence from mouse studies that once developed, IBD is transmissible. Fostering or co-caging healthy mice with IBD-predisposed mice was sufficient to cause IBD development in the healthy mice and was correlated with the transfer of the enteric bacterial species *Klebsiella pneumoniae* and *Proteus mirabilis* from the IBD mice to the healthy mice. In humans, metagenomic analyses of healthy subjects and patients with IBD shows that the gut microbiota of IBD patients shares fewer genes in common with healthy subjects, relative to the number of genes shared among healthy subjects. The microbial community of IBD patients also tends to have significantly reduced functional capacity, as reflected by a reduction in the number of nonredundant (functionally unique) genes relative to subjects that do not have IBD (**Figure 24.24**).

The mechanism of IBD formation is unclear but may be the result of disruption of mucosal barrier integrity (leaky gut) by a gut pathogen or a toxic insult, permitting commensal bacteria to interact with and activate the adaptive immune system (Chapter 27). This stimulates the proliferation and differentiation of T cells into commensal-specific effector cells that can persist in the intestine long after resolution of the infection. For example, the IBD syndromes of *Crohn's disease* and *ulcerative colitis* are known to be associated with a T cell response to intestinal commensal bacteria.

There is also a strong correlation between IBD and diet. With the typical Western diet rich in animal protein, carbohydrates are first fermented in the upper colon (Figure 24.3). As digesta move further along the colon, protein and amino acids are then fermented, generating potentially harmful metabolites such as ammonia, phenols, trimethylamine (TMA), and hydrogen sulfide (H_2S) that have been implicated in promoting IBD as well as playing a role in colon cancer. Animal studies have shown that these compounds can promote the development of a leaky gut and gut inflammation, as well as cardiovascular disease (CVD). TMA is converted to trimethylamine oxide (TMAO) in the liver, and TMAO contributes to coronary thrombosis by increasing blood platelet hyperresponsiveness. A high concentration of TMA accelerates atherosclerosis (clogging and stiffening of the arteries) development in mice and is correlated with a higher incidence of CVD in humans. In contrast, a diet rich in plant-based foods (high fiber) works against development of these pathologies, highlighting the importance of a fiber-rich diet and carbohydrate-based fermentation in the healthy gut. Finally, IBD does not always affect identical twins; this observation argues against a strong genetic connection to IBD and supports the hypothesis that the environment and diet are the major triggers of this intestinal condition.

The Role of the Gut Microbiota in Obesity: Mouse Models

Obesity is a significant health risk that contributes to secondary health issues such as high blood pressure, cardiovascular disease, and type 2 diabetes. More than 600 million adults and 100 million children worldwide are obese (defined as a body mass index of >30), and because of this, several studies have focused on possible correlations between the gut microbiome and obesity.

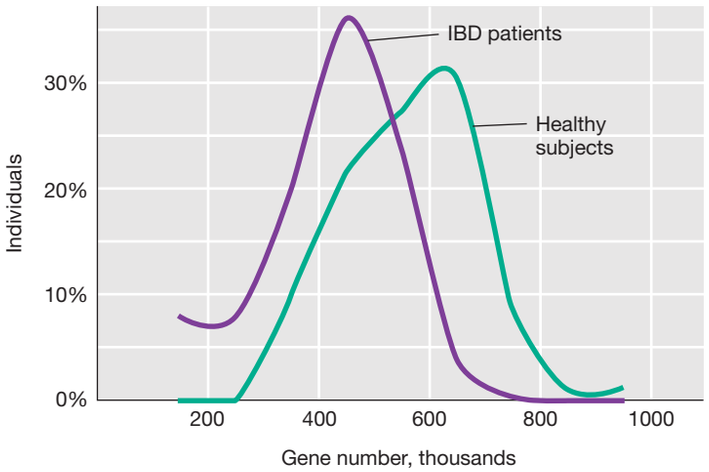


Figure 24.24 Reduced functional capacity of the gut microbiome of patients with inflammatory bowel disease. Metagenomic analysis of human gut microbiota in healthy subjects and patients with inflammatory bowel disease (IBD) revealed a tendency toward fewer nonredundant bacterial genes in patients with IBD.

Initial evidence linking the gut microbiota to host fat accumulation in humans came from studies using germ-free (microbe-free) mice. In these experiments, normal mice had 40% more total body fat than those raised under germ-free conditions, although both mouse populations were fed the same amount and type of food. After germ-free mice were inoculated with fecal material from a normal mouse (a fecal transplant, see later), they developed a gut microbiota and their total body fat increased, although there had been no changes in food intake or energy expenditure.

Experimental colonization of germ-free mice with individual microbial species or microbial communities have demonstrated that colonization triggers the expression of *host* genes for glucose uptake and lipid absorption and transport in the ileum. This indicates that there is likely a link between gut microbial composition and the ability of the host to harvest energy from its diet, ultimately contributing to obesity. This has been demonstrated in experiments with mice. Mice that are genetically obese have microbial gut communities that differ from those of normal mice, with 50% fewer *Bacteroidetes*, a proportional increase in *Firmicutes*, and a greater number of methanogenic *Archaea*. It has been hypothesized that methanogens increase the efficiency of microbial conversion of fermentable substrates to volatile fatty acids (VFAs) by consuming molecular hydrogen (H_2), as also occurs in the rumen (◀ Section 23.16); this makes more VFAs available for absorption by the mouse. In the non-obese mouse, by contrast, fewer H_2-consuming methanogens are present, and the buildup of H_2 functions as a partial inhibitor of microbial fermentation. This favors growth of *Bacteroidetes* species, making fewer VFAs from *Firmicutes* fermentation available for the mouse (**Figure 24.25*a***). What triggers this difference in the methanogen content of lean versus obese mice is not well understood. However, a similar scenario but involving different microbes also appears to be a factor in human obesity (see next subsection).

The importance of the gut community to a predisposition to obesity in mice has also been demonstrated by *fecal transplant studies*, transplanting a small sample of the gut contents from a set of paired

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Art Activity: Figure 24.19 Differences in gut microbial communities between lean and obese mice

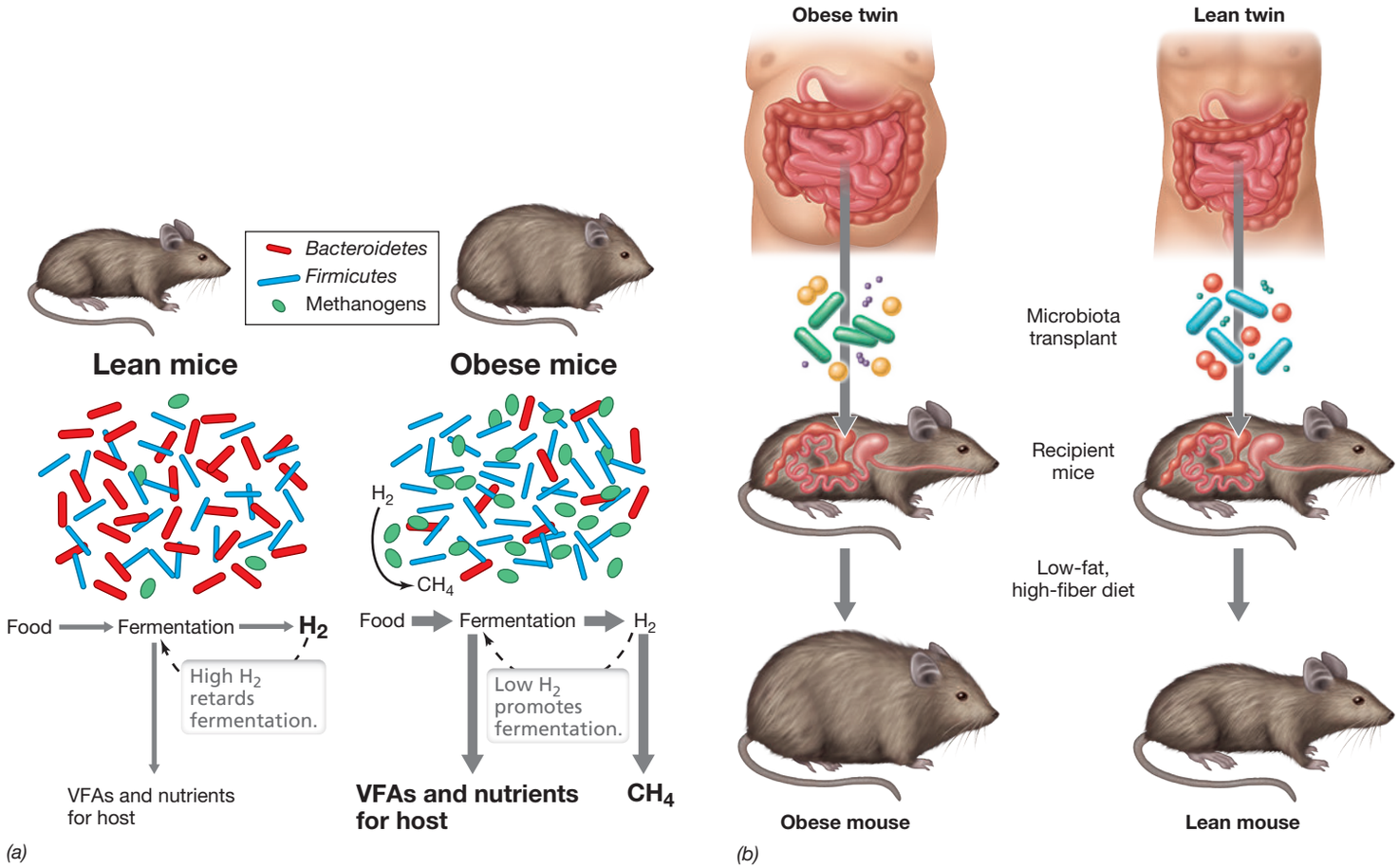

Figure 24.25 Fermentation in the colon of lean and obese mice and transfer of an obese condition by fecal transplant. *(a)* The greater abundance of methanogens in the gut of obese mice is thought to promote fermentation by consuming H_2. *(b)* Transplanting fecal material from the gut contents of a paired identical human twin study group (one twin was obese and the other lean) to germ-free mice showed that the obese twin microbiota made the mouse obese. Conversely, transfer of gut contents from the lean twin did not contribute to an obese phenotype. Part *b* adapted from Ridaura, V.K., et al. 2013. *Science 341* doi:10.1126/science.1241214.

human twins (one obese and one lean) into germ-free mice (see Section 24.11 for more coverage of fecal transplants). Although the recipient mice were fed identical high-fiber diets, the mice receiving fecal contents from the obese twin gained significantly more weight than the mice receiving fecal contents from the lean twin (Figure 24.25*b*). This is direct evidence that a lean or obese body type can be altered by changes in the gut microbiota, even when the microbiota originates from a different species. Put another way, specific but widely distributed gut microbes exist that can control an animal's metabolism to yield a lean or obese body type.

The Gut Microbiota and Human Obesity

Animal model inferences have been more difficult to demonstrate with human subjects, since strict control of diet and host genotype is not feasible and gut microbiota manipulation is more difficult to achieve. Although studies of humans could not confirm the *Bacteroidetes–Firmicutes* relationship established in mice, they have shown that obese humans are more likely to harbor species of *Prevotella* and methanogenic *Archaea* than are lean humans, suggesting that a version of the mouse model (Figure 24.25*a*) is likely applicable to humans. In humans, the methanogens are proposed to remove H_2 produced by *Prevotella*, facilitating fermentation by *Prevotella* and increasing nutrients to the host. This model is also supported by the study of germ-free mice colonized with *Bacteroides thetaiotaomicron* (having a metabolism similar to *Prevotella*) and the methanogen *Methanobrevibacter smithii*. Relative to controls containing just one of these species, co-colonized mice have a higher number of total gut bacteria, higher acetate levels in the intestinal lumen and blood, and greater body fat.

This relatively simple explanation for the cause of obesity is insufficient to fully explain microbial contributions to obesity. For example, the gut microbiota in lean mice produce greater amounts of the fatty acids propionate and butyrate and actually digest *more* of the plant fiber than do the microbiota of obese mice. Similarly, although a fiber-rich diet increases the amount of material available for fermentation in the human large intestine, such a diet also reduces the risk of obesity. As we now know, this may be an attribute of healthy gut homeostasis (Figure 24.23) where binding of SCFAs to specific intestinal cell surface receptors elevates production of hormones affecting energy recovery and sensations of hunger. Similarly,

obesity in humans is also associated with a variety of metabolic complications, including low-grade inflammation exacerbated by reduced production of SCFAs from a diet low in fiber. These factors tend to complicate the simple microbiota models of obesity proposed to date.

An influence of host physiology on the gut community is also suggested by changes during pregnancy in humans. The period between the first and third trimesters of pregnancy is associated with a decrease in gut microbial diversity and enrichment in the gut community of species of *Proteobacteria* and *Actinobacteria*. These changes are associated with the increased body fat and insulin insensitivity that develop later in gestation. A simple interpretation of these findings is that a pregnant woman's body in some way manipulates her gut microbiome as part of its preparations for a greater demand on stored energy reserves. If true, this once again underscores the complex interplay between the gut microbiota and multiple host variables (genetic, physiological, behavioral, and environmental) that may be contributing factors in the development of human obesity and associated metabolic disorders.

Check Your Understanding

- What is dysbiosis? How might this condition lead to inflammatory bowel disease?
- How are higher numbers of gut *Prevotella* species linked to obesity in humans?
- Why are the gut microbiota results obtained in mice more difficult to confirm through human studies?

24.10 Syndromes Linked to the Oral, Skin, and Vaginal Microbiota

Imbalances in the normal microbiota have been linked to human health issues other than those affecting the gut. These include in particular diseases of the oral cavity, skin, and vagina, and we consider some of these important disease centers here.

Dental Caries and Periodontitis

Dental caries and periodontal diseases are among the most common chronic human maladies. We consider dental caries in Chapter 25, where we examine how bacteria attach to solid surfaces. The mechanisms by which oral bacteria stick to the teeth and gums has been used as a major model system for bacterial attachment to solid surfaces. The attached cells eventually form a diverse microbial community in dental plaque and this leads to dental disease from the generation of lactic acid by fermentation (▶ Section 25.2 and Figures 25.7 and 25.8). Although *Streptococcus mutans* is a major contributor to dental caries, colonization of tooth surfaces (**Figure 24.26**) with organisms other than *S. mutans* can also lead to caries. In the absence of *S. mutans*, other acidogenic and acid-tolerant species can initiate caries.

Species of the bacterial genera *Streptococcus, Granulicatella,* and *Actinomyces* are typically elevated in children who develop severe early dental caries, whereas caries-free children have a higher relative abundance of *Aestuariimicrobium*. In older children, caries is associated with decreasing bacterial diversity and increasing prevalence of *Porphyromonas* and *Prevotella* species. Similarly, dental plaque is more microbially complex in healthy subjects than in those with caries. Chronic periodontitis (inflammation and loss of tissue and bone that supports the teeth) is also associated with decreased microbial diversity, although no single bacterial phylotype seems to be directly associated with disease.

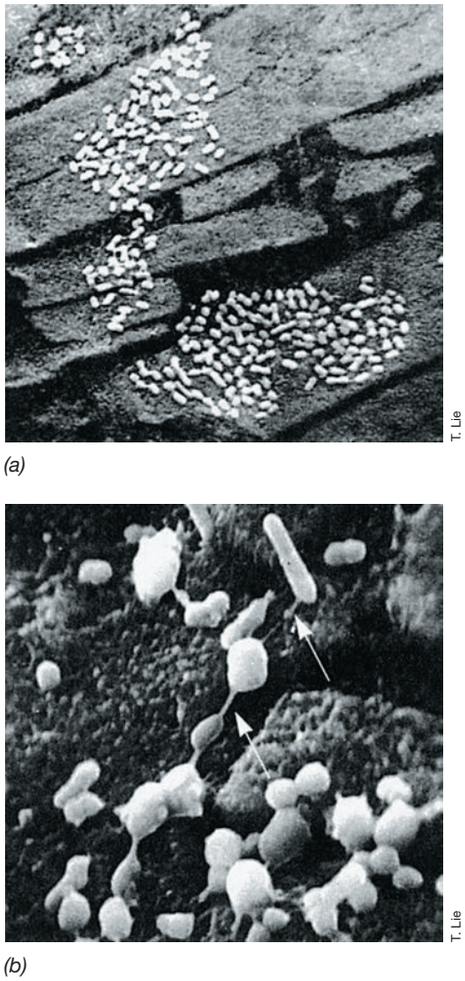

Figure 24.26 Colonization of tooth surfaces. *(a)* The colonies are growing on a model tooth surface inserted into the mouth for 6 h. *(b)* Higher magnification of the preparation in part *a*. Note the diverse morphology of the organisms present and the slime layer (arrows) holding the organisms together.

Collectively, these observations suggest that no single pathogen leads to the formation of dental caries or periodontal disease, but rather that community-wide changes in microbial composition trigger the diseases. Periodontal disease is particularly serious because it is thought to contribute to several debilitating systemic conditions, including cardiovascular disease, diabetes, pneumonia, and arthritis. Although the etiology of these systemic pathologies is unclear, they appear to be associated at least in part with dysbiosis of the oral cavity similar to that observed in dysbiosis of the gut microbiota in inflammatory bowel disease (Section 24.9).

Acne Vulgaris

The interrelationship of species of the bacterial genus *Propionibacterium* with the innate and adaptive arms of immunity (Chapters 26

and 27, respectively) has been examined extensively because of the association of this bacterium with *acne vulgaris*, usually just called *acne*, a cutaneous inflammatory disease of the skin that affects more than 85% of adolescents worldwide. However, the suspected causative agent of acne, *P. acnes*, is also a member—and a dominant member—of the normal cutaneous microbiota (Figures 24.2 and 24.15–24.17) and thus is also present in individuals without inflammatory disease.

Although *Propionibacterium* species have the capacity to elicit an inflammatory response, this alone is insufficient to demonstrate a causal association with disease. A better understanding of the cause(s) of acne will likely emerge from a better understanding of variation among strains of *P. acnes*. Notably, experiments using metagenomic and multilocus sequence analysis (◀ Section 13.12) suggest that a specific strain of *P. acnes* in susceptible individuals may trigger the acne condition, whereas a much greater diversity of strains is observed among individuals having good skin health. Because of the scarcity of nutrients available for the skin microbiota, even minor differences in nutritional requirements between strains may favor the development of one or another *P. acnes* strain. If true, this might lead to therapies for acne using probiotics or prebiotics (see Section 24.12) that foster the growth of harmless strains or the growth of other species that displace *P. acnes* through competition for space and nutrients. For example, a lotion could be applied to the skin that contains a nonpathogenic strain or a nutrient selective for protective strains as a means to prevent or limit colonization by pathogenic strains.

Vaginal Conditions

Dysbiosis of the normal vaginal microbiota (Section 24.4 and Figure 24.14) is associated with either an inflammatory infection (*vaginitis*) or a less severe clinical form (*vaginosis*) that may be asymptomatic or associated with malodor and discharges. Common types of vaginitis result from the overgrowth of species of the yeast *Candida* (candidiasis) or from infection by the sexually transmitted protozoan *Trichomonas vaginalis*, both discussed in Chapter 34. Diagnosis of the less highly inflammatory vaginosis in a clinical research lab is commonly based on a microscopic scoring of a Gram-stained vaginal smear. However, culture-independent molecular methods (Chapter 19) are increasingly being employed to more precisely define vaginal communities associated with health, disease, and predisposition to disease, including neonatal infections, miscarriage, pre-term birth, and increased susceptibility to HIV and sexually transmitted infections.

Longitudinal studies of individual women over multiple months have revealed that the vaginal microbial communities of some women are highly dynamic, changing in composition over relatively short time periods, whereas the communities of others are relatively stable (**Figure 24.27**). Most vaginal microbial communities appear to be stable, displaying *resilience* (the capacity to return to the pre-disturbance community structure following disruption, such as menstruation) or *resistance* (the capacity to resist community disruption in response to disturbance). For example, a low pH–tolerant, *Lactobacillus crispatus*–dominated vaginal community is relatively stable (Figure 24.27), showing either resilience (subject 1) or resistance (subject 2). By contrast, other vaginal communities are more prone to converting to a vaginosis form. For example, the healthy *L. crispatus*–dominated community can convert to a healthy *L. iners*–dominated community before returning to *L. crispatus* (Figure 24.27). However, although both the *L. crispatus* and *L. iners* communities are associated with vaginal health, an *L. iners*–dominated community is more likely to transition to vaginosis. Vaginal bacterial diversity also waxes and wanes over shorter time periods, such as a woman's menstrual cycle. Higher bacterial diversity is observed during days of menstruation than in intervening days, with diversity following a cycle inversely related to that of the estrogen hormone estradiol.

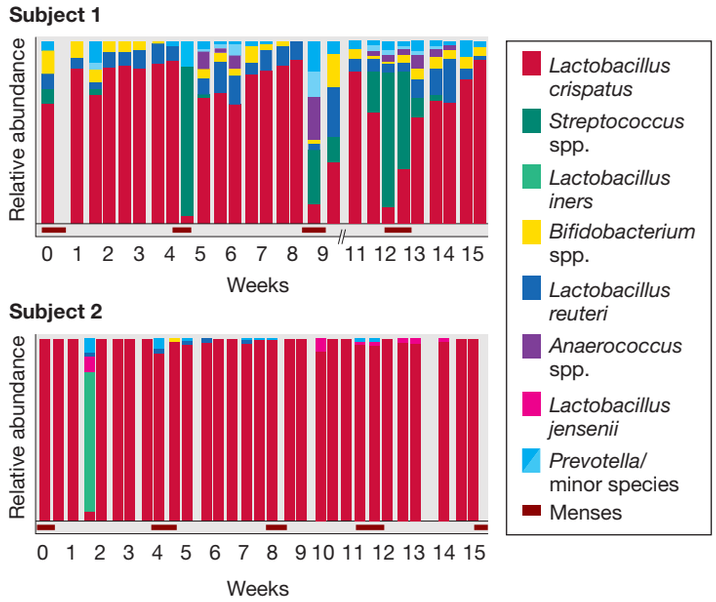

Figure 24.27 Resilience of the normal vaginal microbiota in two subjects. Both vaginal communities were dominated by *Lactobacillus crispatus* and demonstrated resilience to perturbation, most notably the disruption associated with menstruation (indicated by horizontal red bars) in subject 1.

As for most ongoing human microbiome research, molecular microbial community analyses of the vagina have revealed connections between the microbial diversity of the vagina, vaginal health, and the susceptibility to vaginal diseases. However, we do not yet fully understand how the vaginal community is established and maintained or how vaginal dysbiosis develops and resolves. Nevertheless, if some community types are associated with clinical symptoms or are associated with higher risk for vaginal disorders, then early intervention may be important to maintaining women's health and the health of a mother and baby in particular.

Check Your Understanding

- What observations indicate that dental caries are not due solely to *Streptococcus mutans*?
- Why might it be possible to have a high abundance of *Propionibacterium acnes* without developing acne vulgaris?
- What are some clinical advantages of a community-structure-based evaluation of vaginal health?

V • Modulation of the Human Microbiome

Gut dysbiosis can be resolved by introducing microbes from a healthy gut by fecal transplant. Selective modulation of the gut community can be accomplished by the use of probiotics and prebiotics. Early intervention with such therapies can reduce the incidence of serious intestinal disease in infants.

One important basic-science goal of human microbiome studies is to understand how the microbial composition and activity of human-associated microbes promotes health or predisposes the body to a variety of health disorders. Here we look at how the introduction of specific microbes or even transplants of the entire gut community can be used to promote health.

24.11 Antibiotics and the Human Microbiome

Antibiotics are naturally produced antimicrobial substances whose efficacy varies against different bacteria (Chapter 28). However, when an antibiotic is taken orally it kills or inhibits to at least some extent the normal microbiota as well as the targeted pathogen(s). Antibiotic treatment can lead to a significant loss of the normal gut microbiota (Section 24.2). When antibiotic therapy ends, the normal intestinal microbiota is usually, but not always, reestablished spontaneously in adults.

Use of antibiotics during the first few months of life is a particular problem for the developing normal microbiota. This disruption can affect normal development of the immune system and predispose the infant to later autoimmune disorders, such as IBD and allergies (Section 24.9). Research has also shown that early disruption of the gut microbiota influences host energy metabolism. For example, antibiotic exposure during the first 6 months of life is associated with increased weight gain in infants between 10 and 38 months of age compared to those not receiving antibiotics. With growing concerns about the frequency of childhood obesity leading to adult obesity along with the growing number of childhood autoimmune disorders, it is clearly important to avoid, if possible, disruption of the normal development of the human gut microbiota early in life.

Clostridioides difficile Infections

Sometimes antibiotic-resistant opportunistic pathogens become established in young children or the elderly following antibiotic treatment that disrupts the normal microbiota. A particularly problematic complication of antibiotic therapy is infection with toxigenic *Clostridioides difficile* (▶ Section 30.7) and the inability to resolve infections with repeated follow-on administrations of antibiotics (**Figure 24.28**). There is a strong association between antimicrobial therapy and the subsequent development of *C. difficile* infection, and the risk of infection is greater if *C. difficile* is resistant to the antimicrobial agents used in therapy.

In those infected with *C. difficile*, symptoms vary from a mild diarrhea to severe abdominal pain and fever. The most severe complications are inflammatory lesions and bowel perforation; as a result of these, septic shock (▶ Section 25.2) and death are possible. In the past, if intravenous fluids and antibiotics did not resolve the infection, then surgical removal of the colon was a last-resort lifesaving measure.

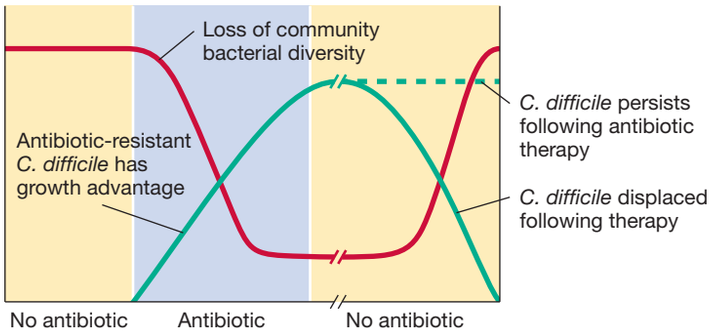

Figure 24.28 Antibiotic treatment increases the risk of *Clostridioides difficile* infection. During antibiotic therapy the displacement of sensitive gut populations results in a significant loss of community diversity, allowing antibiotic-resistant populations such as toxigenic *C. difficile* to increase in abundance. When antibiotic therapy ends, *C. difficile* may be displaced (solid green line) by reestablishment of a normal gut microbiota, or alternatively it may persist (dotted line), causing intestinal disease.

However, the prognosis for recovery from a *C. difficile* infection today has been dramatically improved by a novel nondrug and nonsurgical therapy: the infusion of fecal material from the gut contents of a healthy donor into the gut of a *C. difficile*–infected patient—more commonly called a *fecal transplant*. In most cases such treatment has been shown to restore a healthy colon in patients suffering recurring *C. difficile* infections, and we examine this procedure now.

Fecal Transplants

The goal of a **fecal transplant** is to reintroduce normal microbiota into the gut of someone with an intestinal disease of bacterial origin and have the transplanted microbial community become established and exclude the disease-causing organisms. Although it has taken the medical community some time to adopt this therapy, the dramatic improvement in the rate of resolution from *C. difficile* infection by fecal transplant from a healthy donor has prompted widespread adoption of this procedure.

As we have seen, once established, *C. difficile* is a particularly difficult pathogen to eradicate (Figure 24.28). However, the percentage of otherwise nonresponsive patients cured of *C. difficile* infection without relapse is nearly 90% with fecal transplant therapy versus only about 25% with standard antibiotic treatment. It has also been shown that fecal transplants can alleviate some forms of metabolic syndrome, a condition that is characterized by elevated blood pressure and glucose levels, excess belly fat, and abnormal cholesterol levels and that is a frequent precursor of type 2 (insulin-nonresponsive) diabetes. Metabolic syndrome patients receiving a fecal transplant from a lean donor had significantly increased insulin sensitivity, and also showed increased fecal levels of butyrate, greater overall gut microbial diversity, and increased abundance of the gut bacterium *Roseburia intestinalis*, thought to be a beneficial microbe because of its butyrate production (Figure 24.23).

Fecal transplants require that the donor be healthy and free of infection. And, because feces are considered a body fluid, screening and testing of the donor is essential. Potential donors must complete a screening questionnaire similar to that required for donating blood, and any prospective donors who have risk factors for HIV (AIDS) or hepatitis infections or any history of gastrointestinal

UNIT 6

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Art Activity: Figure 24.23 Antibiotic treatment increases the risk of *Clostridium difficile* infection

disease, autoimmune disease, or cancer are screened out. Prospective fecal donors must also undergo blood tests for a suite of pathogens, and their feces are tested for fecal bacterial pathogens and parasites. If everything checks out, samples of their feces are frozen in small vials and then thawed and used when needed. Fecal transplant recipients typically receive the transplant via a saline solution during colonoscopy to ensure that the transplanted feces actually reach the upper colon and that the colon has previously been cleansed to remove as much of the previous microbial community as possible.

Although a fecal transplant may seem somewhat crude and an essentially uncontrolled or nonprecise therapy, studies are accumulating that show that the appropriate manipulation of the gut microbiota can have significant health benefits and that gut microbiota transplants can be maintained for long periods. More generally, the success of fecal transplants has shown that manipulation of the human microbiome for improved health is feasible and that with greater understanding of these complex communities, more targeted intervention may be possible, as we consider in the next section.

Check Your Understanding

- Why do healthy adults usually not contract *Clostridioides difficile* infections?
- Why is it unsafe for a fecal transplant recipient to receive feces from an unscreened donor?

24.12 Probiotics, Prebiotics, and Synbiotics

We close this chapter with a brief discussion of how ingestion of living microbial cultures (*probiotics*), particular nutrients typically derived from plants (*prebiotics*), or a combination of probiotic and prebiotic (called *synbiotic*) supplements could be used to treat disease and promote health by modulating the gut microbial community.

Probiotics

The United Nations Food and Agriculture Organization and the World Health Organization define a **probiotic** as a "live microorganism which, when administered in adequate amounts, confers a health benefit on the host." Species of *Bifidobacterium* and *Lactobacillus* bacteria are the most commonly discussed probiotics. They are usually delivered to the gastrointestinal system by ingestion of a fermented milk product, such as yogurt (**Figure 24.29**), with the hope of suppressing gut or urogenital disturbances.

Most physicians and scientists agree that ingestion of probiotic foodstuffs probably has limited therapeutic value. Nevertheless, human microbiome research has spurred a renaissance in probiotics research and investment, especially for the development of effective and more targeted probiotics. The remarkable success of fecal transplants (Section 24.11) points to the therapeutic potential of modification of the gut microbiome. However, although fecal transplants have proven very effective, there is minimal understanding of the mechanisms behind their successes and also why they do not work in a limited number of cases.

Molecular analyses of *Clostridioides difficile* infections have highlighted the importance of a mechanistic understanding for the development of effective probiotic therapies. Resistance to *C. difficile* infection following antibiotic therapy (Figure 24.28) in both mice and humans is correlated with the presence of a *Clostridium* species,

Deborah O. Jung and John Martinko

Figure 24.29 Examples of some probiotic foods and supplements widely available worldwide. Some probiotic products also contain prebiotics (specific polysaccharides obtained from various plants and other sources).

C. scindens. Administration of this species to mice infected with *C. difficile* has been shown to be an effective probiotic therapy; suppression has been linked to the ability of *C. scindens* to convert cholic acid (found in bile) into deoxycholic acid, a growth inhibitor of *C. difficile*. In similar studies, recovery from diarrhea caused by *Vibrio cholerae* (cholera, ▶ Section 33.3) has been associated with an increased abundance of the bacterium *Ruminococcus obeum*. Mouse studies demonstrated that production of a quorum-sensing autoinducer (AI-2) (◀ Section 7.7) by *Ruminococcus* suppressed expression of *V. cholerae* colonization factors and effectively prevented the cholera bacterium from establishing an infection.

The promise of effective probiotic therapies suggested from mouse studies have been supported in several human studies. For example, the fortuitous ingestion of a *Bacillus* strain present in a rural Thai environment was shown to prevent colonization by pathogenic *Staphylococcus aureus.* Similar to the mechanism of action observed in *R. obeum*, the *Bacillus* prevents colonization by interfering with the *S. aureus* quorum-sensing signaling system, ultimately preventing transcription of genes encoding virulence (**Figure 24.30**). This particular *Bacillus* strain could thus be marketed as an effective probiotic to eliminate intestinal as well as nasal *S. aureus* colonization. This is important because treatment of *S. aureus* infections has become increasingly difficult due to the prevalence of multi-antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA; ▶ Section 31.9). Thus, an effective probiotic therapy that targets a specific pathogen and avoids the disruptive impact of broad-spectrum antibiotics on the human microbiome (see Section 24.11) would be a powerful addition to clinical medicine.

Probiotics have also been shown effective in reducing the incidence of necrotizing enterocolitis (NEC), a severe inflammatory injury to the intestines of low-birthweight premature infants. NEC affects as many as 10% of premature infants, and about a quarter of those affected will die. Molecular characterization by 16S rRNA gene sequencing (◀ Section 19.6) of the developing gut communities in these infants showed that during the first 60 days of life, species of *Veillonellaceae* (*Negativicutes*) and *Clostridia* increased in infants not developing NEC but were displaced by increasing numbers of *Enterobacteriaceae* species in those developing NEC (**Figure 24.31**). Thus, NEC is a type of dysbiosis, the disruption of the normal sequence of gut colonization.

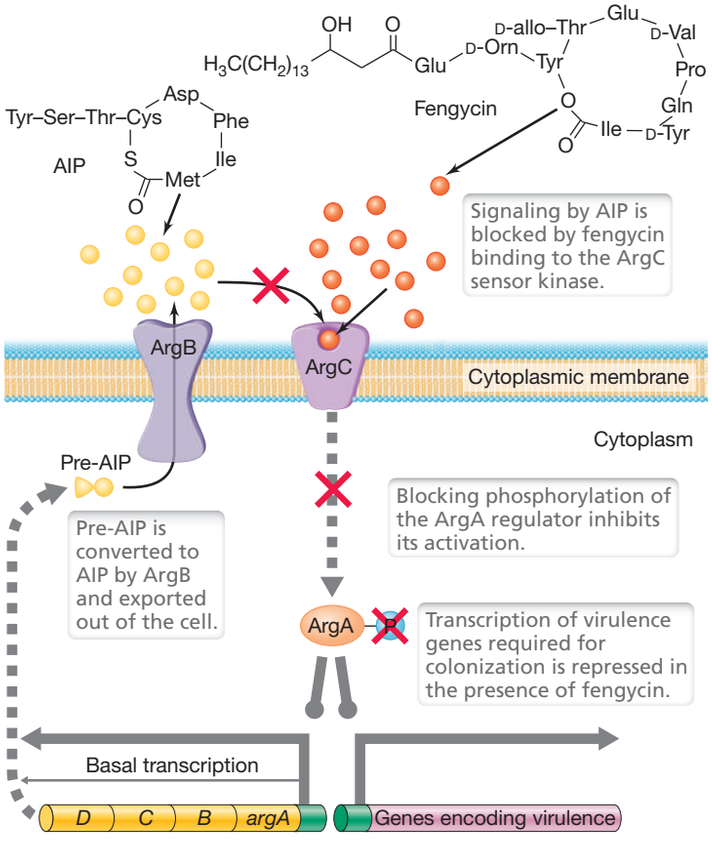


Figure 24.30 Probiotic inhibition of *Staphylococcus aureus* intestinal colonization. Some intestinal *Bacillus* species prevent colonization of the opportunistic pathogen *S. aureus* by producing fengycin, an inhibitor of quorum sensing that is structurally similar to the autoinducing peptide (AIP) made by *S. aureus*. Fengycin binding to the ArgC sensor kinase (◀ Figure 7.20) inhibits AIP binding but does not induce the signal transduction required for virulence gene expression and colonization of the gut by *S. aureus*.

The most effective preventive treatment of NEC has been the probiotic feeding of commercially available species of *Bifidobacterium* and *Lactobacillus*; early treatment with these bacteria reduced the risk of death by 25%, likely by competing for space and resources with pathogenic enteric bacteria. From these promising results, current research is focused on identifying the most effective species and strains in hopes of more precisely targeting probiotic treatment of NEC and getting the gut microbiome of premature infants back on a normal track (Section 24.8).

Prebiotics and Synbiotics

Probiotics should not be confused with *prebiotics*. The **prebiotic** approach to developing a healthy normal microbiota promotes the ingestion of certain nutrients as microbial growth stimulants with the idea that they will nurture particular bacterial species already present in the gut and known to be associated with a healthy colon. Prebiotics are typically carbohydrates that are indigestible by the body but are excellent carbon and energy sources for certain fermentative gut bacteria. Fructooligosaccharides, polymers of the sugar fructose present in many vegetables, are thought to stimulate desirable gut microbes, and the complex polysaccharide *inulin* is widely promoted commercially as a major prebiotic.

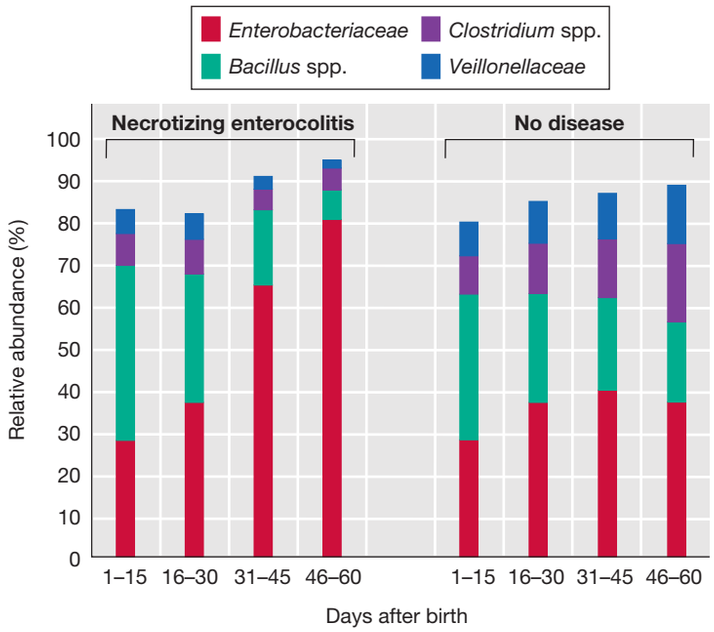


Figure 24.31 Dysbiosis contributing to necrotizing enterocolitis of premature infants. Inflammation and necrosis of the infant's intestine is associated with an overgrowth of enteric bacteria, displacing protective species such as those of the *Veillonellaceae* and the genus *Clostridium*.

Some probiotic formulations (such as certain yogurts) contain prebiotics as well to supply both beneficial aspects in a single treatment; these combined formulations are called **synbiotics**. The prebiotic in a synbiotic is added to promote the growth and colonization of the probiotic organism. The most dramatic success of synbiotics has been in the treatment of sepsis in young children. Sepsis in newborns, a systemic infection originating from an inflammation of the gut similar to NEC, is a major cause of child illness and death in the developing world, causing over half a million deaths a year. A synbiotic containing *Lactobacillus plantarum* and fructooligosaccharide given orally to newborns for 7 days beginning 2 days after birth reduced the risk of death from sepsis by 40% in a trial in India. This again demonstrates the importance of early intervention in gut colonization for preventing dysbiosis with its complications of possible sepsis and later life health problems.

The remarkable success of these treatment examples offers tremendous hope for future development of effective and inexpensive therapeutic agents for the prevention of disease and improved human health. The most successful advances to date have come from careful scientific studies based on our steadily improving understanding of the developing and mature microbiome and its relationship with its human host.

Check Your Understanding

- What is a probiotic? How does it differ from a prebiotic and a synbiotic?
- Why do you think fecal transplants have stimulated renewed research into the development of probiotic and prebiotic therapies?
- Explain how probiotics based on interference of quorum sensing can suppress infection.

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Structure and Function of the Healthy Adult Gastrointestinal and Oral Microbiomes

24.1 The human body is colonized by a diverse assemblage of microorganisms in numbers that approximately equal the total number of cells in the human body. Different body sites provide distinctive habitats that influence the types of microorganisms inhabiting each site. Collectively, these microbes constitute the human microbiome, now recognized to be intimately associated with human health and disease.

Q Why is understanding the nature of the human microbiome such a complex process?

24.2 The human gastrointestinal tract is composed of many segments that differ dramatically in their nutritional and pH characteristics and support quite distinct bacterial populations.

Q How do microbial diversity and abundance vary along the length of the gastrointestinal tract?

24.3 Distinctive groups of microorganisms colonize the mucous membranes and hard surfaces of the mouth and are constantly shed in saliva. The different microhabitats of the mouth (hard and soft surfaces, gingival crevice) sustain a high diversity of both aerobic and anaerobic populations.

Q Why does saliva not serve as a good microbial growth medium?

II • Urogenital Tract and Skin Microbiomes and the Human Viral Microbiome

24.4 With the exception of the vagina and distal urethra, the male and female urogenital tracts harbor no or few microorganisms. The healthy vaginal microbial community is dominated by *Lactobacillus* species, which suppress the growth of other populations by lowering the vaginal pH to about 5 through fermenting glycogen to lactic acid.

Q How does the composition of different species of *Lactobacillus* in the vaginal community impact predisposition to infection?

24.5 The skin is a complex human organ covering about 2 square meters of the body and hosting about 10^{10} microorganisms in the healthy adult. Microbial community composition varies among the diversity of skin sites, generally categorized as moist, dry, or oily. The most common skin microorganisms are bacteria of the phyla *Actinobacteria* and *Firmicutes*.

Q What characteristic of *Propionibacterium* species accounts for their colonization of the sebaceous gland system?

24.6 The human virome consists of the vast diversity of animal and bacterial viruses that colonize the body and its microbiome. The greatest diversity and abundance of viruses is found in the gut, consisting of many novel bacteriophages that infect that complex microbial community. Unlike the environmental virosphere, where phage virions greatly exceed microbial cell numbers, most gut phages are lysogenic and have unknown impact on the gut microbial ecology.

Q What accounts for the low ratio of virions to bacteria in the gut compared to the open environment, such as in the ocean (◀ Section 20.13)?

III • From Birth to Death: Development of the Human Microbiome

24.7 Our current understanding of relationships between the human microbiome and health status is derived from complementary studies of select human study groups and experimental manipulation of animal models, in particular the mouse. Human studies are limited by incomplete control of lifestyle and genetic factors influencing the microbiome. Animal studies are limited by differences from humans in microbiology, anatomy, and physiology.

Q What are the major anatomical differences between mouse and human gastrointestinal systems, and how might those differences influence microbial composition?

24.8 The newborn gut microbial community evolves into a more complex adultlike community over the first 2–3 years of life. Initial colonization is influenced by vaginal or C-section delivery and by feeding of breast milk or formula. The unique oligosaccharides in human breast milk suppress pathogen colonization and select for beneficial microorganisms such as *Bifidobacterium* species. Changes in the gut microbial community with age have been associated with frailty in the elderly.

Q When a child is transitioned from breast milk to solid food, what are the associated changes in the gut microbial community and what physiological differences do those changes reflect?

IV • Disorders Attributed to the Human Microbiome

24.9 The two major disorders associated with changes in the human gut microbial community are inflammatory bowel disease and obesity. Inflammatory bowel disease is associated with dysbiosis, a breakdown in the normal interactions between the human host and its intestinal microbiota reflected by reduced diversity of microbial genes in the diseased state. Obesity is associated with the microbial community composition of the gut, which influences host energy recovery, but obesity is likely not determined by a single contributing microbial or host factor.

Q How are short-chain fatty acids associated with good health as well as with inflammatory bowel disease and obesity?

24.10 Dental caries and periodontitis are diseases of the mouth caused by the combined activities of multiple microbial species, not a single pathogen. Strain variation among *Propionibacterium* species is associated with ability to cause acne, a common cutaneous inflammatory disease of adolescents. The vaginal community is dominated by *Lactobacillus* species that limit colonization by other organisms through lowering pH by production of lactic acid. Although generally very resilient to perturbation, the vaginal community transitions to a more diverse community during menstruation and during incidents of vaginitis or vaginosis.

Q Why might a therapy based on colonization of the skin by selected *Propionibacterium* strains be effective in preventing the development of acne?

V • Modulation of the Human Microbiome

24.11 Antibiotic therapy may predispose an individual to infection by *Clostridiodes difficile*, a toxigenic bacterium that causes severe intestinal disease, because oral antibiotics reduce competition from the normal microbiota of the gut. Infections that cannot be resolved by standard or repeated antibiotic therapy have been shown to respond to fecal transplants, which introduce fecal material from a healthy donor into the gut of the infected individual.

Q Why has the success of fecal transplants in treating *C. difficile* infection encouraged the development of therapies based on microbial ecology for other disorders associated with the human microbiome?

24.12 Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. New understanding of the mechanisms by which organisms such as *Clostridium scindens* and *Bacillus* species suppress pathogen colonization show that targeted probiotics can likely be developed to prevent or treat certain diseases. Prebiotics are nutritional supplements thought to promote the growth of beneficial gut microbes. Synbiotics combine a prebiotic with a probiotic to enhance the efficacy of the probiotic.

Q What is the mechanism by which certain *Bacillus* species suppress colonization by pathogenic *S. aureus*?

APPLICATION QUESTION

1. You are told that you must be placed on a high dose of a broad-spectrum antibiotic to treat a serious infection. You are concerned that this therapy will seriously disrupt your intestinal microbiota, possibly leading to intestinal disease such as that caused by *Clostridiodes difficile*. In advance of the treatment, what might you do to ensure that your normal intestinal microbiota is restored after extended antibiotic therapy? Discuss two ways this restoration could be accomplished, one that would return your exact microbiota and one that would return some representative species.

CHAPTER GLOSSARY

Dysbiosis an alteration or imbalance of an individual's microbiome relative to the normal, healthy state, primarily observed in the microbiota of the digestive tract or the skin

Fecal transplant the transfer of microbiota from the colon of one individual into the colon of another

Host an organism that can harbor pathogenic or beneficial (micro)organisms

Human microbiome the total microbial content in and on the human body

Lower respiratory tract the trachea, bronchi, and lungs

Microbiome a functional collection of different microbes in an environmental system such as the human body

Microbiota the types of organisms present in an environmental habitat, such as the human skin or the human gastrointestinal tract

Mucin a secretion from specialized epithelial cells containing water-soluble glycoproteins and proteins, forming the mucus that retains moisture and impedes microbial invasion on mucosal surfaces

Normal microbiota microorganisms that are usually found associated with healthy body tissue

Prebiotic food additive that promotes the growth and activity of beneficial microorganisms, usually in the gastrointestinal tract

Probiotic a live microorganism that, when administered in adequate amounts, confers a health benefit on the host

Short-chain fatty acids (SCFAs) also called volatile fatty acids or VFAs, these are the major fatty acids (acetate, propionate, and butyrate) produced during fermentation in the large intestine of monogastric animals and the rumen or cecum of herbivores

Synbiotic a mixture of probiotic and prebiotic that beneficially affects the host by altering the microbiome

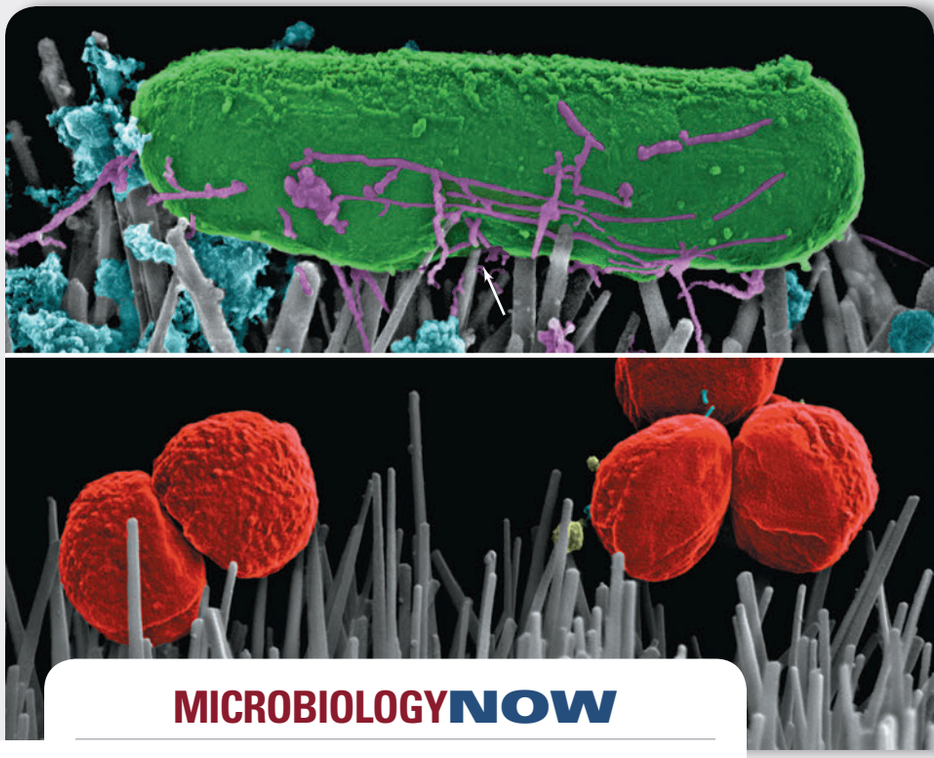
Upper respiratory tract the sinuses, nasal and oral cavities, and structures of the throat—the pharynx, tonsils, and larynx

Virome the entire population of viruses, including animal, microbial, and plant viruses, in an environmental system such as in and on the human body

25 Microbial Infection and Pathogenesis

I Human–Pathogen Interactions 851

II Enzymes and Toxins of Pathogenesis 858



MICROBIOLOGYNOW

Killing Pathogens on Contact

The adherence of pathogenic bacteria to surfaces plays a crucial role in their ability to colonize areas of the body and cause disease. In addition to natural tissue surfaces in the body, such as tooth enamel and mucous membranes, pathogens can also adhere to artificial surfaces associated with implanted medical devices, including catheters, prosthetic heart valves, and artificial joints. The adherence of bacteria to these sites can lead to the formation of biofilms, which are notoriously difficult to treat using antibiotics.

Because of the tendency of pathogenic bacteria to colonize artificial surfaces in the body, prosthetic joint infections (PJIs) are a constant threat in orthopedic medicine. Considering the ineffectiveness of antibiotics in treating these infections, successful treatment of PJIs often requires physical removal of the colonized biofilm from the prosthesis and surrounding tissues in a surgical procedure called *debridement*. To avoid the complications of treating infection, can we find ways to prevent the formation of biofilms that lead to PJIs in the first place?

Taking a lesson from nature itself, researchers are turning to the field of *biomimetics* to address these issues. Several animal and plant tissue surfaces exhibit antibacterial properties, including shark skin, insect wings, and lotus leaves. Such natural surfaces are composed of nanostructures that kill bacteria on contact, likely by disrupting the integrity of their cytoplasmic membranes. Borrowing this concept, antibacterial nanofabrication methods have centered on titanium dioxide (TiO_2) as a potential substrate to mimic natural contact killing. Although TiO_2 has shown limited potential for killing gram-positive cocci, such as *Staphylococcus aureus* (bottom photo), it has shown considerable promise in destroying gram-negative bacteria on contact, including *Escherichia coli*, in which the spiky nanostructures cause indentation and eventual puncture of the cells (arrow, top photo).

If artificial joints and other medical implants can be manufactured with surfaces having antibacterial nanostructures, PJIs and other biofilm-associated infections may become much easier to prevent and control—no antibiotics necessary.

Source: Jenkins, J., et al. 2018. Characterization of bactericidal titanium surfaces using electron microscopy. *Microsc. Anal. (Am. Ed.).* *32*: 15.

I • Human–Pathogen Interactions

To induce pathogenesis and begin to parasitize the host, pathogenic microbes must first adhere to host tissues and overcome immune defenses that seek to inhibit microbial colonization and invasion.

Humans are exposed to microorganisms of all sorts in their environment. Whether one is walking outdoors, sitting indoors, or participating in any type of physical activity, environmental microbes and humans interact. The human body is also a natural home to enormous numbers of microorganisms, as we saw in Chapter 24. Most of these are harmless, and in fact essential, with only a small percentage capable of causing disease. However, those that do, along with pathogens that are not part of the normal human microbiota, possess specific traits that underlie their pathogenic lifestyles. A major focus of this chapter will be a consideration of these specific traits and how they induce the diseased state.

25.1 Microbial Adherence

In the world of infectious diseases, the term **infection** is used to imply the growth of microorganisms on or in the host, whereas the term **disease** is reserved for actual tissue damage or injury that impairs host function. If a **pathogen** gains access to the specific tissues it infects, disease will occur only if it first adheres to those tissues, multiplies to yield many cells or viral particles, and then proceeds to damage tissues (or the entire organism) by the release of toxic or invasive substances (**Figure 25.1**). Adherence is the first step, and although adherence is *required* to initiate disease, it is not *sufficient* to initiate disease because the host has many innate defenses that can thwart infection; we consider these in Chapter 26.

Adherence Molecules

Pathogens typically adhere to epithelial cells through specific interactions between molecules on the pathogen and molecules on the host tissues. In addition, pathogens may adhere to each other, forming biofilms (◀ Sections 4.9 and 20.4), with the biofilm itself adhering to specific tissues. In medical microbiology, **adherence** is the enhanced ability of a microorganism to attach to a cell or a surface. Pathogens gain access to host tissues by way of a *portal of entry* of one sort or another. These include mucous membranes, the skin surface, or under mucous membranes or the skin during penetration of these sites from puncture wounds, insect bites, cuts, or other abrasions. The portal of entry may be critical for the establishment of an infection because a pathogen that gains access to incompatible tissues is typically ineffective. For example, if cells of the bacterium *Streptococcus pneumoniae* are swallowed, they will be killed by the strong acidity of the stomach, whereas if the same cells reach the respiratory tract, they could trigger a fatal case of pneumonia.

Receptor molecules coating the surfaces of both the pathogen and cells of its host are often critical for adhering the pathogen to host tissues. Specific receptors can be important for the binding of any type of pathogenic microbe, including bacteria, viruses, and parasites (**Figure 25.2**). Receptors on pathogens have evolved to bind specifically to complementary molecules in the host, and the complementary nature of the receptors on pathogen and host cells alerts the pathogen that it has arrived on a suitable infection site. Receptors on the pathogen surface are called **adhesins** and are composed of glycoprotein or lipoprotein covalently bound to the outer

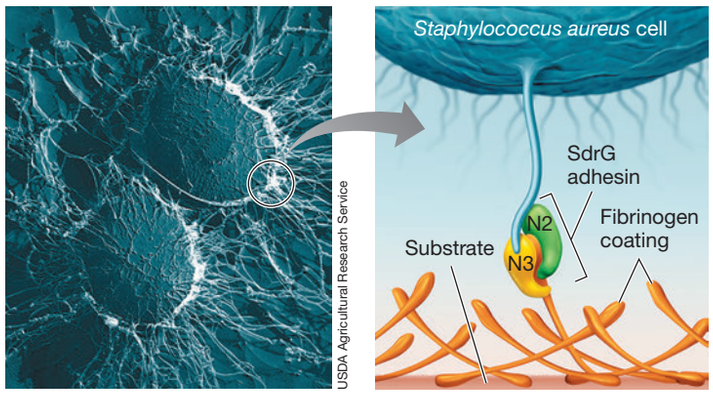


Figure 25.2 Bacterial adherence. A bacterial pathogen attaches specifically to host tissues by way of complementary receptors on the bacterial and host surfaces. *(a)* Cryogenic transmission electron micrograph of the gram-positive bacterium *Staphylococcus aureus* adhering to an extracellular matrix of fibrinogen, which the cells convert to a fibrin clot as a defense against host immune responses (see also Figure 25.12*b*). The *S. aureus* cells are about 0.75 μm in diameter. *(b)* Expanded diagram of *S. aureus* binding fibrinogen using the N2 and N3 domains of its SdrG adhesin protein.

Mastering Microbiology
Art Activity: Figure 25.1 Microbial pathogenesis

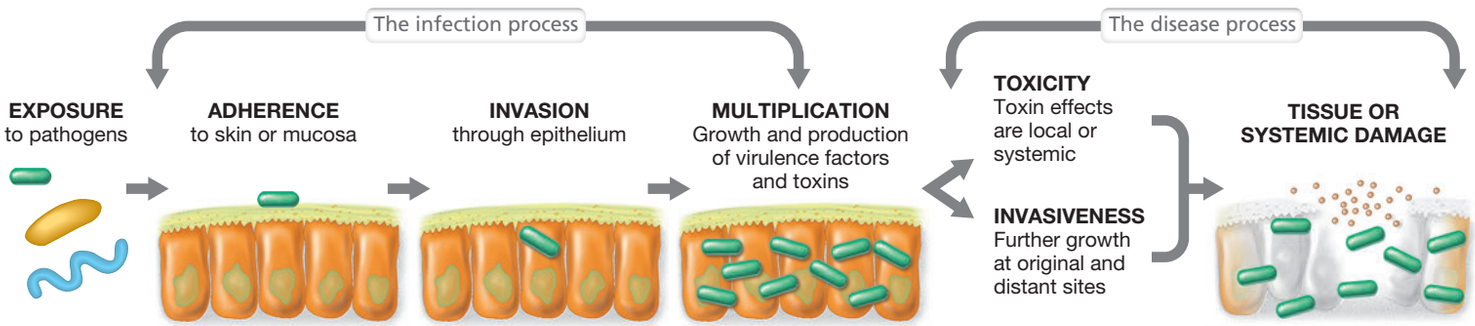


Figure 25.1 Microbial pathogenesis. Following exposure to a pathogenic microbe, a series of events leads to infection, and a further series of events results in disease.

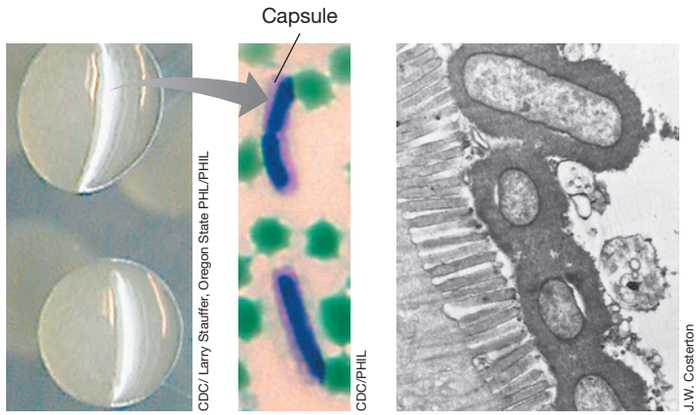


Figure 25.3 The bacterial capsule as a facilitator of pathogen attachment. *(a) Bacillus anthracis* growing on an agar plate. The mucoid colonies of encapsulated cells are about 0.5 cm in diameter. *(b)* Light micrograph of cells of *B. anthracis* growing in horse blood and stained to show the capsule (dark pink surrounding blue cell). Cells are about 1 μm in diameter. *(c)* Cells of enteropathogenic *Escherichia coli* attached to the brush border of intestinal microvilli by way of a distinct capsule. The *E. coli* cells are about 0.5 μm in diameter.

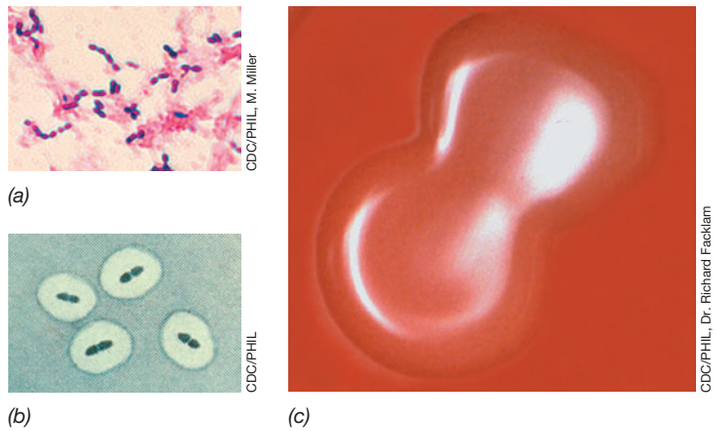


Figure 25.4 Capsules and colonies of *Streptococcus pneumoniae*. *(a)* Gram stain of *S. pneumoniae* cells; capsules are not visible. *(b) S. pneumoniae* treated with anticapsular antibodies (Quellung reaction) that make the capsule visible. *(c)* Colonies of encapsulated *S. pneumoniae* cells grown on blood agar show a mucoid morphology with a sunken center. The colonies are about 2–3 mm in diameter and a single cell of *S. pneumoniae* is about 0.8 μm in diameter.

layer of the cell (Figure 25.2*b*). In addition to extracellular matrices, such as the fibrinogen shown in Figure 25.2, host receptors for bacterial adhesins include cell surface glycoproteins or complex membrane lipids, such as gangliosides or globosides (both are sphingolipids containing sugars and other molecules).

Adherence Structures: Capsules, Fimbriae, Pili, and Flagella

Some adhesins form part of an outer cell surface structure that may or may not be covalently linked to components of the cell wall. For example, some notable pathogenic bacteria form a **capsule**. In *Bacillus anthracis* (the bacterium that causes anthrax), the capsule is composed of polypeptide containing only the amino acid D-glutamate. The capsule of *B. anthracis* can be seen in cells by light microscopy, and the encapsulated cells form smooth, slimy colonies when grown on agar plates (**Figure 25.3**). The electron microscope can also clearly reveal bacterial capsules (Figure 25.3*c*). The capsule surface contains specific receptors that facilitate adherence to host tissues, but the inherently sticky nature of the capsule itself also assists in the overall attachment process. Although capsules are important for adherence of some pathogens to host tissues, many important pathogens, such as *Vibrio cholerae*, the causative agent of the disease cholera, lack them.

Besides adherence, capsules are important for protecting pathogenic bacteria from host defenses. For example, the only known virulence factor for *Streptococcus pneumoniae* (bacterial pneumonia) is its polysaccharide capsule (**Figure 25.4**). Encapsulated strains of *S. pneumoniae* grow abundantly in the lungs where they initiate host responses that interfere with lung function, cause extensive host damage, and can cause death (▶ Section 31.2). By contrast, nonencapsulated strains of *S. pneumoniae* are quickly and efficiently ingested and destroyed by phagocytes, white blood cells that ingest and kill bacteria by a process called *phagocytosis* (▶ Sections 26.5–26.7).

Many pathogens selectively adhere to particular types of cells through cell surface structures other than adhesins, capsules, or slime layers. For example, *Neisseria gonorrhoeae*, the pathogen that causes the sexually transmitted disease gonorrhea (▶ Section 31.13), adheres specifically to mucosal epithelial cells in the genitourinary tract, eye, rectum, and throat; by contrast, other tissues are not infected. *N. gonorrhoeae* has a cell surface protein called Opa (*o*pacity *a*ssociated *p*rotein) that binds specifically to a host protein found only on the surface of epithelial cells of these body regions, allowing adherence of the pathogen to host cells. Likewise, influenza virus targets upper respiratory tract mucosal cells and attaches specifically to these and later to lung epithelial cells by way of the protein hemagglutinin present on the virus surface (◀ Section 11.9 and ▶ Section 31.8).

Fimbriae and pili are bacterial cell surface protein structures (◀ Section 2.6) that function in attachment (**Figure 25.5**). For instance, along with Opa, the pili of *Neisseria gonorrhoeae* play a key role in attachment to urogenital epithelia, and fimbriated strains of *Escherichia coli* are more frequent causes of urinary tract infections than strains lacking fimbriae. Among the best-characterized fimbriae are the *type I fimbriae* of enteric bacteria (*Escherichia, Klebsiella, Salmonella,* and *Shigella*), which are uniformly distributed on the surface of cells (Figure 25.5). Pili are typically longer and fewer in number than fimbriae, and in addition to attachment, some pili function in the bacterial genetic transfer process of conjugation (◀ Section 9.8). Both pili and fimbriae function by specifically binding to host cell surface glycoproteins, thereby initiating adherence. Flagella may also facilitate adherence of bacterial cells to host cells (Figure 25.5), although their role is thought to be less important than that of fimbriae and pili.

Check Your Understanding

- Distinguish between infection and disease.
- What event is required but not sufficient to cause an infectious disease?
- Describe the molecules or structures that facilitate pathogen adherence to host tissues.

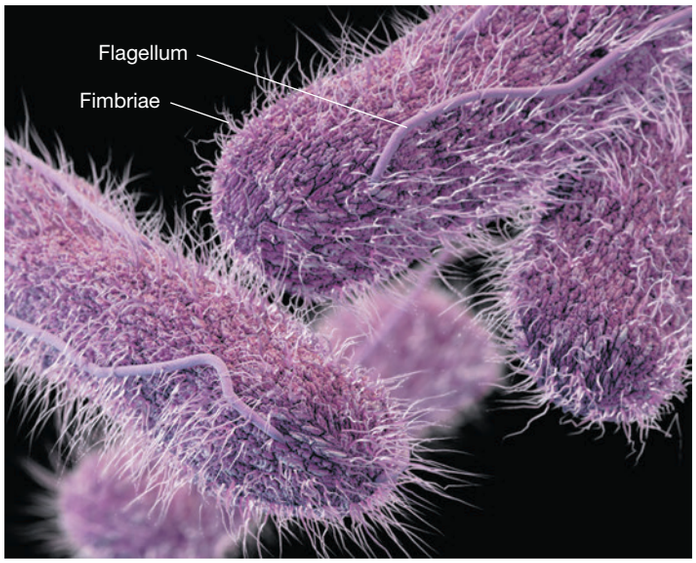


Figure 25.5 Fimbriae. Computer-generated image of a scanning electron micrograph of cells of *Salmonella enterica* (*typhi*) showing the numerous thin fimbriae and the much thicker peritrichously arranged flagella. A single cell is about 1 μm in diameter.

25.2 Colonization and Invasion

If a single pathogenic virus or cell attaches to its specific host tissue, it alone is insufficient to cause disease; the pathogen must establish residence there and multiply. **Colonization**, the growth of a microorganism after it has gained access to host tissues, begins at birth as a newborn child is naturally exposed to a suite of harmless (and in many cases necessary) bacteria and viruses that will be the infant's initial normal microbiota (Chapter 24).

The human body is rich in organic nutrients and provides conditions of controlled pH, osmotic pressure, and temperature that are favorable for the growth of microorganisms. However, each body region, such as the skin, respiratory, gastrointestinal, and genitourinary tracts, differs chemically and physically from others, and thus provides a selective environment for the growth of certain microbes and not others. The result is that pathogens show rather rigid tissue specificities (▶ Table 26.1), and this reality is often helpful in the diagnosis of microbial infections.

Colonization typically begins at sites in the **mucous membranes** (**Figure 25.6**). Mucous membranes are composed of *epithelial cells*, tightly packed cells that interface with the external environment. They are found throughout the body, lining the urogenital, respiratory, and gastrointestinal tracts. The epithelial cells in mucous membranes secrete **mucus**, a thick liquid secretion that contains water-soluble proteins and glycoproteins. Mucus retains moisture and naturally inhibits microbial attachment because most microbes are swept away by physical processes, such as swallowing or sneezing. Nevertheless, some microbes—both pathogens and nonpathogens—adhere to the epithelial surface and colonize. If these attached microbes are pathogens, it sets the stage for infection, invasion, and disease (Figure 25.1). Nonpathogenic microbes that penetrate mucous membranes include large numbers—potentially many billions—of innocuous bacteriophages that contribute to what has been called the "intrabody phageome." Although poorly understood, the human phageome may play a role in modulating inflammation and other immune responses, which may have implications in controlling autoimmune diseases such as type 1 diabetes and Crohn's disease (▶ Section 28.1).

Growth of the Microbial Community: An Example from Human Dental Caries

Infection requires growth of the pathogen after it has attached to and colonized a surface (Figures 25.1 and 25.6), and the actual disease process may not be the result of a single type of microbe but of a community of interacting microorganisms. An example of this is found in the oral microbial disease **dental caries** (tooth decay), where attachment and infection have been well studied as models of these key events in the disease process.

Even on a freshly cleaned tooth surface, acidic glycoproteins from the saliva form a thin organic film several micrometers thick; this film provides an attachment site for bacterial cells, and oral streptococci quickly colonize it. These include in particular the two *Streptococcus* species most often implicated in tooth decay: *S. sobrinus* and *S. mutans*. Both of these organisms produce a capsule (Section 25.1). The *S. sobrinus* capsule contains adhesins specific for host salivary glycoproteins (**Figure 25.7*a, b***), whereas *S. mutans* resides in crevices and small fissures where it relies on dextran—a strongly adhesive exopolysaccharide—that it produces to secure cells to the tooth and gum surface (Figure 25.7*c, d*). Both *S. sobrinus* and *S. mutans* are lactic acid bacteria (◀ Section 16.6) that ferment glucose to lactic acid, the agent that destroys tooth enamel. However, the trigger for decay activities is *sucrose* (table sugar), since it is sucrose that allows these species to produce the dextran exopolysaccharide and capsules necessary for attachment and colonization.

Extensive bacterial growth of these oral streptococci results in a thick biofilm called **dental plaque** (Figure 25.7). Using phylogenetic probes, it has been possible to more thoroughly explore the microbial diversity of dental plaque, and it is clear that the two *Streptococcus* species are not the entire story. Many other gram-positive and gram-negative *Bacteria* are present in plaque, including species of *Corynebacterium, Porphyromonas, Leptotrichia, Neisseria*, the

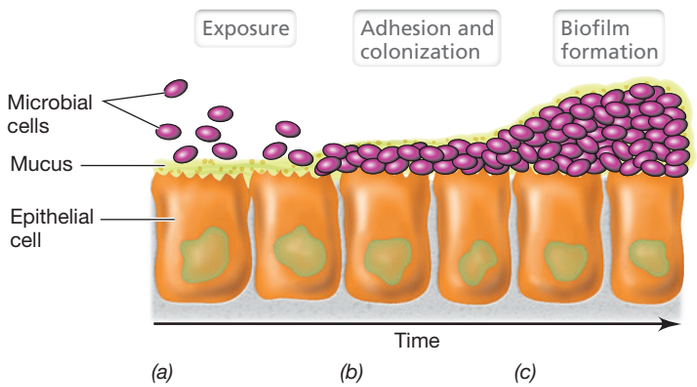


Figure 25.6 Bacterial interactions with mucous membranes. *(a)* Loose association upon initial exposure. *(b)* Penetration of mucus leading to adhesion and colonization. *(c)* Further colonization leading to biofilm formation.

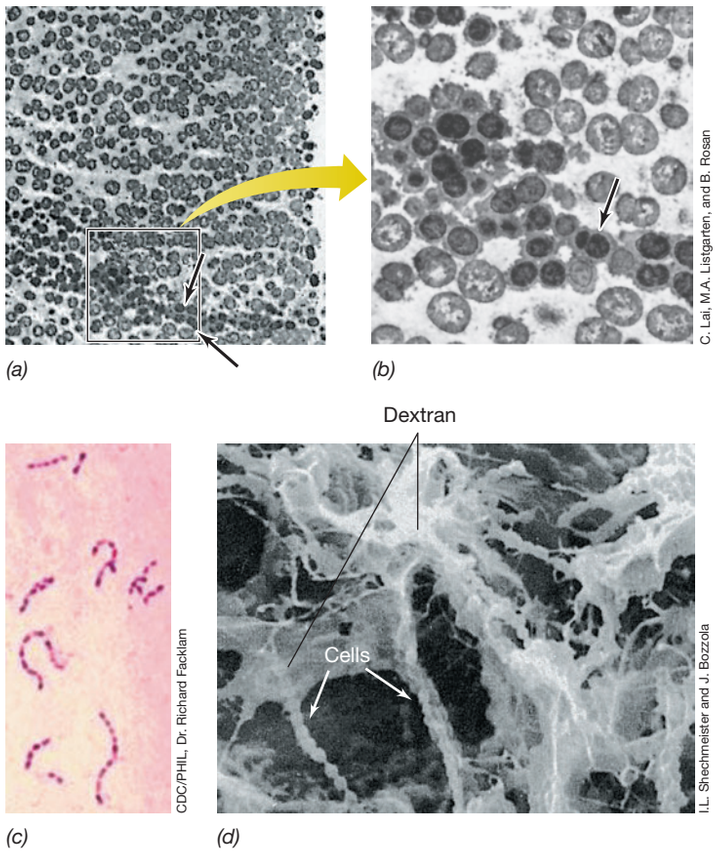


Figure 25.7 Cariogenic *Streptococcus* spp. and dental plaque. *(a)* The low-magnification micrograph shows predominantly streptococcal cell morphology embedded in dental plaque. The species *Streptococcus sobrinus* (arrows) appears darker from a specific staining technique. *(b)* Higher-magnification micrograph showing a region in the plaque with *S. sobrinus* cells (dark, arrow). Note the extensive capsule (lighter grey area) surrounding the *S. sobrinus* cells. *(c)* Light micrograph of a *Streptococcus mutans* culture showing the characteristic cell chains of streptococci. *(d)* Scanning electron micrograph of the sticky dextran material that holds cells together in filaments. Individual cells of both *S. sobrinus* and *S. mutans* are about 1 μm in diameter.

filamentous anaerobe *Fusobacterium*, and many others (**Figure 25.8**). Moreover, the different species likely play specific structural and functional roles in mature dental plaque. This can be seen in FISH-stained (◀ Section 19.5) sections of plaque, where filamentous streamers of cells of *Corynebacterium* attached to a thin biofilm on the tooth surface anchor cells of *Streptococcus* and other bacteria a short distance away from the tooth surface (Figure 25.8). Such an arrangement probably allows *Streptococcus* cells to extend out from the tooth surface into regions of the oral cavity where saliva, sugars, and other nutrients are more abundant.

Dental plaque is thus a complex mixed-culture biofilm composed of several different genera of *Bacteria* and their accumulated products. A few *Archaea* are also present in dental plaque, primarily methanogenic species such as *Methanobrevibacter oralis*. As dental plaque accumulates, the microbiota secrete locally high concentrations of lactic acid that decalcifies tooth enamel, resulting in dental caries. Tooth enamel is strongly calcified tissue, and the ability of microbes to invade this tissue plays a major role in the extent of dental caries and related, often more serious, oral pathologies, including *gingivitis* and *periodontitis*, bacterial diseases that erode the tooth-supporting gum and bone tissues.

Invasion and Systemic Infection

In the case of dental caries, the bacterial infection primarily resides on the tooth and gum surfaces. By contrast, in most infectious diseases, the pathogen must invade past the tissue surface in order to promote disease. **Invasion** is the ability of a pathogen to enter into host cells or tissues, spread, and cause disease. Some pathogens remain localized after initial entry, multiplying and invading at a single focus of infection, such as the boil that may arise from *Staphylococcus* skin infections (▶ Section 31.9). However, sometimes the pathogens enter the bloodstream, from where they can travel to distant parts of the body. Depending on the pathogen and the condition of an individual and that individual's immune system, the presence of bacteria in the blood can have mild or highly severe consequences.

The mere presence of bacteria in the blood is called **bacteremia**; this condition is typically self-limiting and asymptomatic, as the bacterial cells do not grow in the bloodstream, and the immune system quickly removes them. By contrast, in **septicemia** bacteria multiply in the bloodstream, and the organism spreads systemically

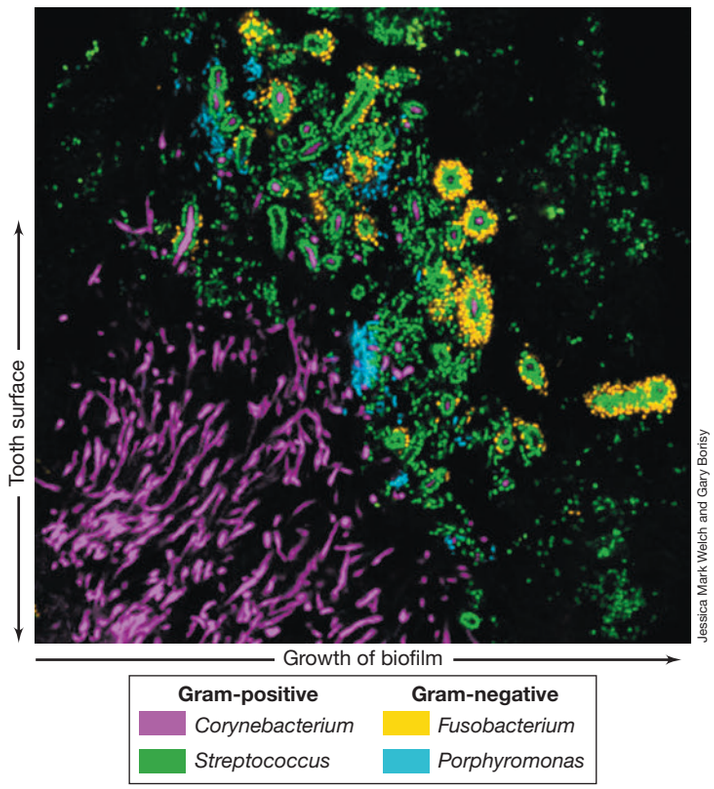


Figure 25.8 Bacterial diversity of dental plaque. Confocal micrograph of a FISH-stained (◀ Section 19.5) section through human dental plaque using a suite of phylogenetic probes containing different fluorescent tags. Color matching to specific groups is shown below the photo. The tooth surface would be on the left with the filamentous *Corynebacterium* species (purple) containing attached streptococci (green) flaring out from an attachment site on the tooth surface.

from an initial focus and produces toxins or other poisonous substances. Septicemia usually begins as an infection in a specific organ such as the intestine, kidney, or lung, and then spreads rapidly throughout the body from there. Septicemia is typically associated with major symptoms and may lead to massive inflammation, culminating in septic shock (sepsis) and death. *Viremia* is the term used to describe viruses present in the bloodstream, and measles, a highly infectious disease in those not vaccinated (▶ Section 31.6), is a good example of a systemic viremia.

A pathogen that causes disease in a given host can trigger mild or severe outcomes depending on its inherent capacity to elicit disease. We consider the principles that govern these capacities now with a focus on the important infectious disease concept called *virulence*.

Check Your Understanding

- At what body sites do pathogens typically attach and colonize?
- How does the capsule of *Streptococcus mutans* assist in the formation of dental caries?
- Which is the more serious condition, bacteremia or septicemia, and why?

25.3 Pathogenicity, Virulence, and Virulence Attenuation

Unique properties of each pathogen contribute to its **pathogenicity**, the overall ability to cause disease. The measure of pathogenicity is called **virulence**, the relative ability of a pathogen to cause disease. Pathogenicity and virulence are not uniform properties of a given pathogen and can differ dramatically between different strains of the same bacterial species or virus. Highly virulent strains of a given pathogen tend to emerge every so often and when they do, they often trigger a particularly severe course of disease that is rapid and widespread, potentially resulting in an epidemic or pandemic (Chapter 30). The virulence of a given pathogen depends on a number of factors including its relative abilities to adhere, colonize, and invade (Figure 25.1), as well as its arsenal of virulence factors.

Virulence

Virulence is the net outcome of host–pathogen interactions, a dynamic relationship between the two organisms influenced by ever-changing conditions in the pathogen, the host, and the environment. Host damage in an infectious disease is mediated by **virulence factors**, toxic or destructive substances produced by the pathogen that directly or indirectly enhance invasiveness and host damage by facilitating and promoting infection. The second part of this chapter is devoted to major virulence factors.

Virulence is a quantifiable entity, especially if a pathogen is lethal and an experimental animal model is available. For example, the LD_{50} (LD stands for "lethal dose") is defined as the number of cells of a pathogen (or virions, for a viral pathogen) that kills 50% of the population of the host organism in a test group. Highly virulent pathogens frequently show little difference in the number of cells required to kill 100% of a test group as compared with the LD_{50}. To illustrate this, recall the foundational work of the British microbiologist Frederick Griffith (◀ Section 1.14 and Figure 1.38). Griffith worked with the gram-positive bacterium *Streptococcus pneumoniae* and discovered that strains of *S. pneumoniae* that contained a capsule ("smooth" strains because they formed smooth colonies on plates) were highly virulent for mice, whereas mutant derivatives lacking a capsule ("rough" strains) were not. The *S. pneumoniae* capsule is the primary virulence factor of this bacterium because it helps the bacterium evade immune surveillance. Griffith's key discovery was that the smooth phenotype could be transferred to rough cells by treating rough cells with an extract from smooth cells (◀ Figure 1.38). This was the first experimental example of transformation, a bacterial genetic transfer process (◀ Section 9.6), and the transforming constituent in the extract was later shown (by other scientists) to be DNA.

Griffith's choice of experimental organism was fortuitous because *S. pneumoniae* is both readily transformable and highly virulent for mice. Only a few cells of an encapsulated strain of *S. pneumoniae* can establish a fatal infection and kill all mice in a test population. As a result, the LD_{50} for *S. pneumoniae* in mice is not proportional to the number of cells delivered (**Figure 25.9**). By contrast, the number of cells of a less virulent pathogen, such as the gram-negative enteric bacterium *Salmonella enterica* (*typhimurium*), necessary to kill all of the mice in the test population is about 10,000-fold greater than the highly virulent *S. pneumoniae* cells, and the LD_{50} is proportionally related to the number of cells of the pathogen injected into the mice (Figure 25.9).

Mastering Microbiology
Art Activity: Figure 25.9 Microbial virulence

There are many examples of highly virulent human pathogens, especially among viruses. For example, some strains of the influenza virus are so highly virulent that only a few virions can initiate

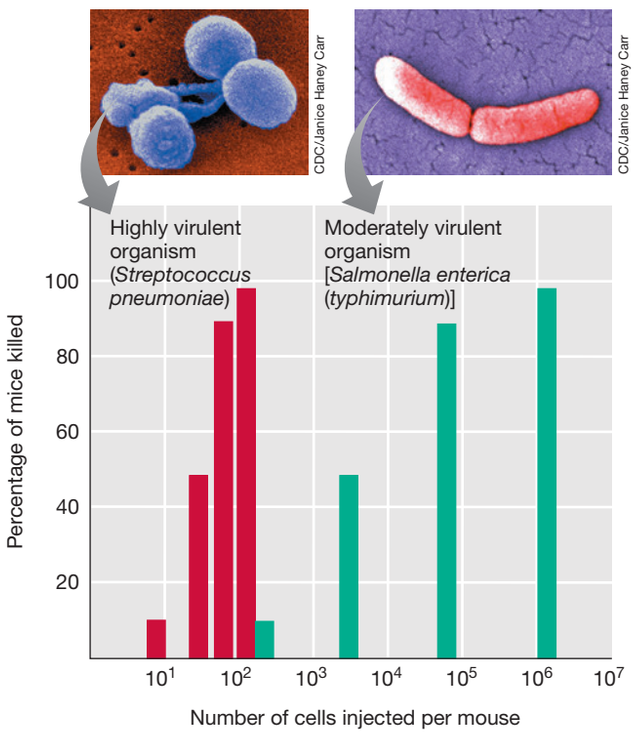


Figure 25.9 Microbial virulence. Differences in microbial virulence demonstrated by the number of cells of *Streptococcus pneumoniae* (red bars) and *Salmonella enterica* (*typhimurium*) (green bars) required to kill mice. A colorized scanning electron micrograph of each bacterium is shown above its respective graph.

disease even though mortality rates are typically low. Ebola virus is also highly virulent, and the tiny inoculum necessary to initiate disease often results in a fatal infection. By contrast, the bacterial pathogen *Vibrio cholerae* (which causes cholera) is not especially virulent, as a large inoculum of this intestinal pathogen is necessary to initiate disease (▶ Section 33.3).

Attenuation

The virulence of a pathogen can change. *Attenuation* is the decrease or loss of virulence of a pathogen (**Figure 25.10**). When pathogens are kept in laboratory culture rather than isolated from diseased animals, their virulence often decreases, or may be completely lost. Strains that have either a reduced virulence or are no longer virulent are said to be **attenuated**. Attenuation is thought to occur because nonvirulent or weakly virulent mutants grow faster than virulent strains in laboratory media where virulence has no selective advantage. After successive transfers in fresh media, such mutants are therefore selectively favored. However, if an attenuated culture is reinoculated into an animal, the organism may regain its original virulence, especially with continued in vivo passage as more-virulent strains are naturally selected. In some cases, though, the loss of virulence is permanent. For example, if a deletion mutation led to a major modification of a required receptor molecule (Figure 25.2*b*) or to the inability to produce a key virulence factor, such as the production of a toxin or invasive enzyme, then the mutant strain would be permanently attenuated.

Attenuated strains of various pathogens are valuable to clinical medicine because they are often used for the production of vaccines, especially viral vaccines. This principle was first demonstrated in the 1880s by Louis Pasteur with his remarkable development of the first rabies vaccine (◀ Section 1.11 and Figure 1.32*a*), and since that time, attenuated viral strains have also been employed in the production of vaccines for measles, mumps, rubella, chicken pox/shingles, and yellow fever (Figure 25.10). Although attenuated viruses are "live" in the sense that, unlike "killed" strains, they are capable of replication and could in principle become virulent once again, properly attenuated virus vaccines (those free of any unattenuated virions) typically show greater efficacy and generate an overall stronger immune response than do killed virus vaccines.

Check Your Understanding

- What are virulence factors? How can the LD_{50} test be used to define virulence of a pathogen?
- What circumstances can contribute to attenuation of a pathogen?

25.4 Genetics of Virulence and the Compromised Host

The virulence of a bacterial pathogen and the eventual outcome of an infectious disease are the net result of genetic and physiological features of both the pathogen and the host. In the case of the host, a pathogen may infect a healthy, well-rested young adult or an individual compromised by an ailment, such as a physiological condition (old age, hospitalization, immune suppression), an ongoing infectious disease (for example, acquired immunodeficiency syndrome [AIDS] caused by the human immunodeficiency virus [HIV]), or a genetic disease (for example, cystic fibrosis). The outcome of infection—health or disease—may be very different in these different individuals, even if they are infected by the same strain of a viral or bacterial pathogen.

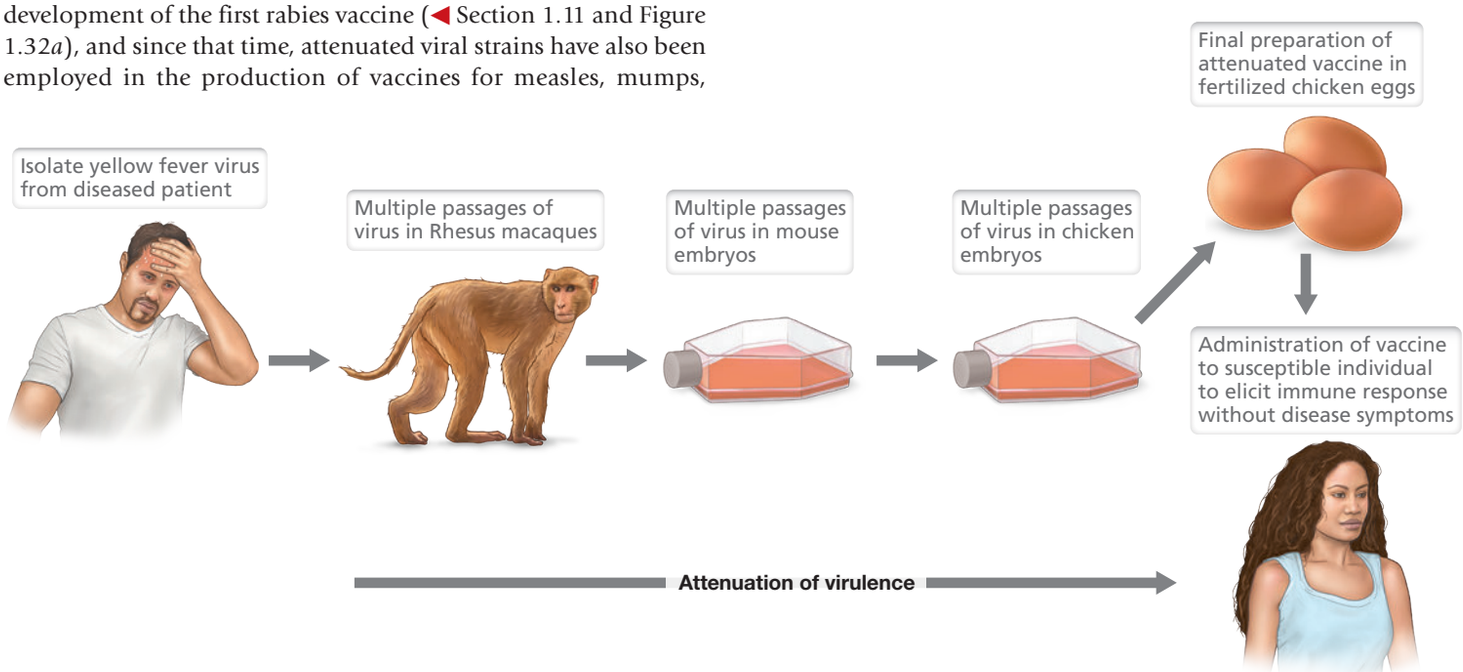


Figure 25.10 Attenuation of virulence in vaccine production. To produce the yellow fever vaccine, the isolated virus is attenuated by dozens of passages in hosts that are increasingly dissimilar to the original human host, with the final preparation occurring in embryonated chicken eggs. With each successive passage, the virus gradually becomes more adapted to its new host, while simultaneously becoming less virulent to the original human host. When introduced to a susceptible individual, the weakened viral strain elicits a protective immune response to yellow fever without causing symptoms of the disease itself.

The virulence of a pathogen may be encoded by firmly entrenched chromosomal genes or by highly mobile genetic elements. For example, the gram-negative bacterium *Bordetella pertussis*, the causative agent of whooping cough (pertussis, ▶ Section 31.3), makes several toxins including pertussis toxin, a potent AB-type exotoxin (Section 25.6); collectively, these toxins trigger the symptoms of whooping cough. Other species of *Bordetella* do not make pertussis toxin, and the chromosomal gene encoding pertussis toxin does not readily move from *B. pertussis* to other species. But in contrast to *B. pertussis*, some bacterial pathogens routinely exchange genes encoding virulence factors with different bacterial species or even genera, and thus highly related versions of their most potent weapons may appear in several different pathogens. *Salmonella* is a well-studied example of the genetic transfer of virulence factors, and we focus on this bacterium now.

Virulence in *Salmonella*: Pathogenicity Islands and Plasmids

Salmonella species infect humans, leading to various gastrointestinal illnesses (▶ Section 33.10), and they encode a large number of virulence factors that are important in disease. These include type I fimbriae (Section 25.1) to facilitate attachment of cells to gastrointestinal tissues; several different classes of exotoxins (Section 25.6); antiphagocytic proteins that block engulfment of bacterial cells by host phagocytes; proteins that promote survival if the bacterium does get phagocytosed; siderophores, organic molecules that bind iron tightly and, in pathogenic bacteria, allow the bacteria to outcompete host sequestration systems for iron; and endotoxin (Section 25.8). With the exception of endotoxin, many of these virulence factors are encoded by genes present on mobile DNA rather than on the cell's chromosome (**Figure 25.11**).

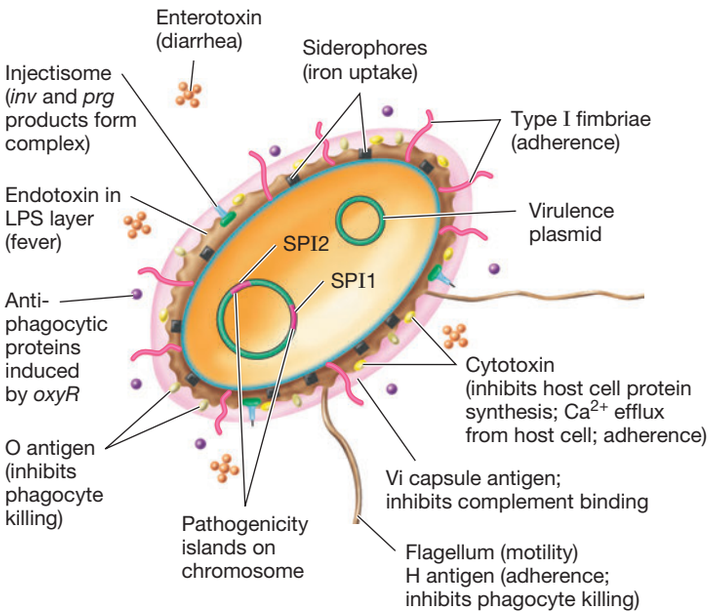


Figure 25.11 Virulence factors in *Salmonella*. Factors important for virulence and the development of pathogenesis in this gram-negative enteric pathogen are shown. Genes encoding many of the factors reside on the pathogenicity islands or plasmids.

Several genes that encode these virulence factors in *Salmonella* and related gram-negative pathogens, such as pathogenic strains of *Escherichia coli* (▶ Section 33.11), are found clustered together on the chromosome as *pathogenicity islands*. *Salmonella* pathogenicity island 1 (SPI1) is a cluster of genes that encodes over 10 distinct proteins that promote virulence and invasion. One of these is *invH*, a gene encoding a surface adhesion protein (Section 25.1). Several *inv* genes encode proteins important for trafficking of virulence factors. For example, the InvJ regulator protein controls assembly of structural proteins InvG, PrgH, PrgI, PrgJ, and PrgK, which form a type III secretion system called the *injectisome*, an organelle in the bacterial envelope that allows for the direct transfer of virulence proteins into host cells through a needle-like assembly (◀ Section 6.13 and Figures 6.43 and 6.44).

A second *Salmonella* pathogenicity island, SPI2, contains genes that are responsible for causing more systemic than localized disease and resistance to host defenses. In addition, several plasmid-encoded virulence factors, such as antibiotic resistance genes encoded on R plasmids (◀ Section 6.2), can spread between *Salmonella* species and other genera of enteric bacteria. Pathogenicity islands and R plasmids allow for the facile and rapid transfer of virulence factors. It is thus not uncommon for genes encoding factors in one pathogen to be similar, if not identical, to those in another because of transfer of parts or all of the islands or plasmids between species by horizontal gene exchange (Chapter 9).

In the well-known opportunistic pathogen (see next subsection) *Pseudomonas aeruginosa*, pathogenicity islands can also contain genes encoding antibiotic resistance. As for *Salmonella*, many cases of multiple antibiotic resistance in *P. aeruginosa* are linked to plasmids. However, in some strains of *P. aeruginosa*, genomic islands containing transposable elements (◀ Section 9.11) are present. By transposition these islands have "captured" multiple antibiotic resistance genes and can then disseminate them to other organisms; the genomic islands are present in *P. aeruginosa* in place of or in addition to resistance plasmids. Regardless of how they are encoded, these strains have become resistant to virtually all of the clinically useful antibiotics that have traditionally been used to control *P. aeruginosa* infections. This is a particularly serious problem for the compromised host and in the hospital environment, and we consider these issues now.

Mastering Microbiology
Art Activity: Figure 25.10 Virulence factors in *Salmonella*

The Compromised Host

Some individuals are simply more susceptible to infection than others for reasons that have little to do with the virulence of the pathogen. These so-called *compromised hosts* are individuals in which one or more mechanisms of resistance to disease are inactive and in whom the probability of infection is therefore increased. Many hospital patients with noninfectious diseases (for example, cancer and heart disease) acquire microbial infections more readily because they are compromised hosts. Such *healthcare-associated infections* (also called *nosocomial infections*; ▶ Section 29.2) affect up to 2 million individuals each year in the United States, with a nearly 5% mortality rate. Invasive medical procedures such as catheterization, hypodermic injection, spinal puncture, biopsy, and surgery may unintentionally introduce

microorganisms into the patient. The stress of surgery and the anti-inflammatory drugs given to reduce pain and swelling can also reduce host resistance.

Some factors can compromise host resistance outside the hospital, including lifestyle choices that affect major organs of the body, such as intravenous drug usage, tobacco use, and excessive consumption of alcohol, or genetic diseases that eliminate parts of the immune system (▶ Section 28.2). People that are physically compromised for any of a number of reasons may be more susceptible to infections, not only because they are physically weakened but also because their living conditions and lifestyle choices may put them in more continual contact with infectious agents. For example, infection with the human immunodeficiency virus (HIV) predisposes an individual to infections from **opportunistic pathogens**, microbes that cause disease only in the absence of normal host resistance. HIV causes AIDS by destroying a specific class of immune cell, the CD4 T lymphocytes (▶ Section 31.15), which are key to an effective immune response. The reduction in CD4 T cells reduces immunity, and an opportunistic pathogen—one that does not cause disease in a healthy, uninfected host—can then cause serious disease or even death. Individuals with immunodeficiencies from underlying genetic causes rather than infection are also more susceptible to opportunistic infections because part of their immune system is either nonfunctional or suboptimal.

The outcome of an infectious disease thus depends on several factors, both host and pathogen related. Two individuals exposed to the same pathogen in the same way may well show different outcomes. But once an infection has proceeded to the actual stage of disease, the symptoms that appear are due to harmful products of the pathogens, and we turn our attention to these now.

Check Your Understanding

- What major virulence factors are produced by *Salmonella*?
- What is an opportunistic pathogen? What steps can a person take to help avoid opportunistic infections?
- What is a nosocomial infection?

II • Enzymes and Toxins of Pathogenesis

Many pathogenic microbes increase their competitiveness by synthesizing potent products—enzymes and toxins—that allow them to access nutrients in the host. These virulence factors may help the pathogen in initial invasion, be the final result of successful colonization, or be secreted into food or other nonliving substances later ingested by the host.

Bacterial pathogens damage host tissues (or the entire host) in two major ways: (1) by secreting tissue-destroying enzymes and (2) by secreting or shedding toxins that target specific host tissues or the entire host. In contrast to bacterial pathogens, most viral pathogens damage host tissues by lysing cells directly, although some viruses are nonlytic and instead introduce genes into host cells that may eventually harm the host (◀ Section 5.7).

We turn our focus now to the enzymes and toxins of well-studied pathogenic bacteria, contrasting their potency and modes of action. Some of these virulence factors cause only minor disease symptoms, whereas others are among the most poisonous substances known.

25.5 Enzymes as Virulence Factors

Following adherence, colonization, and infection by a pathogen, invasiveness requires the breakdown of host tissues and access to nutrients released from host cells. In many classical bacterial pathogens, this is accomplished through the activity of *enzymes* that attack and destroy cells in one type of tissue or another (Table 25.1).

Tissue-Destroying Enzymes

Many virulence factors are enzymes. For example, streptococci, staphylococci, and certain clostridia produce *hyaluronidase* (Table 25.1), an enzyme that promotes spreading of organisms in tissues by breaking down the polysaccharide hyaluronic acid. Among other functions, hyaluronic acid is a component of the extracellular matrix and functions as a type of "intercellular cement" in animal connective tissues, helping to maintain the organization of

TABLE 25.1 Enzyme virulence factors of some well-known gram-positive bacterial pathogens

Organism	Disease	Enzyme[a]	Enzyme activity
Staphylococcus aureus	Pus-forming infections	Coagulase	Induces fibrin clotting; allows bacterial cells to remain at site of infection (prevents access to pathogens by cells of the immune response)
		Nuclease; lipase	Break down nucleic acids or lipids
Streptococcus pyogenes	Pus-forming infections; scarlet fever; strep throat	Hyaluronidase	Dissolves hyaluronic acid in connective tissues; allows bacterial cells to spread (enhances pathogen invasion)
		Streptokinase	Dissolves fibrin clots; allows bacterial cells to spread
Clostridium perfringens	Gas gangrene; food poisoning	Collagenase	Breaks down collagen (a protein), allowing the bacterium to spread to other tissues
		Protease	Breaks down proteins

[a]The activities of coagulase, hyaluronidase, and streptokinase are depicted in Figure 25.12.

individual cells into tissues. The activity of hyaluronidase causes host cells to slough apart, allowing pathogens at an initial colonization site to spread between host cells to attack subsurface tissues (**Figure 25.12*a***). Similarly, the clostridia that cause gas gangrene produce *collagenase*, an enzyme that destroys collagen (a major protein of connective tissues in muscle and other body tissues); collagenase enables these bacteria to gain access to deeper host tissues and spread through the body. Recall that clostridia are anaerobes, and colonizing deeper tissues allows them to reach less oxic conditions and provides a ready source of nutrients from destroyed tissues (gangrene clostridia are typically proteolytic species, ◀ Section 16.8 and ▶ Section 32.9). Many pathogenic streptococci and staphylococci also produce proteases, nucleases, and lipases that degrade host proteins, nucleic acids, and lipids, respectively (Table 25.1).

Two virulence factors are enzymes that affect fibrin, the insoluble blood protein that triggers blood clots, but the activities of the enzymes yield opposing results (Figure 25.12*b*). Blood clotting is triggered by tissue injury and functions not only to stop blood loss but also, in the case of a bacterial infection, to isolate the pathogen, limiting the infection to a local region. Some pathogens counter this host protective mechanism by producing fibrinolytic enzymes, such as *streptokinase* produced by *Streptococcus pyogenes*. This bacterium is often associated with pus-forming wounds and secretes streptokinase to dissolve fibrin clots and make further invasion possible (Table 25.1, Figure 25.12*b*). Streptokinase specifically activates the host to produce plasmin, an enzyme that degrades fibrin blood clots. Because of this powerful activity, streptokinase also has a medically beneficial function. The protein is marketed as a pharmaceutical and administered intravenously to dissolve clots for conditions in which normal blood flow is blocked by blood clots, such as from heart attacks, deep vein thromboses, or embolisms.

Mastering Microbiology
Art Activity: Figure 25.11*a* Activity of some enzyme virulence factors

In contrast to the fibrin-*destroying* activity of streptokinase, some pathogens produce enzymes that actually *promote* the formation of fibrin clots. These clots protect the pathogen from host responses. For example, *coagulase* (Table 25.1), produced by virulent *Staphylococcus aureus*, converts fibrinogen to fibrin, resulting in the clotting of blood and the formation of fibrin surrounding the *S. aureus* cells; this blanket of fibrin protects the *S. aureus* cells from attack by cells of the host's immune system (Figure 25.12*b*). The fibrin matrix produced as a result of coagulase activity may also account for the localized nature of many staphylococcal infections, as is typically seen in boils and pimples (▶ Section 31.9). Coagulase-positive *S. aureus* strains are typically more virulent than coagulase-negative strains, a likely reflection of the former's ability to evade innate immune responses such as phagocytosis (Chapter 26) and continue growth and tissue destruction for a longer period.

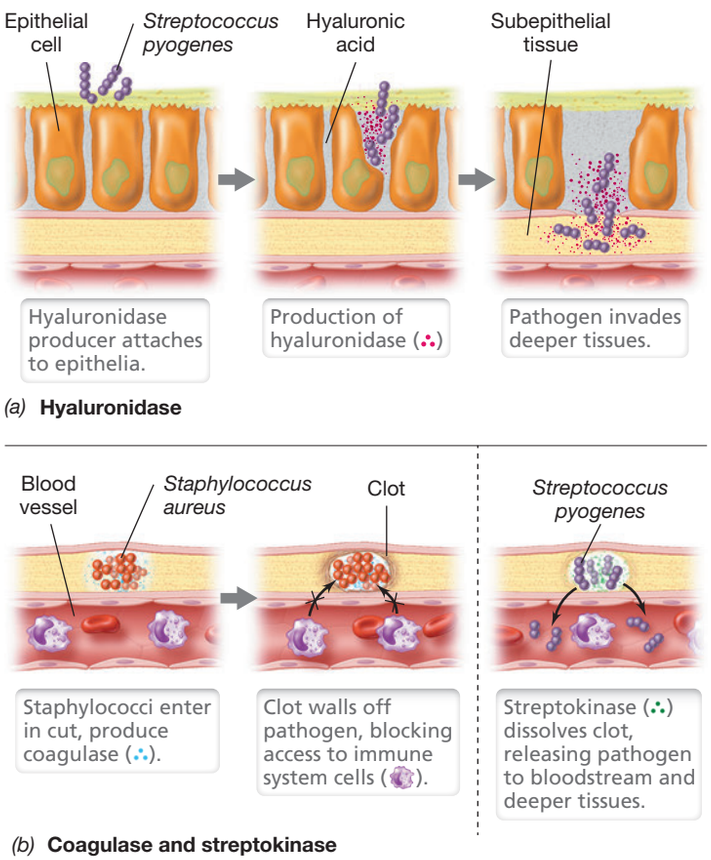


Figure 25.12 Activity of some enzyme virulence factors. *(a)* Hyaluronidase. *(b)* Coagulase (left) and streptokinase (right). Along with some other bacterial pathogens, virulent strains of *Streptococcus pyogenes* typically produce hyaluronidase and streptokinase, and virulent strains of *Staphylococcus aureus* typically produce coagulase.

Enzyme Activities at the Host's Mucosal Surface

Host mucosal surfaces are bathed in immune substances including enzymes such as lysozyme, an enzyme that cleaves the peptidoglycan of bacterial cells and promotes their osmotic lysis (◀ Section 2.3). Virulence factors produced by the gram-positive bacterium *Enterococcus faecalis*—a major cause of bacteremia, surgical wound infections, and urinary tract infections—subvert the protective role of lysozyme by altering the structure of the bacterium's peptidoglycan such that lysozyme can no longer recognize its substrate.

Antibodies are also present on mucosal surfaces, in particular a class of antibody called *IgA* (▶ Section 27.3). These "secretory antibodies," as they are called, help prevent pathogen adherence to host tissues (Section 25.1). However, certain pathogenic bacteria counter this protective role by producing enzymes that specifically cleave IgA (IgAases), rendering this host defense useless; *Neisseria* species such as *N. gonorrhoeae* (gonorrhea) and *N. meningitidis* (meningitis) are particularly notorious in this regard.

We thus see that pathogens can produce enzymes both as offensive weapons—to destroy host tissues—and as defensive weapons—to destroy or inactivate defensive weapons of the host. Both strategies accomplish a similar objective: The invasiveness of the pathogen is increased, and this allows it to ultimately extract more resources from its host.

Check Your Understanding

- Identify host factors that limit or accelerate infection of a microorganism at selected local sites.
- How do streptokinase and coagulase promote bacterial infection and invasion?
- What is an IgAase, and why would a bacterial pathogen produce one?

25.6 AB-Type Exotoxins

Toxicity is the ability of an organism to cause disease by means of a toxin that inhibits host cell function or kills host cells (or the host itself). **Exotoxins** are toxic *proteins* secreted by the pathogen as it grows. These toxins travel from a site of infection and cause damage at distant sites. Some exotoxins are **enterotoxins**, toxic proteins whose site of action is the small intestine, generally causing secretion of fluid into the intestinal lumen, resulting in vomiting and diarrhea. Exotoxins fall into three categories in terms of their mechanism: *AB toxins*, *cytolytic toxins,* and *superantigen toxins*. In this section we consider the AB toxins, and in Section 25.7 we focus on cytolytic and superantigen toxins.

Mastering Microbiology
Art Activity: Figure 25.12 The activity of diphtheria toxin

As the name implies, AB toxins consist of two subunits, A and B. The B component binds to a host cell surface molecule, facilitating the transfer of the A subunit across the cytoplasmic membrane, where it damages the cell. Some of the best-known and most potent exotoxins are AB toxins, including those expressed in diphtheria, tetanus, botulism, and cholera (Table 25.2).

Diphtheria Exotoxin: Blockage of Protein Synthesis

The diphtheria toxin produced by the aerobic gram-positive bacterium *Corynebacterium diphtheriae* is an AB toxin and an important virulence factor of the pathogen (▶ Section 31.3). Diphtheria toxin inhibits protein synthesis in eukaryotic cells. Rats and mice are relatively resistant to diphtheria toxin, whereas humans are very susceptible, with only a single molecule of toxin sufficient to kill a cell. Diphtheria has a significant mortality rate, especially in the young, and death ensues from tissue destruction in vital organs such as the heart and liver as diphtheria toxin blocks protein synthesis.

Cells of *C. diphtheriae* secrete diphtheria toxin as a single polypeptide. One component of the toxin, subunit B, specifically binds to a host cell receptor protein on eukaryotic cells, the heparin-binding epidermal growth factor (Figure 25.13). After binding, proteolytic cleavage between subunit B and the remaining portion of the protein, subunit A, allows subunit A to move across the host cytoplasmic membrane into the cytoplasm. Here subunit A disrupts protein synthesis by blocking transfer of an amino acid from tRNA to growing polypeptide chains. Diphtheria toxin specifically inactivates elongation factor 2 (EF-2), a protein that functions in growth of the polypeptide chain, by catalyzing the attachment of adenosine diphosphate (ADP) ribose from NAD^+. Following ADP-ribosylation, the activity of the modified EF-2 decreases dramatically, and protein synthesis stops (Figure 25.13).

Diphtheria toxin is not encoded by the bacterium but instead by a viral gene called *tox* present in the genome of the lysogenic bacteriophage β. Lysogenic phages are those whose genomes have become

TABLE 25.2 Some classic exotoxins and cytotoxins produced by human bacterial pathogens

Organism	Disease	Toxin[a]	Activity[b]
Bacillus anthracis	Anthrax	Lethal factor Edema factor Protective antigen (AB)	Combine to cause cell death
Bordetella pertussis	Whooping cough	Pertussis toxin (AB)	Blocks G protein function; kills cells
Clostridium botulinum	Botulism	Botulinum toxin (AB)	Causes flaccid paralysis
Clostridium tetani	Tetanus	Tetanospasmin (AB)	Causes spastic paralysis
Clostridium perfringens	Gas gangrene	α, β, γ, δ toxins (AB)	Hemolysis, lecithin destruction
	Food poisoning	Enterotoxin (CT)	Alters intestinal tract permeability
Corynebacterium diphtheriae	Diphtheria	Diphtheria toxin (AB)	Inhibits eukaryotic protein synthesis
Escherichia coli (enterotoxigenic strains only)	Gastroenteritis	Shiga-like (*E. coli*) toxin (AB)	Inhibits protein synthesis, induces bloody diarrhea
Pseudomonas aeruginosa	Burn and certain wound and ear infections; cystic fibrosis lung infections	Exotoxin A (AB)	Inhibits eukaryotic protein synthesis
Salmonella sp.	Gastroenteritis	Enterotoxin (AB)	Lyses cells; inhibits protein synthesis
		Cytotoxin (CT)	Induces fluid loss from intestine
Shigella dysenteriae	Gastroenteritis	Shiga toxin (AB)	Bloody diarrhea and hemolytic uremic syndrome
Staphylococcus aureus	Pyogenic (pus-forming) wounds; food poisoning, toxic shock	α, β, γ, δ toxins (CT)	Hemolysis, leukolysis, cell death
		Toxic shock toxin (SA)	Systemic shock
		Enterotoxins A–E (SA)	Vomiting, diarrhea, systemic shock
Streptococcus pyogenes	Pyogenic infections; strep throat; scarlet fever	Streptolysins O, S (CT)	Hemolysis
		Erythrogenic toxin (SA)	Causes scarlet fever
Vibrio cholerae	Cholera	Cholera (AB)	Induces fluid loss from intestine

[a]AB, AB toxin; CT, cytotoxin; SA, superantigen.
[b]See Figures 25.13–25.18 for the mode of action of some of these toxins.

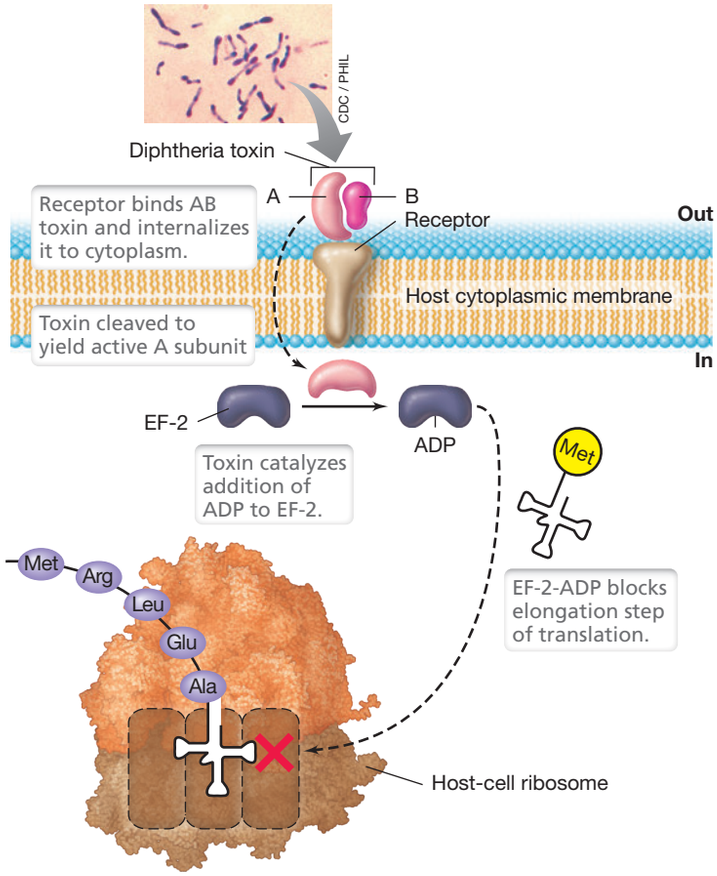


Figure 25.13 The activity of diphtheria toxin. Diphtheria toxin, an AB toxin produced by cells of *Corynebacterium diphtheriae*, binds to the host cytoplasmic membrane by way of its B subunit. Cleavage of the toxin allows the A subunit to enter the cell where it catalyzes ADP-ribosylation of elongation factor 2 (EF-2), a key factor in translation. The modified elongation factor no longer binds to the ribosome, resulting in the cessation of protein synthesis and host cell death. Inset photo: Light micrograph of Gram-stained cells of *Corynebacterium diphtheriae*. A single cell is about 0.75 μm in diameter.

integrated into their host's chromosome (◀ Section 5.6). Toxigenic, pathogenic strains of *C. diphtheriae* are infected with phage β and hence produce the toxin. Nontoxigenic, nonpathogenic strains of *C. diphtheriae* can be converted to pathogenic strains by infection with phage β, a process called *phage conversion* (◀ Section 9.7).

Exotoxin A of *Pseudomonas aeruginosa* functions similarly to diphtheria toxin, also modifying EF-2 by ADP-ribosylation. The enterotoxin produced by *Shigella dysenteriae*, called *Shiga toxin*, and the Shiga-like toxin produced by enteropathogenic *Escherichia coli* O157:H7 (▶ Section 33.11) are also AB toxins (Table 25.2). Shiga and Shiga-like toxins target cells of the small intestine near where the pathogen colonized, shutting down protein synthesis. This leads to cell death, bloody diarrhea, and hemolytic uremic syndrome, a kidney disease that can trigger kidney failure, especially in children.

Neurological Exotoxins: Botulinum and Tetanus Toxins

Clostridium botulinum and *Clostridium tetani* are endospore-forming bacteria commonly found in soil and which cause the serious and potentially fatal diseases botulism and tetanus, respectively; both diseases are caused by the secretion of highly poisonous AB exotoxins that function as *neurotoxins* (▶ Sections 32.9 and 33.9). Neither *C. botulinum* nor *C. tetani* is highly invasive, and therefore pathogenicity is almost exclusively due to neurotoxicity.

C. botulinum sometimes grows directly in the intestine, causing infant or wound botulism. Most frequently for adults, however, botulism results from cells of *C. botulinum* growing and producing toxin in improperly preserved foods, such as home-canned vegetables. Thus, infection and growth of the pathogen in the body are unnecessary. Botulinum toxins consist of seven related but distinct AB toxins, and of these, at least two are encoded on lysogenic bacteriophages specific for *C. botulinum*. Botulinum toxin and tetanus toxin both block the release of neurotransmitters that control muscle activities, but as we will see, the mode of action and symptoms of botulism and tetanus are quite distinct.

Normal transmission of a nerve impulse to a muscle cell at a neuromuscular junction requires interaction of the neurotransmitter acetylcholine with a muscle receptor (**Figure 25.14*a***). Botulinum toxins, which consist of both a heavy chain that binds the neuronal surface and a light-chain endopeptidase, prevent this interaction. After binding the motor end plate—the presynaptic membrane on the terminus of the stimulatory motor neuron—the toxin enters the cell by endocytosis. The heavy chain of the toxin then translocates the light chain across the vesicle membrane into the cytoplasm of the neuron. Depending upon which of the seven forms of botulinum toxin has entered the cell, the proteolytic light chain then cleaves any of several proteins that coordinate release of the

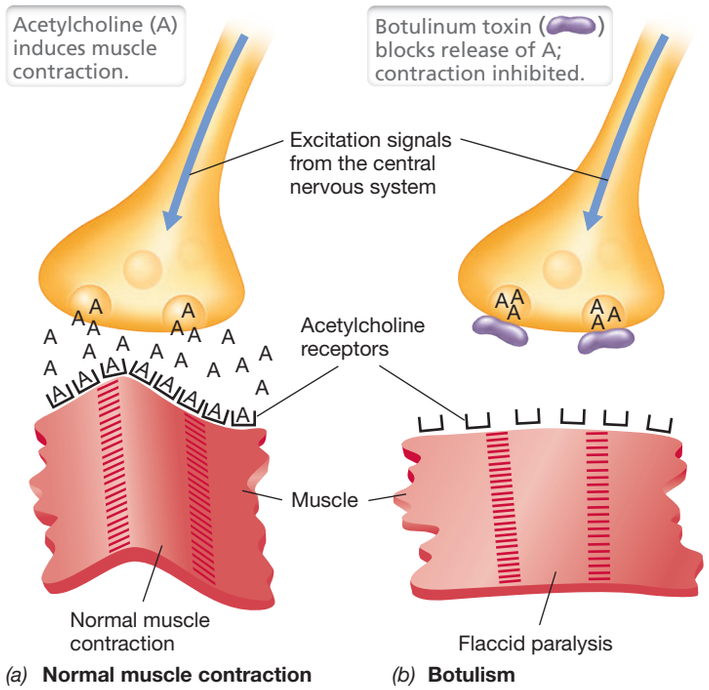


Figure 25.14 The activity of botulinum toxin. *(a)* Upon stimulation of peripheral and cranial nerves, acetylcholine (A) is normally released from vesicles at the neural side of the motor end plate. Acetylcholine then binds to specific receptors on the muscle, inducing contraction. *(b)* Botulinum toxin acts at the motor end plate to prevent release of acetylcholine from vesicles, resulting in a lack of stimulus to the muscle fibers, irreversible relaxation of the muscles, and flaccid paralysis.

Mastering Microbiology
Art Activity: Figure 25.15 The activity of cholera enterotoxin

neurotransmitter into the synaptic space, thus blocking the excitatory acetylcholine signal and preventing muscle contraction (Figure 25.14*b*). This is recognized as *flaccid paralysis* in a botulism victim, and it can lead to death by suffocation if the diaphragm muscles are severely affected.

Botulinum toxins are the most potent biological substances known; just one nanogram (10^{-9} g) of botulinum toxin is sufficient to kill a guinea pig. Thus, it is perhaps surprising that botulinum toxins have proved beneficial in the treatment of chronic pain. Because chronic pain, such as that associated with ongoing back and neck pain or frequent migraines, is often caused by neuropathy or stress-induced muscle tension, low-dose injections of dilute botulinum toxin (Botox®) can relax the affected tissues and effectively mitigate pain. This is especially useful considering that botulinum toxin treatments are not linked to the unwanted side effects that commonly accompany traditional drug treatments for these conditions.

In contrast to *C. botulinum*, *C. tetani* grows in the body in deep wounds that become anoxic, such as punctures. *C. tetani* cells rarely leave the wound where they were first introduced, growing relatively slowly at the wound site. On contact with the nervous system, tetanus toxin, called *tetanospasmin*, is transported through the motor neurons to the spinal cord, where it binds specifically to ganglioside lipids at the termini of the inhibitory interneurons (Figure 25.15). The inhibitory interneurons normally work by releasing an inhibitory neurotransmitter, typically the amino acid glycine, which binds to receptors on the motor neurons. Glycine from the inhibitory interneurons then stops the release of acetylcholine by the motor neurons and inhibits muscle contraction, allowing relaxation of the muscle fibers. However, if tetanus toxin blocks glycine release, the motor neurons cannot be inhibited, resulting in the continual release of acetylcholine and uncontrolled contraction of the muscle fibers (Figure 25.15*b*). Thus, in contrast to botulinum toxin, which prevents muscle *contraction* (Figure 25.14), tetanus toxin prevents muscle *relaxation* (Figure 25.15).

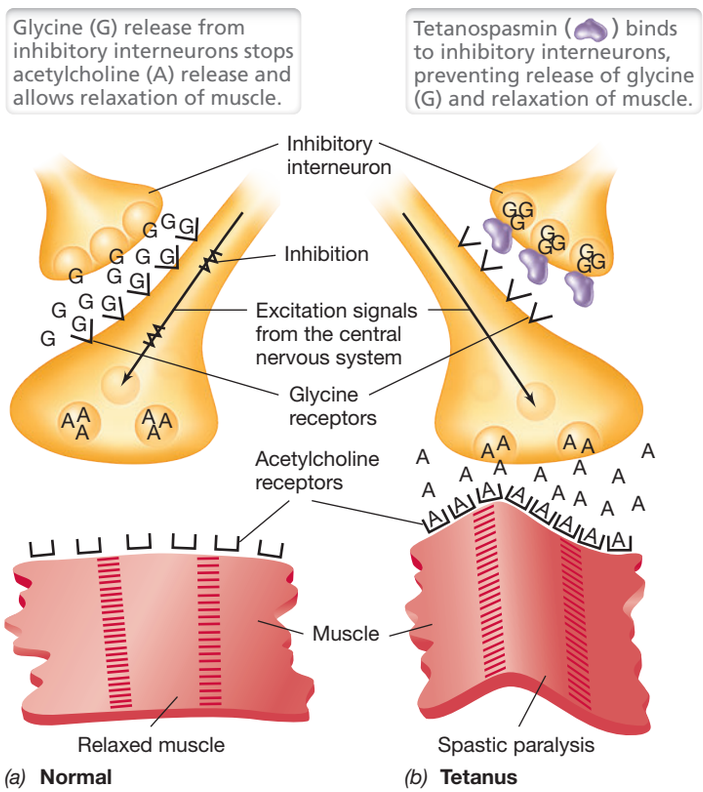


Figure 25.15 The activity of tetanus toxin. *(a)* Muscle relaxation is normally induced by glycine (G) release from inhibitory interneurons. Glycine acts on the motor neurons to block excitation and release of acetylcholine (A) at the motor end plate. *(b)* Tetanus toxin binds to the interneuron to prevent release of glycine from vesicles, resulting in a lack of inhibitory signals to the motor neurons, constant release of acetylcholine to the muscle fibers, irreversible contraction of the muscles, and spastic paralysis. For the purpose of illustration, the inhibitory interneuron is shown near the motor end plate, but it is actually in the spinal cord.

The outcome in a case of tetanus is a twitching, *spastic paralysis*—the hallmark of tetanus (▶ Section 32.9 and Figure 32.23*b*)—as affected muscles are constantly contracting. If the muscles of the mouth are involved, as is commonly the case, the prolonged contractions restrict the mouth's movement, resulting in a condition called *lockjaw*. If the diaphragm is affected, its prolonged contraction may result in death due to asphyxiation.

Cholera Enterotoxin: Intestinal Distress

Cholera enterotoxin is an AB-type exotoxin produced by *Vibrio cholerae*, the causative agent of the waterborne disease cholera (▶ Section 33.3). Cholera is characterized by massive fluid loss from the intestines, resulting in severe diarrhea, life-threatening dehydration, and electrolyte depletion (Figure 25.16). Cholera starts by ingestion of *V. cholerae* cells from food or water contaminated with human feces. The organism travels to the small intestine, where it colonizes and secretes cholera toxin. In the small intestine, the B subunit of cholera toxin, consisting of five identical monomers, binds specifically to GM1 ganglioside, a complex glycolipid found in the cytoplasmic membrane of intestinal epithelial cells (Figure 25.16).

The B subunit targets cholera toxin specifically to receptors in the intestinal epithelium but has no toxicity itself; toxicity is a function of the A subunit, which crosses the cytoplasmic membrane and activates adenylate cyclase, the enzyme that converts ATP to cyclic adenosine monophosphate (cAMP). This molecule is a cyclic nucleotide (◀ Figure 7.22) that mediates several regulatory systems in cells, including ionic balance. The increased cAMP induced by cholera enterotoxin blocks Na^+ uptake by small intestine epithelial cells and induces secretion of chloride and bicarbonate (HCO_3^-) into the intestinal lumen. This change in ion concentrations leads to the secretion of large amounts of water; the rate of water loss into the small intestine is greater than the possible reabsorption of water by the large intestine, resulting in a large net fluid loss and watery diarrhea. If untreated, cholera victims can die within hours of the major onset. However, if lost fluids are replaced with an oral rehydration solution (the main treatment for cholera), the effects of cholera toxin can be neutralized, and a cholera victim can return to normal in just a few days.

A few other enterotoxins, most notably the Shiga-like toxin of enterotoxigenic strains of *Escherichia coli* (▶ Section 33.11) and enterotoxins of *Shigella* and *Salmonella*, are also of the AB type (Table 25.2), and all of these function by inhibiting protein synthesis. This leads to major bouts of diarrhea, typically bloody and foul smelling, and

severe dehydration. In addition, some cytolytic enterotoxins are known, and the powerful enterotoxins of *Staphylococcus aureus* (Table 25.2) are of the superantigen type. We consider these dangerous toxins now.

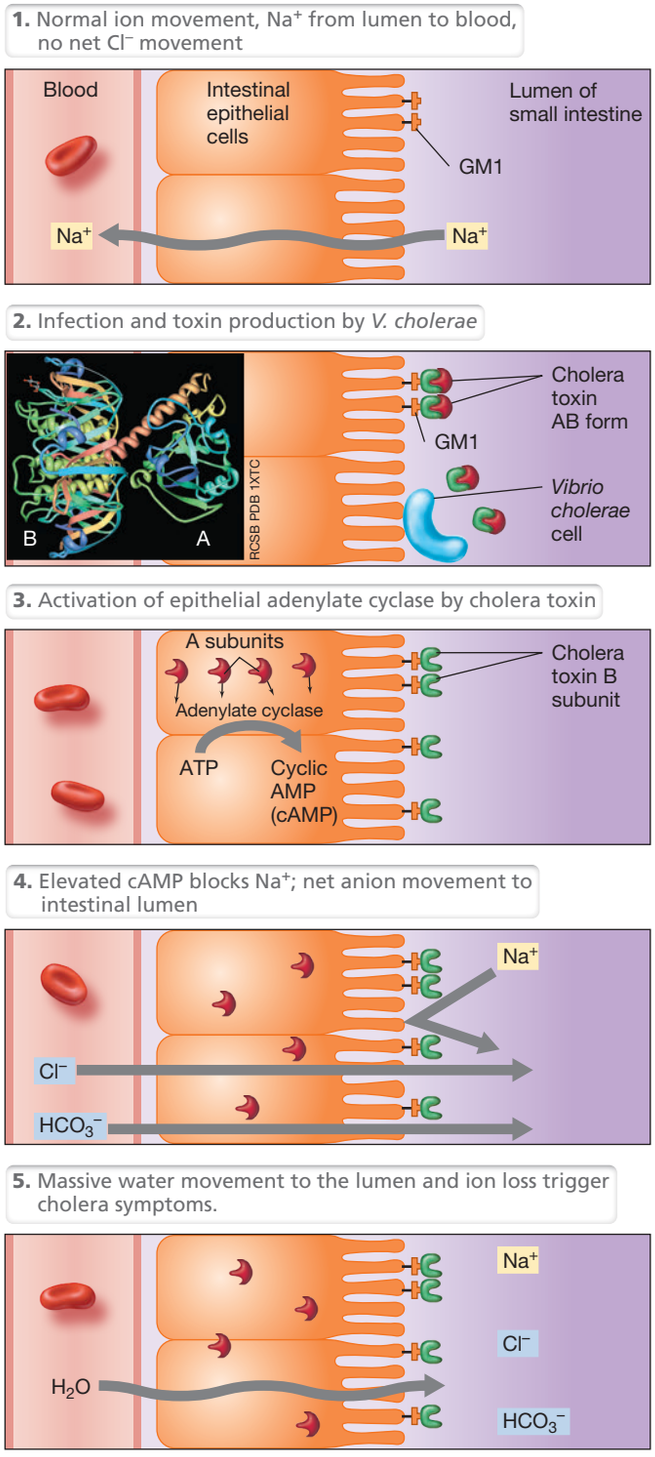


Figure 25.16 The activity of cholera enterotoxin. Cholera toxin is an AB enterotoxin that activates a second-messenger pathway, disrupting normal ion flow in the intestine, resulting in potentially life-threatening diarrhea. The thumbnail photo of the three-dimensional structure shows a side view of the toxin, with the separate cell-binding B subunit and the enzymatically active A subunit.

Check Your Understanding

- What key features are shared by all AB exotoxins?
- Are bacterial growth and infection in the host necessary for the production of toxins? Explain and cite examples for your answer.
- Why do botulism and tetanus show such opposing symptoms?

25.7 Cytolytic and Superantigen Exotoxins

The pathogenesis of exotoxins such as the cytolytic and superantigen toxins differs from those of the classical AB toxins. Cytolytic and superantigen exotoxins function by destroying host cells, such as from the activity of hemolysins, or by triggering a massive immune response, such as in the case of toxic shock exotoxin, the cause of toxic shock syndrome. As for other exotoxins, however, these proteins are produced by the pathogen to increase its invasiveness and release host cell resources that can benefit the pathogen.

Cytolytic Exotoxins

Cytotoxins (also called cytolytic exotoxins) are soluble proteins secreted by a variety of pathogens (Table 25.2). Cytotoxins damage the host cytoplasmic membrane, causing cell lysis and death. Because the lytic activity of these toxins is most easily observed in assays that use red blood cells (erythrocytes), the toxins are often called *hemolysins* (Table 25.2). However, hemolysins also lyse cells other than erythrocytes. The production of hemolysins can be demonstrated by streaking the pathogen on a blood agar plate (a rich medium containing 5% sterile blood). During growth of the colonies, hemolysin is released and lyses the surrounding red blood cells, releasing hemoglobin and creating a clear area, called a zone of *hemolysis*, around the growing colonies (**Figure 25.17*a***).

Some hemolysins attack the phospholipid of the host cytoplasmic membrane. Because the phospholipid lecithin (phosphatidylcholine) is often used as a substrate, these enzymes are called *lecithinases* or *phospholipases*. An example is the α-toxin of *Clostridium perfringens*, a lecithinase that dissolves membrane lipids, resulting in cell lysis (Table 25.2, Figure 25.17*b*). Because the cytoplasmic membranes of all organisms contain phospholipids (◀ Section 2.1), phospholipases can destroy bacterial as well as animal cell cytoplasmic

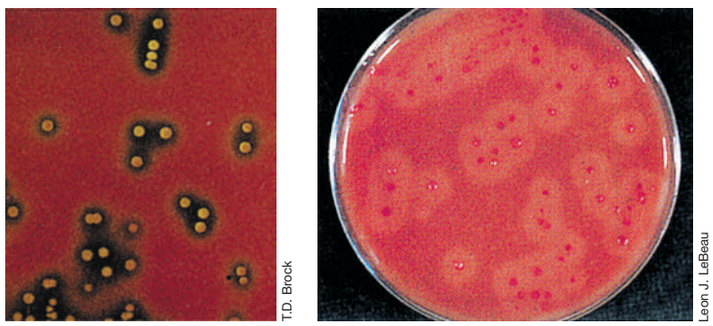


Figure 25.17 Hemolysis. *(a)* Zones of hemolysis around colonies of *Streptococcus pyogenes* growing on a blood agar plate. *(b)* Activity of lecithinase, a phospholipase, around colonies of *Clostridium perfringens* growing on an agar medium containing egg yolk, a source of lecithin. Lecithinase dissolves the cytoplasmic membranes of red blood cells, producing cloudy zones of hemolysis around each colony.

membranes. In the case of *C. perfringens*, a major cause of gas gangrene, the activity of its lecithinase helps destroy tissues and release proteins that are then fermented by the bacterium in energy metabolism. Since *C. perfringens* lecithinase is secreted immediately after synthesis and is unable to reenter the cell, the enzyme does not affect the phospholipids in *C. perfringens* cells.

Some hemolysins, however, are not phospholipases. Streptolysin O, a hemolysin produced by streptococci, affects the sterols of the host cytoplasmic membrane. *Leukocidins* lyse white blood cells and thereby decrease the host immune response. Staphylococcal α-toxin (**Figure 25.18** and Table 25.2) destroys both nucleated cells and erythrocytes. To do this, the seven α-toxin subunits first bind to the phospholipid bilayer. The subunits then combine to form into nonlytic heptamers, now associated with the membrane. Each heptamer then undergoes conformational changes to produce a membrane-spanning pore that releases cytoplasmic contents and allows the influx of extracellular materials, thus killing the cell (Figure 25.18).

Superantigen Exotoxins

The gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* are major producers of exotoxin superantigens. We consider the mode of action of superantigens in Section 28.2 in the context of the immune response and focus here only on some basic disease symptoms.

Superantigen poisoning can be triggered by certain types of food poisoning (in particular, that caused by the enterotoxins of *S. aureus*), by toxic shock syndrome, or by pyrogenic fever (fever induced either from an internal source, such as small immune system proteins called cytokines, or from an external substance, such as endotoxin; see Section 25.8). Reactions to superantigen poisoning are severe and can even be fatal in some individuals, particularly those whose immune systems and overall health are weakened from cancer, drug treatments, HIV infection, or old age. Toxic shock syndrome (TSS) is the classic example of the systemic effects of a toxic superantigen and results from exposure to any of a series of exotoxins secreted during infection by certain strains of *S. aureus* or *S. pyogenes* (▶ Sections 31.9 and 31.2, respectively).

S. aureus TSS commonly originates as a result of a localized rather than a generalized infection. By contrast, *S. pyogenes* TSS is typically the result of a systemic infection where bacteremia or septicemia (Section 25.2) is present, and tissue damage including extensive tissue necrosis occurs (▶ Figure 31.10); as a result, mortality rates from *S. pyogenes* TSS are considerably higher than from *S. aureus* TSS. In both cases, however, the symptoms of TSS are triggered when the immune system recognizes the superantigen toxin, but rather than activating just a small subset of T lymphocytes (key cells in adaptive immunity, Chapter 27) as usually occurs in the adaptive immune response, a large proportion of the entire T lymphocyte pool becomes activated. It is the structure of the superantigen itself that leads to this overblown immune response, a result of which is widespread release of cell-signaling cytokines and systemic inflammation that leads to hypotension (low blood pressure), intestinal disruption, organ failure, and eventually systemic shock.

In a clinical diagnosis, either staphylococcal or streptococcal TSS is suspected when an individual has a fever of 39°C or greater, systolic blood pressure of < 90 mm Hg, and functional disruption of three or more organ systems, most often gastrointestinal, kidney, and liver. In severe cases of TSS, intensive care hospitalization may be needed with intravenous administration of antibiotics.

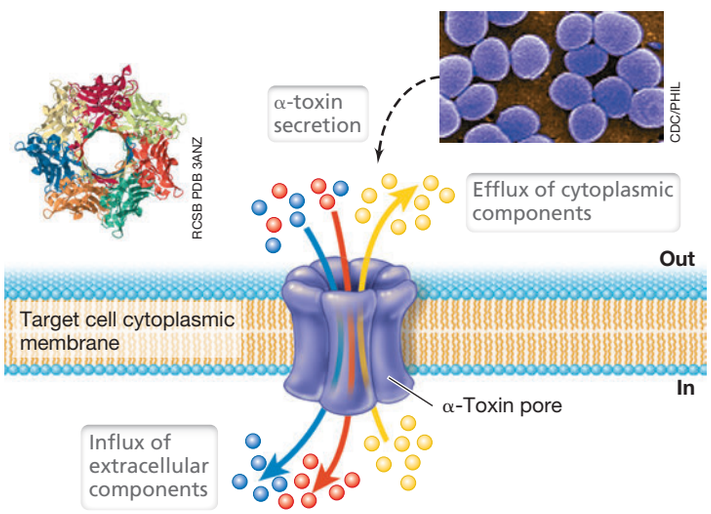


Figure 25.18 Staphylococcal α-toxin. Staphylococcal α-toxin is a pore-forming cytotoxin that is produced by growing *Staphylococcus aureus* cells. Released as monomers, seven identical protein subunits oligomerize in the cytoplasmic membrane of target cells to form a pore, releasing the contents of the cell. In red blood cells, hemolysis occurs, visually indicating cell lysis. The inset image on the top left shows the structure of α-toxin looking down through the pore; each of the seven identical subunits is shown in a different color. The inset photo on the top right is a scanning electron micrograph of *S. aureus* cells.

Check Your Understanding

- Give an example of a cytolytic exotoxin and a superantigen exotoxin, as well as the bacteria that produce each.
- How can activity of a hemolytic exotoxin be detected?

25.8 Endotoxins

Endotoxins are the toxic lipopolysaccharides found in the cell walls of most gram-negative *Bacteria*. Endotoxins are not proteins but are structural components of the gram-negative outer membrane (◀ Section 2.4). In contrast to exotoxins, which are the secreted products of living cells, endotoxins are cell bound and released in toxic amounts only when the cells lyse. The basic properties of exotoxins and endotoxins are compared in **Table 25.3**.

Endotoxin Structure and Biology

A major component of the gram-negative cell outer membrane is lipopolysaccharide (LPS) (◀ Figures 2.12 and 2.13). LPS consists of three covalently linked subunits: the membrane-distal O-specific polysaccharide, a membrane-proximal core polysaccharide, and lipid A—a phosphoglycolipid and the membrane-anchoring portion of LPS (**Figure 25.19**). The lipid A portion of LPS is responsible for toxicity, whereas the polysaccharide fraction by itself is nontoxic. The polysaccharide functions to make the entire LPS complex soluble and immunogenic, and thus both the lipid and polysaccharide fractions must be delivered as a unit for toxicity to occur.

TABLE 25.3 Properties of exotoxins and endotoxins

Property	Exotoxins	Endotoxins
Chemistry	Proteins, secreted by certain gram-positive or gram-negative *Bacteria;* generally heat-labile	Lipopolysaccharide–lipoprotein complexes, released on cell lysis as part of the outer membrane of gram-negative *Bacteria;* extremely heat-stable
Mode of action; symptoms	Specific; usually bind to specific cell receptors or structures; either cytotoxin, enterotoxin, or neurotoxin with defined, specific action on cells or tissues	General; fever, diarrhea, vomiting
Toxicity	Often highly toxic in picogram to microgram quantities, sometimes fatal	Moderately toxic in tens to hundreds of microgram amounts, rarely fatal
Immune response	Highly immunogenic; stimulate the production of neutralizing antibody (antitoxin)	Relatively poor immunogens; immune response not sufficient to neutralize toxin
Toxoid potential[a]	Heat or chemical treatment may destroy toxicity, but treated toxin (toxoid) remains immunogenic	None
Fever potential	Nonpyrogenic; do not produce fever in the host	Pyrogenic; often induce fever in the host
Genetic origin	Often encoded on extrachromosomal elements or lysogenic bacteriophages	Encoded by chromosomal genes

[a]A toxoid is a modified toxin that is no longer toxic but can still elicit an immune response against the toxin (▶ Section 28.3).

Endotoxins have been well studied in the bacteria *Escherichia*, *Shigella*, and especially in *Salmonella*, where they are another of the many virulence factors that contribute to pathogenesis (Figure 25.11). The biosynthesis pathway of the toxic component of endotoxin, lipid A, is known and is a highly conserved process among gram-negative bacteria. Nevertheless, not all lipid A is structurally the same, as the molecule can be modified using enzymes that catalyze postsynthesis modifications that control the presence, absence, or number of phosphate groups, and the chemistry and number of fatty acid side chains (Figure 25.19). These subtle but important alterations affect the properties of the LPS molecule and are a virulence strategy that certain pathogens have evolved to either evade recognition by the host immune system or increase the toxicity of the molecule. However, some lipid A alterations affect toxicity in a negative way. For example, the phosphate groups (Figure 25.19) are essential for binding lipid A to animal cell receptors, and although phosphate-free lipid A can evade immune surveillance, its toxicity is greatly reduced.

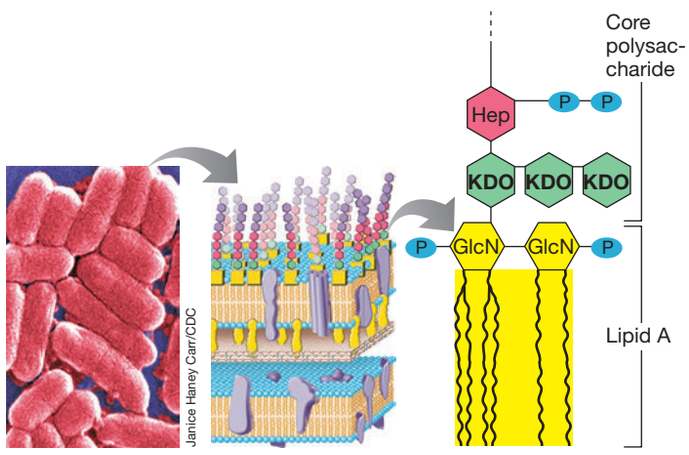


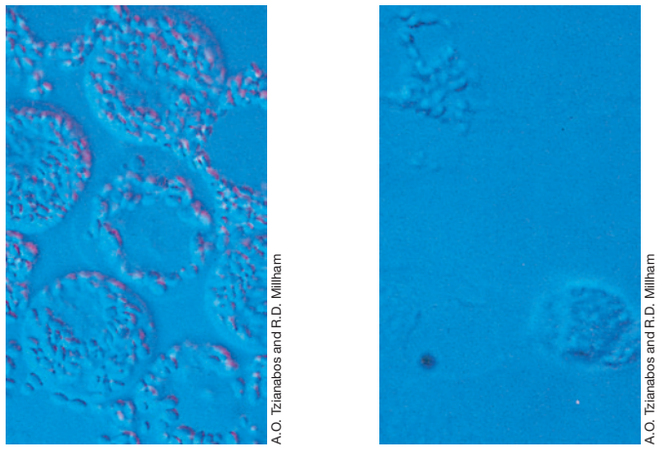
Figure 25.19 Endotoxin. Left to right: Scanning electron micrograph of cells of the gram-negative bacterium *Escherichia coli*; structure of the gram-negative cell wall including the lipopolysaccharide (LPS) outer membrane; detailed structure of lipid A, the toxic portion of LPS, along with part of the core polysaccharide of LPS.

Endotoxins cause a variety of physiological effects. Fever is an almost universal result of endotoxin exposure because endotoxin stimulates host cells to release cytokines, soluble proteins secreted by certain cells of the immune system that function as *endogenous pyrogens*, proteins that affect the temperature-controlling center of the brain, causing fever. Cytokines released as a result of endotoxin exposure can also cause diarrhea, increased heart rate (tachycardia), a rapid decrease in the numbers of lymphocytes and platelets, and generalized inflammation (▶ Section 26.8). Other physiological consequences of endotoxin exposure include activation of the complement cascade (complement is a series of immune system proteins, ▶ Section 26.9), which also triggers inflammation, and activation of the blood coagulation cascade, which can lead to blood clots and reduced blood flow. Large doses of endotoxin can cause death from hemorrhagic shock and kidney failure.

Although significant virulence factors, endotoxins are generally less toxic than most exotoxins and rarely cause symptoms that can lead to death in an otherwise healthy individual if exposure is from a gastrointestinal source (Table 25.3). For instance, in mice the LD_{50} (see Figure 25.9) for endotoxin is 200–400 *micro*grams per animal, whereas the LD_{50} for botulinum exotoxin is about 25 *pico*grams, about 10 million times less. By contrast, intravenous administration of endotoxin, for example from a heavily contaminated intravenous solution, could have fatal consequences, and we discuss how such faulty solutions can be identified now.

Limulus Amoebocyte Lysate Assay for Endotoxin

Because endotoxins induce fever and can trigger other, more serious symptoms, pharmaceuticals such as injectable antibiotics and intravenous solutions must be free of endotoxin. An endotoxin assay of high sensitivity and specificity in widespread use employs lysates of



(a) (b)

Figure 25.20 *Limulus* amoebocyte assay for endotoxin. *(a)* Normal amoebocytes from the horseshoe crab *Limulus polyphemus*. *(b)* Amoebocytes following exposure to bacterial lipopolysaccharide (LPS). LPS induces degranulation and lysis of the cells.

amoebocytes from the horseshoe crab *Limulus polyphemus* (amoebocytes are mobile cells in the blood and fluids of invertebrates that are analogous to the white blood cells of vertebrates). Endotoxin specifically causes lysis of the amoebocytes (**Figure 25.20**). In the *Limulus* amoebocyte lysate (LAL) assay, *Limulus* amoebocyte extracts are mixed with the solution to be tested. If endotoxin is present, the amoebocyte extract forms a gel and precipitates, causing a change in turbidity. This reaction is measured quantitatively with a spectrophotometer and can detect as little as 10 picograms of LPS in a 1-ml sample.

The LAL assay is also used to detect endotoxin in clinical samples such as serum or cerebrospinal fluid, and a positive result provides presumptive evidence for infection by gram-negative bacteria. Drinking water, water used for formulation of injectable drugs, and injectable aqueous solutions are routinely tested using the LAL assay to identify and eliminate endotoxin contamination from gram-negative bacteria. A commercially available assay uses horseshoe crab factor C made by recombinant DNA techniques (factor C is the key protein activated by endotoxin in the LAL assay). Rather than relying on amoebocytes collected from harvested horseshoe crabs, the recombinant protein is just as sensitive but less expensive, allows for a more standardized assay protocol, and is totally free of animal products.

Check Your Understanding

- What part of the *Escherichia coli* cell contains endotoxin? Why do gram-positive bacteria not produce endotoxins?
- Why is it necessary to test for endotoxin in water used for injectable drug preparations?

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CHAPTER REVIEW

I • Human–Pathogen Interactions

25.1 If a pathogen gains access to the specific tissues it infects, disease will occur only if it first adheres to those tissues. Although adherence is required to initiate disease, it is not sufficient to initiate disease; colonization, invasion, and production of toxic substances are also required.

Q What are adhesins, and what are they typically composed of?

25.2 Each body region differs chemically and physically from others, and thus provides a variety of selective environments for the growth of certain microbes but not others. Colonization of tissues by a pathogenic microbe followed by growth to form populations sufficient to trigger a biological effect is necessary before disease symptoms appear.

Q Where are mucous membranes found in the body, and what do they consist of? How do they prevent pathogen invasions in the human body?

25.3 The pathogenicity of a pathogen is a function of its virulence, its relative ability to cause disease. Virulence is a quantitative measure that can be assayed in terms of the number of cells (or virions, if a viral pathogen) required to infect or kill 50% of a given population—the LD_{50}. Attenuated pathogens are strains with diminished virulence and are quite useful for preparing vaccines.

Q What virulence factor, present in *Streptococcus pneumoniae* but absent from *Salmonella enterica*, makes *S. pneumoniae* so highly virulent for mice?

25.4 The genetics and physiology of both the pathogen and the host affect the outcome of an infectious disease. In *Salmonella* species, chromosomal islands or conjugative plasmids are present that encode several virulence factors; these mobile genetic elements can quickly spread a suite of virulence factors to other gram-negative bacteria. Susceptibility to an infectious disease is increased in a compromised host.

Q What factors might diminish the ability of a host to fight off an infectious disease?

II • Enzymes and Toxins of Pathogenesis

25.5 The virulence of a pathogen depends on the number and kinds of virulence factors it produces. Some pathogenic bacteria produce enzymes that function to either destroy host tissues or disarm host defenses. The activity of these enzymes releases nutrients to support growth of the pathogen and facilitate further invasiveness.

Q Give two reasons why a pathogen might benefit from the secretion of an enzyme that destroys host tissue integrity.

25.6 Exotoxins are toxic proteins and major virulence factors. Each exotoxin affects a specific host cell function. Enterotoxins are exotoxins that affect the small intestine. The clostridial botulinum and tetanus exotoxins are among the most poisonous substances known.

Q Assuming a person was poisoned with either botulinum toxin or tetanus toxin, how could the person's physical state signal the type of poisoning that had occurred?

25.7 Cytotoxins and superantigens are toxic proteins that lyse host cells and trigger a massive immune response against host tissues, respectively. Hemolysis on a blood agar plate is a classic cytotoxic effect, whereas toxic shock syndrome, a potentially fatal condition, is one result of superantigen activity.

Q Distinguish between the mechanism of cytotoxins and AB toxins and provide one example of each.

25.8 Endotoxins are lipopolysaccharides derived from the outer membrane of gram-negative bacteria. Both the lipid and the polysaccharide components of endotoxin are necessary for toxicity. Symptoms of endotoxin poisoning include fever and intestinal distress.

Q Identify the structural features, origins, and major effects of endotoxins.

APPLICATION QUESTIONS

1. Coagulase is a virulence factor for *Staphylococcus aureus* that acts by causing clot formation at the site of *S. aureus* growth. Streptokinase is a virulence factor for *Streptococcus pyogenes* that acts by dissolving clots at the site of *S. pyogenes* growth. Reconcile these opposing strategies for enhancing pathogenicity.
2. Although mutants incapable of producing exotoxins are relatively easy to isolate, mutants incapable of producing endotoxins are much harder to isolate. From what you know of the structure and function of these types of toxins, explain the differences in mutant recovery.

CHAPTER GLOSSARY

Adherence the enhanced ability of a microorganism to attach to a cell or surface
Adhesins glycoproteins or lipoproteins covalently bound to the outer layer of the pathogen that function in attachment to host tissues
Attenuate to decrease or eliminate the virulence of a pathogen or virus
Bacteremia the presence of bacteria in the blood
Capsule a polysaccharide or protein outermost layer, usually rather slimy, present on some bacteria
Colonization the growth of a microorganism after it has gained access to host tissues
Dental caries tooth decay resulting from bacterial infection
Dental plaque a bacterial biofilm found on the teeth and consisting of cells embedded in a matrix of extracellular polymers and salivary products
Disease an injury to a host organism, caused by a pathogen or other factor, that is accompanied by specific signs and symptoms that affect host function
Endotoxin the lipopolysaccharide portion of the outer membrane of most gram-negative *Bacteria*, which is a toxin when solubilized
Enterotoxin a protein that is released extracellularly by a microorganism as it grows and that produces immediate damage to the small intestine of the host
Exotoxin a protein that is released extracellularly by a microorganism as it grows and that produces immediate damage to the host
Infection an event during which a microorganism not a member of the local microbiota is established and grows in a host, regardless of whether the host is harmed
Invasion the ability of a pathogen to enter into host cells or tissues, spread, and cause disease
Mucous membrane a layer of mucus-covered epithelial cells in the body that communicates with the external environment
Mucus a viscous liquid composed of mucin secreted by specialized epithelial cells that contains water-soluble glycoproteins and proteins that retain moisture and aid in resistance to microbial invasion on mucosal surfaces
Opportunistic pathogen an organism that causes disease only in the absence of normal host resistance
Pathogen a disease-causing microorganism
Pathogenicity the ability of a pathogen to cause disease
Septicemia a bloodborne systemic infection
Toxicity the ability of an organism to cause disease by means of a preformed toxin that inhibits host cell function or kills host cells
Virulence the relative ability of a pathogen to cause disease
Virulence factors substances or strategies of a pathogen that indirectly or directly enhance invasiveness and host damage by facilitating and promoting infection

26 Innate Immunity: Broadly Specific Host Defenses

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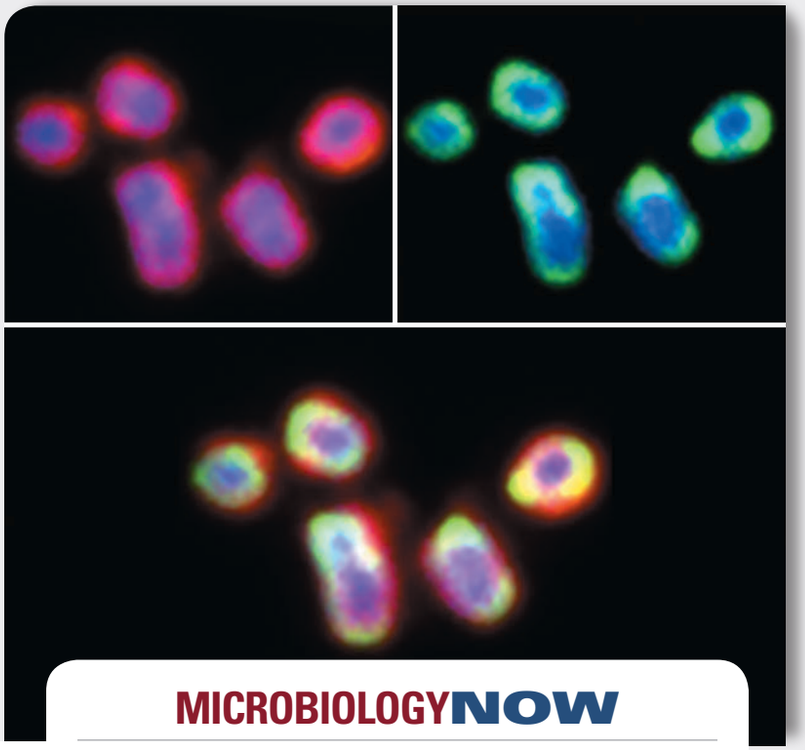
IV Other Innate Host Defenses 882



MICROBIOLOGY**NOW**

Periodontal Disease and Alzheimer's: Evidence for Causation?

Alzheimer's disease (AD) is characterized by the presence of insoluble aggregates of amyloid-β protein (Aβ) in the brain, producing plaques that interfere with cognitive brain function. Although Aβ has traditionally been viewed as an injurious substance, studies have now shown that it may function as an innate antimicrobial defense to protect the brain from bacteria that cross the blood–brain barrier. In such cases, the invading cells become entombed in a meshwork of Aβ, minimizing their growth and dissemination in the brain (see Figure 26.3). Chronic or heavy bacterial infections are believed to initiate the Aβ plaques that lead to Alzheimer's symptoms, and a recent report has implicated a specific bacterium in the development of these plaques: *Porphyromonas gingivalis*, the causative agent of periodontal disease.

It has long been recognized that Alzheimer's patients also often suffer from chronic gum disease, including gingivitis and periodontitis, and multiple reports are emerging that suggest the onset of gum disease is a risk factor that precipitates AD. In one study, researchers introduced oral *P. gingivalis* infection in mice and subsequently documented *P. gingivalis* colonization of the brain. There, the invading bacteria produced neurotoxic proteases called gingipains (red, on cells of *P. gingivalis*, top left photo). This triggered inflammation and accumulation of Aβ (green, on cells, top right photo), which colocalized with the gingipains on cells of *P. gingivalis* (bottom photo), ultimately promoting Aβ plaque formation. However, when gingipain inhibitors were administered, bacterial numbers diminished, inflammation and production of Aβ subsided, and neurons in the hippocampus were restored, suggesting that such inhibitors may reduce Aβ plaque formation and serve as a new weapon against AD.

Although the study is not conclusive, the results indicate that *P. gingivalis* infection may be a contributing factor in the development of AD. Further study along these lines may eventually yield a more complete understanding of AD pathology, as well as more effective treatment options.

Source: Dominy, S.S., et al. 2019. *Porphyromonas gingivalis* in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci. Adv. 5*. doi: 10.1126/sciadv.aau3333.

We began this unit by considering the associations of microbes with humans, and in the last chapter we discussed pathogenicity, virulence, and risk factors for infection. In this and the next chapter, we focus on mechanisms used by vertebrates to resist pathogens and the diseases they cause, the science of *immunology*. In this chapter, we present concepts of **innate immunity**, inborn host defenses against a *broad range* of pathogens. In Chapter 27 we discuss **adaptive immunity**, the essential second tier of the immune system that targets *specific* pathogens to minimize their harmful effects.

I • Fundamentals of Host Defense

The human body has both innate (broadly targeted) and acquired (highly specific) immune mechanisms to either prevent or respond to invasion by pathogens. This highly developed "search and destroy" system maintains human health from potentially harmful microbes in the environment.

We begin with an overview of innate and adaptive immunity and follow this with a consideration of the human body's significant natural barriers to invasion by pathogens.

26.1 Basic Properties of the Immune System

Immunity is the ability of an organism to resist infection. The human immune system employs a two-pronged defense against invading pathogens. *Innate immunity*, the first of these interconnected defensive mechanisms, is the built-in capacity of the immune system of multicellular organisms to target pathogens that are seeking to colonize the host. Depending on the virulence of the pathogen, the infectious dose, and the immune competence of the individual, innate mechanisms alone may be insufficient to eliminate the pathogen. In such cases, and assuming that the pathogen has not quickly killed the host, *adaptive immunity* is triggered within the individual against those specific pathogens that have overcome the innate defenses. Each adaptive immune response is specifically targeted to a particular invading pathogen, such as a single strain of virus. Figure 26.1 compares and contrasts the fundamental elements of each of these indispensable branches of the immune system.

Mastering Microbiology
Art Activity: Figure 26.1 Overview of the two-pronged immune response

Principles of Innate Immunity

Innate immunity is a noninducible, preexisting ability of the body to recognize and destroy a broad range of pathogens or their products. Innate immune responses develop quickly, typically within

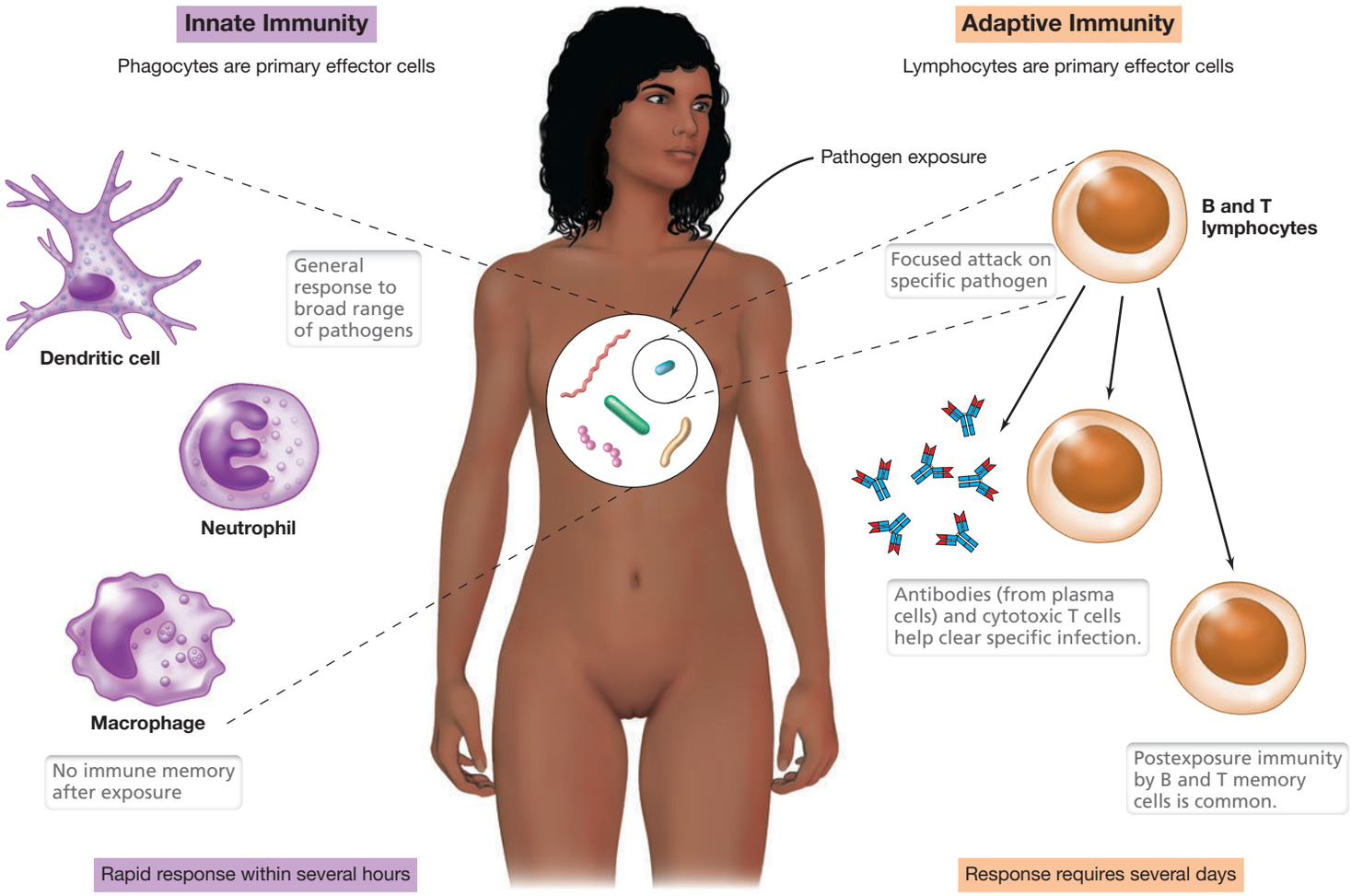


Figure 26.1 Overview of the two-pronged immune response. Principal characteristics of innate (shown in purple) and adaptive (shown in orange) immunity. Phagocytes and lymphocytes are the primary effector cells of innate and adaptive immunity, respectively, but note that not all immune cells are shown (compare to Figure 26.5).

several hours of exposure to a pathogen or its product, and they do not require previous exposure to a pathogen or its products for activation. Eukaryotes have functionally similar pathogen recognition mechanisms that lead to rapid and effective host defense. For example, pathogen recognition in the insect *Drosophila* (fruit fly) is carried out in much the same way as it is in humans, using immune cells that show structural and evolutionary homology.

In addition to a variety of physical and chemical barriers to infection that we will describe in Section 26.2, innate immunity is largely dependent on the activity of **phagocytes** (Figure 26.1), cells that ingest, kill, and digest microbial pathogens. Phagocytes include **neutrophils**, **macrophages**, **dendritic cells**, and **eosinophils**, and we define their distinctive features in Section 26.4. In addition to phagocytes, cells of the innate immune system consist of **mast cells** and **basophils**, both of which trigger inflammation when activated (Section 26.8), and **natural killer (NK) cells**, which identify and destroy host cells that have become compromised and are therefore dangerous to the host, such as cells that are infected by a pathogen or are cancerous (Section 26.10).

Mastering Microbiology
Art Activity: Figure 26.2 Barriers to infection in the human body

Principles of Adaptive Immunity

When innate immune responses fail to eliminate an invading pathogen, adaptive immunity is activated. Adaptive immunity is the *acquired* ability to recognize and destroy a specific pathogen or its products. Adaptive responses show **specificity** because they are directed at unique pathogen components or products called **antigens**, which often define a particular strain of pathogen or type of foreign material. Macrophages and dendritic cells ingest and process pathogens to present peptide antigens to **T lymphocytes (T cells)**, which play a key role in initiating the adaptive response. Another adaptive immune cell, the **B lymphocyte (B cell)**, also presents antigen to T cells. The presented antigen peptides, called **epitopes**, bind specific receptors on the lymphocyte surface, triggering the expression of genes that cause the lymphocytes to proliferate and differentiate. Differentiated B cells called **plasma cells** specialize in the production of antigen-specific proteins called **antibodies** (**immunoglobulins**), which bind the pathogen and mark it for destruction.

Unlike the comparatively rapid innate response, a protective adaptive response usually takes several days to develop, and the strength of the adaptive response increases as the numbers of antigen-reactive lymphocytes increase. Although adaptive immune responses are typically slower than innate response mechanisms, they are highly specific and result in **immune memory**, the ability of lymphocytes to quickly respond after subsequent exposure to a previously encountered antigen.

With this broad overview of inborn and acquired immune responses in place, we now consider the many natural barriers to infection that exist in the healthy human body. It is only after one or more of these barriers has been breached that pathogen invasion can occur and the immune system is brought into play.

Check Your Understanding

- What major class of immune cells mediates an innate immune response? What additional type of immune cells is required for an adaptive immune response?
- What term is used to describe the unique molecules found on the surface of different pathogens?

26.2 Barriers to Pathogen Invasion

Although the vast majority of microorganisms that associate with the human body are not pathogenic, certainly some do cause disease. The best defense against infectious diseases for the human body is to prevent pathogens from colonizing in the first place. Humans have a host of resistance factors that inhibit pathogen colonization and thereby prevent the onset of infectious diseases. These include a variety of physical and chemical barriers to microbial infection. In addition, the condition of the host and the composition of microorganisms that normally inhabit the host are factors that are often the tipping point between health and disease.

Natural Host Resistance

Several resistance factors common to vertebrate hosts inhibit infection by most pathogens in a nonspecific way (Figure 26.2). For example, the presence of normal microbiota in the human body is critically important for resisting pathogen infection, especially on the skin and in the mucosal tissues of the gastrointestinal, respiratory, and reproductive tracts (Chapter 24). Pathogens do not easily infect tissues on which normal microbiota are well established because the harmless microbes limit available nutrients and sites for

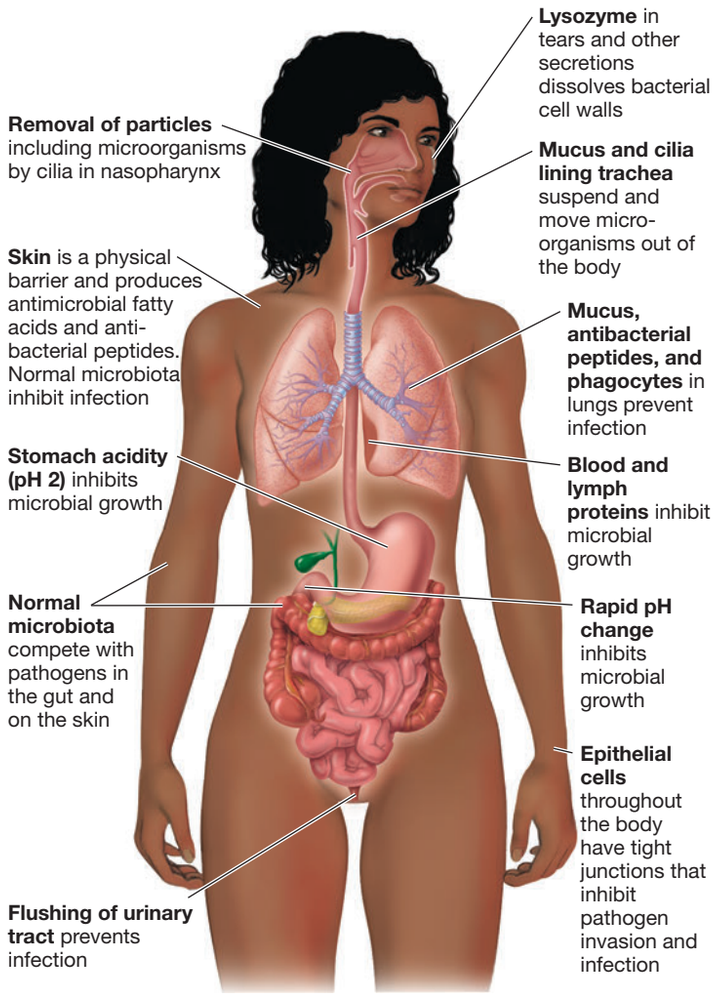


Figure 26.2 Barriers to infection in the human body. These barriers provide natural resistance to colonization and infection by pathogens.

infection by the pathogens. Although the *competitive exclusion* of invading pathogens by resident microbes is technically not a component of the host's immune system, the nonpathogenic normal microbiota found in or on the body play a major role in preventing disease through this principle. It is not uncommon for disruptions to the composition of the normal microbiota, such as that which may occur when antibiotic drug treatments nonspecifically kill harmless and even beneficial microbes in the body, to trigger the onset of disease by opportunistic pathogens.

The ability of a particular pathogen to cause disease in a host is highly variable. For instance, certain animal species, including raccoons and skunks, are much more susceptible to the rabies virus than are others, such as the opossum, which only rarely develops the disease. Anthrax infects many species of animals, causing disease symptoms varying from fatal blood poisoning in cattle to the mild pustules of human cutaneous anthrax. Introduction of the same pathogen by other routes, however, may challenge the resistance of the host. For example, anthrax causes a localized infection when acquired through the skin but a lethal, systemic infection when acquired through the mucous membranes of the lungs (▶ Sections 30.9 and 32.8). Another barrier to infection is the fact that diseases of endothermic ("warm-blooded") animals, such as birds and mammals, are rarely transmitted to ectothermic ("cold-blooded") animals, such as amphibians and reptiles, and vice versa. Presumably, the anatomical and physiological features of one group are not compatible with pathogens that infect the other group.

In addition to these factors, the general condition of the host plays a role in the balance between health and disease. Susceptibility to infections is higher in the very young and the very old, as well as those suffering from nutritional or stress-related problems. For example, the intestinal microbiota matures over time and thus infants under one year of age often contract diarrhea caused by bacterial or viral pathogens more readily than do adults. Conversely, the immune system of older adults may be weakened or compromised from medical procedures or drug therapy, leading to a higher susceptibility to infectious disease. Other problems that affect both the young and the old—smoking, poor diet, intravenous drug use, excessive alcohol consumption, chronic lack of sleep—can all play a role in whether a pathogen can infect a host and cause disease.

Infection Site and Tissue Specificity

Most pathogens must adhere to and colonize the site of exposure to initiate infection. Even if pathogens adhere to an exposure site, the organisms cannot colonize the host if the site is not compatible with the pathogen's nutritional and metabolic needs. For example, if cells of *Clostridium tetani* (cause of the disease tetanus) were ingested, tetanus would not normally result because the pathogen would be either killed by the acidity of the stomach or unable to compete with the well-developed normal intestinal microbiota. If, on the other hand, *C. tetani* cells or endospores were introduced into a puncture wound, the organism would grow and produce tetanus toxin in the anoxic zones created by local tissue death. Conversely, enteric bacteria, such as *Salmonella* and *Shigella*, do not normally cause wound infections but can infect the intestinal tract and cause gastrointestinal illness.

In some cases, pathogens interact exclusively with members of a few closely related host species because the hosts share tissue-specific receptors, important in establishing infection (◀ Section 25.1). For example, human immunodeficiency virus (HIV, the causative agent of AIDS) infects only humans and their closest primate relatives, including the great apes. This is because the HIV-binding cell surface proteins CXCR4, present on human T lymphocytes, and CCR5, present on human macrophages (Section 26.1), are also expressed in great apes. Other animals, including most other primates, lack CXCR4 and CCR5 and are therefore not susceptible to HIV infection. Many other pathogens exhibit tissue specificity to such a degree that they infect only a single species. For example, a rhinovirus that infects nasal epithelial cells in humans, causing symptoms of the common cold, would generally not infect and cause similar symptoms in a nonhuman host. **Table 26.1** presents some other examples of tissue specificity in pathogens.

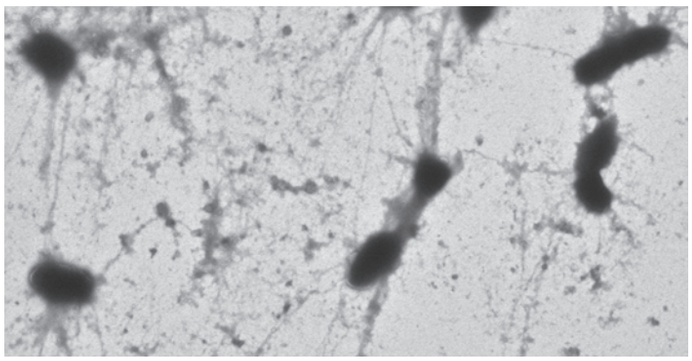
TABLE 26.1 Tissue specificity in infectious disease

Disease	Tissue or cell type infected	Pathogen
Acquired immunodeficiency syndrome (AIDS)	T-helper lymphocytes	Human immunodeficiency virus (HIV)
Botulism	Motor end plate	*Clostridium botulinum*
Cholera	Small intestine epithelium	*Vibrio cholerae*
Dental caries	Oral epithelium	*Streptococcus mutans, S. sobrinus, S. mitis*
Diphtheria	Throat epithelium	*Corynebacterium diphtheriae*
Gonorrhea	Mucosal epithelium	*Neisseria gonorrhoeae*
Influenza	Respiratory epithelium	Influenza A and influenza B viruses
Malaria	Blood (erythrocytes)	*Plasmodium* spp.
Pyelonephritis	Kidney medulla	*Proteus* spp.
Spontaneous abortion (cattle)	Placenta	*Brucella abortus*
Tetanus	Inhibitory interneuron	*Clostridium tetani*

Physical and Chemical Barriers to Infection

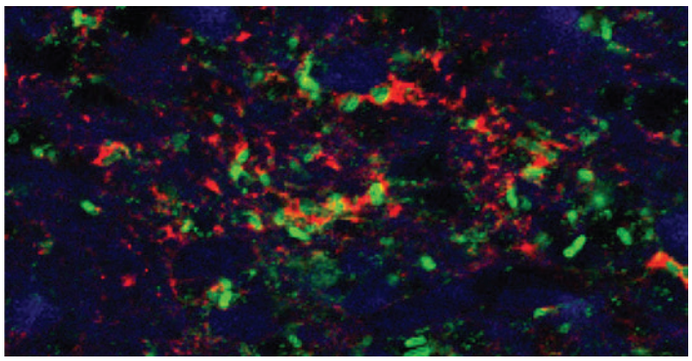
The structural integrity of tissue surfaces poses a barrier to penetration by microorganisms (◀ Section 25.2). The tight junctions between epithelial cells that line body tissues inhibit invasion and infection (Figure 26.2). In the skin and mucosal tissues, potential pathogens must first adhere to tissue surfaces and then grow at these sites before traveling elsewhere in the body. Mucosal surfaces are coated with a layer of protective mucus that traps microorganisms, pollen, and other foreign agents. Epithelial cells underlying the mucus layer have cilia on their surfaces that carry out coordinated movements to expel suspended pathogens and keep them from adhering to tissues. This combination of mucus and ciliary motion plays a key role in removing inhaled microorganisms from the mucosal surfaces of the trachea and bronchial tubes, thus preventing microbial colonization of the lungs.

In the central nervous system (CNS), pathogens that are able to penetrate the blood–brain barrier present a special challenge



Deepak Kumar, Vijaya Kumar, and Robert Moir

(a)



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(b)

Figure 26.3 Amyloid-β (Aβ) protein as an innate defense in the central nervous system. *(a)* Bacteria that breach the blood–brain barrier stimulate production of Aβ protein, which forms a dense network of fibrils to trap the pathogens and minimize their invasiveness. *(b)* Fluorescence micrograph of cells of *Salmonella* bacteria (green) immobilized by a mesh of Aβ fibrils (red), enabling more efficient pathogen destruction by immune mechanisms.

because adaptive immune responses mediated by lymphocytes are less effective at these sites. However, a long-misunderstood protein—amyloid-β (Aβ)—may play a role in innate defense of the brain by containing invading pathogens. As discussed in MicrobiologyNow at the beginning of the chapter and shown in **Figure 26.3**, Aβ proteins form a mesh of fibrils to trap bacteria that are able to invade the CNS. However, in cases of heavy or chronic infection, excessive deposition of Aβ may cause the fibrils to aggregate into dense, insoluble complexes that form amyloid plaques in the brain tissue, one of the hallmarks of Alzheimer's disease.

Sebaceous glands in the skin secrete fatty acids and lactic acid, lowering the acidity of the skin to pH 5 and inhibiting colonization by many pathogenic bacteria (blood and most internal organs are about pH 7.4). Potential pathogens ingested in food or water must survive the strong acidity (pH 2) and digestive enzymes, such as pepsin, in the stomach (Figure 26.2). Although the pH returns to neutral in the lower intestinal tract, pathogens that survive passage through the stomach must then compete with the abundant resident microbiota present in the small and large intestines (◀ Figure 24.3). In addition to the established normal microbiota that exist at many potential infection sites, resistance to infection and invasion is enhanced by antibacterial substances called *defensins*, produced in the skin, lungs, and gut. Finally, the lumen of the kidney, the eye, the respiratory system, and the cervical mucosa are constantly bathed with tears, mucus, or other secretions that contain *lysozyme*, an enzyme that can kill bacteria by digesting the cell wall.

The human body is thus naturally protected by a host of physical barriers, chemicals, and secretions, all of which combine to naturally suppress pathogen invasion and infection. However, breaching of these defenses by a pathogen activates cellular defenses of the immune system, beginning with mechanisms of microbe recognition and innate responses. We now consider the organs and cellular components of the immune system before exploring how this complex and interrelated system works.

Check Your Understanding

- How do pathogens recognize the tissues they infect?
- Identify physical and chemical barriers to pathogens. How might these barriers be compromised?
- What other factors may control the outcome of an infectious disease?

II • Cells and Organs of the Immune System

Cellular components of the immune system are derived from hematopoietic stem cells in the bone marrow. These cells circulate throughout the body in the blood and lymph systems in constant surveillance for foreign cells, viruses, or their antigenic products.

The immune response is a result of the activities of a wide variety of specialized cells that circulate throughout the body, primarily through the blood and *lymph*, a fluid similar to blood but which lacks red blood cells.

26.3 The Blood and Lymphatic Systems

Circulatory systems in the human body include separate vasculature for blood and lymph. It is through circulation of these fluids that cells and proteins of the immune system are transported to the various tissues and organs of the body. All cells found in the blood and lymph are derived from **hematopoietic stem cells** found primarily in the bone marrow but also in the gut. Hematopoietic stem cells continuously divide and differentiate to supply the body with both erythrocytes (red blood cells) and **leukocytes** (white blood cells).

Blood and Lymph Circulation

Blood is pumped by the heart through arteries and capillaries throughout the body and is returned to the heart through the veins (**Figure 26.4*a*, *b***). In the capillary beds, leukocytes and solutes pass to and from the blood into the lymphatic system. Lymph drains from extravascular tissues into lymphatic vessels (lymph capillaries and lymph ducts) and then into **lymph nodes** throughout the lymphatic system (Figure 26.4*b*–*d*). Lymph nodes contain leukocytes arranged to encounter antigens, such as those found on microorganisms, as they travel through the lymphatic circulation. Lymph containing antibodies and immune cells empties into the blood circulatory system via the thoracic lymph duct (Figure 26.4*a*).

UNIT 6

Mastering Microbiology
Art Activity: Figure 26.3*a* The blood and lymphatic systems

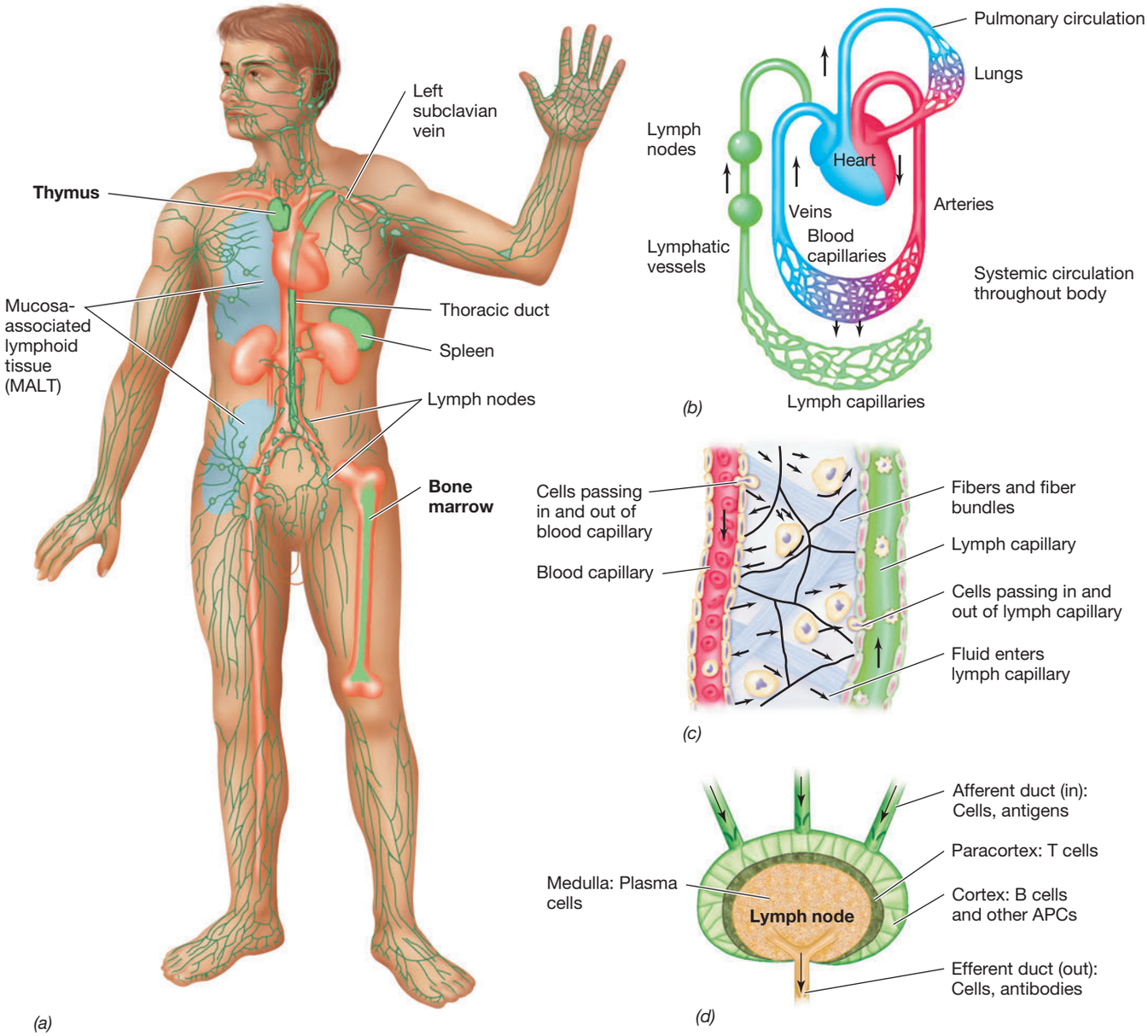


Figure 26.4 The blood and lymphatic systems. *(a)* Blood and lymph circulation in the body. Major blood vessels and associated organs are shown in red. Major lymphatic organs and vessels are shown in green. The primary lymphoid organs are the bone marrow and thymus. The secondary lymphoid organs are the lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT). *(b)* Connections between the lymphatic and blood systems. Blood flows from the veins to the heart, to the lungs, and then through the arteries to capillaries in tissues. Exchange of solutes and cells occurs between blood and lymphatic capillaries. Lymph drains from the thoracic duct into the left subclavian vein of the blood circulatory system. *(c)* The exchange of cells between the blood and lymphatic systems is shown in detail. Both blood and lymphatic capillaries are closed vessels, but cells pass from blood capillaries to lymphatic capillaries and back by the process of diapedesis. *(d)* A secondary lymphoid organ, the lymph node, showing the major anatomical areas and the immune cells concentrated in each area. The anatomy of the MALT and the spleen is analogous to that of the lymph nodes. APCs, antigen-presenting cells.

Mucosa-associated lymphoid tissue (MALT) is another organized lymphoid tissue containing leukocytes that interact with antigens that enter the body through mucous membranes, including those of the gut, the genitourinary tract, and the respiratory tract.

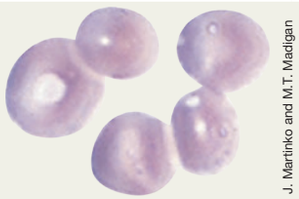
The *spleen* is an important organ in immunity and consists of *red pulp*—rich in red blood cells and macrophages—and *white pulp*, which consists of organized lymphocytes and phagocytes arranged to filter blood in a manner similar to lymph nodes and MALT. Collectively, the lymph nodes, MALT, and spleen are called **secondary lymphoid organs** (or tissues, in the case of MALT, Figure 26.4). The secondary lymphoid organs are the sites where antigens interact with antigen-presenting cells (Section 26.4) and lymphocytes to trigger the adaptive immune response.

Hematopoiesis

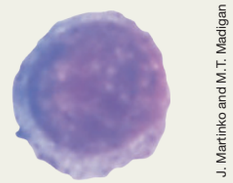
Hematopoietic stem cells are precursor cells found in bone marrow and the gut that can differentiate into any type of blood cell (see Figure 26.5), a process called **hematopoiesis**. Stem cell differentiation in these tissues is influenced by soluble **cytokines** and **chemokines**,

TABLE 26.2 Major cell types found in normal human blood

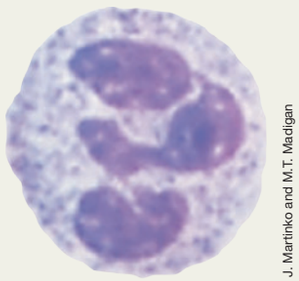
Cell type	Cells per milliliter
Erythrocytes	$4.2–6.2 \times 10^9$
Leukocytes[a]	$4.5–11 \times 10^6$
Lymphocytes	$1.0–4.8 \times 10^6$
Granulocytes and monocytes	Up to 7.0×10^6



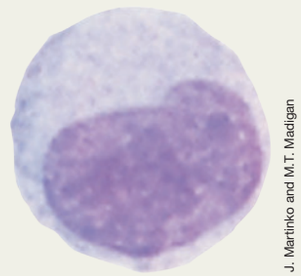
(a) **Red blood cells (erythrocytes)**



(b) **Lymphocyte**



(c) **Neutrophil (a granulocyte)**



(d) **Monocyte**

[a]Leukocytes include all nucleated blood cells (Figure 26.5).

proteins that direct immune cell production, function, and movement, thereby modulating the immune response (Section 26.5). Following differentiation, newly formed blood cells enter the circulation and reach other parts of the body, where the leukocytes further mature and become activated upon antigen encounter.

Blood consists of cellular and noncellular components, including many cells and molecules active in the immune response (Table 26.2). The most numerous cells in human blood are *erythrocytes*, small, nonnucleated cells that carry oxygen from the lungs to the tissues. About 0.1% of the cells in blood, however, are white blood cells—leukocytes—of which there are many different types (Table 26.2). Blood cells are suspended in **plasma**, a liquid that contains proteins and other solutes. Outside the body, blood or plasma quickly forms an insoluble fibrin clot, remaining liquid only when an anticoagulant such as potassium citrate or heparin is added. When blood clots, the insoluble proteins trap the cells in a large, insoluble mass. The remaining fluid, called **serum**, lacks both cells and clotting proteins. Serum does, however, contain a high concentration of other proteins, in particular antibodies, key players in the adaptive immune response, as we will learn in Chapter 27.

Check Your Understanding

- Describe the circulation of a leukocyte from the blood to the lymph and back to the blood.
- What soluble molecules determine whether a particular hematopoietic stem cell will become a phagocyte, lymphocyte, or erythrocyte?

26.4 Leukocyte Production and Diversity

The innate and adaptive immune responses are mediated by several types of leukocytes (Table 26.2). From its hematopoietic stem cell precursor, a leukocyte will differentiate and mature through either the *myeloid* or the *lymphoid* lineage (Figure 26.5). Leukocytes move throughout the body and, through a process called *diapedesis* (also called *extravasation*), pass from blood to interstitial spaces. Leukocytes may then be collected with lymph into lymphatic vessels, where they are eventually returned to the blood circulatory system (Figure 26.4*c*).

Myeloid Cells—Monocytes and Granulocytes

Myeloid cells, the principal effector cells of innate immunity, are derived from myeloid precursor cells. Mature myeloid cells develop from one of two lineages: **monocytes** or **granulocytes** (Figure 26.5 and Table 26.2). Immature monocyte precursor cells circulate in the blood for several days before moving into other tissues and differentiating into *macrophages* or *dendritic cells*. These phagocytic cells function as **antigen-presenting cells (APCs)** because they use a special type of cell surface protein called a class II **major histocompatibility complex (MHC)** to present engulfed and processed antigens to T lymphocytes, which then initiate an adaptive immune response (Figure 26.5). We will revisit MHC proteins later in this chapter and discuss them in detail in Chapter 27.

Macrophages are often the first defensive cells that interact with a pathogen and are abundant in many tissues and organs, especially the spleen, lymph nodes, and MALT, where they constitute up to 15% of total cells. Because they ingest and destroy most pathogens and foreign molecules that invade the body, macrophages are essential to the innate response. Dendritic cells are found throughout the body tissues and are especially abundant along epithelial linings, including the skin and mucous membranes. When dendritic cells ingest antigen, they migrate to nearby lymph nodes (Figure 26.4), where they are extremely efficient at presenting antigen to T lymphocytes. Thus, dendritic cells are an important link between innate and adaptive immunity. The specialized antigen-presenting properties of macrophages and dendritic cells will be examined in Section 27.5.

Granulocytes are the second lineage of cells derived from myeloid precursors. Granulocytes contain cytoplasmic inclusions, or granules, that are visible in stained microscopic preparations. These granules contain toxins or enzymes that are released to destroy target cells. One granulocyte, the *neutrophil*, also called a *polymorphonuclear leukocyte* (*PMN*), is an abundant, highly motile phagocyte that responds rapidly to pathogen challenge, an activity that is central to innate immunity. Neutrophils are found predominantly in the bloodstream and bone marrow, from where they migrate to sites of active infection. *Eosinophils* are a second, much less numerous, type of phagocytic granulocyte. A major function of eosinophils is to destroy larger, extracellular parasites, such as infectious helminths (worms, ▶ Section 34.7), by secreting cytotoxic enzymes and then clearing the debris from the body tissues.

Other granulocytes include *basophils*, which circulate in the blood, and *mast cells*, which reside in host tissues (Figure 26.5). The intracytoplasmic granules of these cells contain histamine and other mediators that function to initiate an inflammatory response when secreted, a process called *degranulation*. Mast cell and basophil degranulation is responsible for certain types of allergic reactions (▶ Section 28.1).

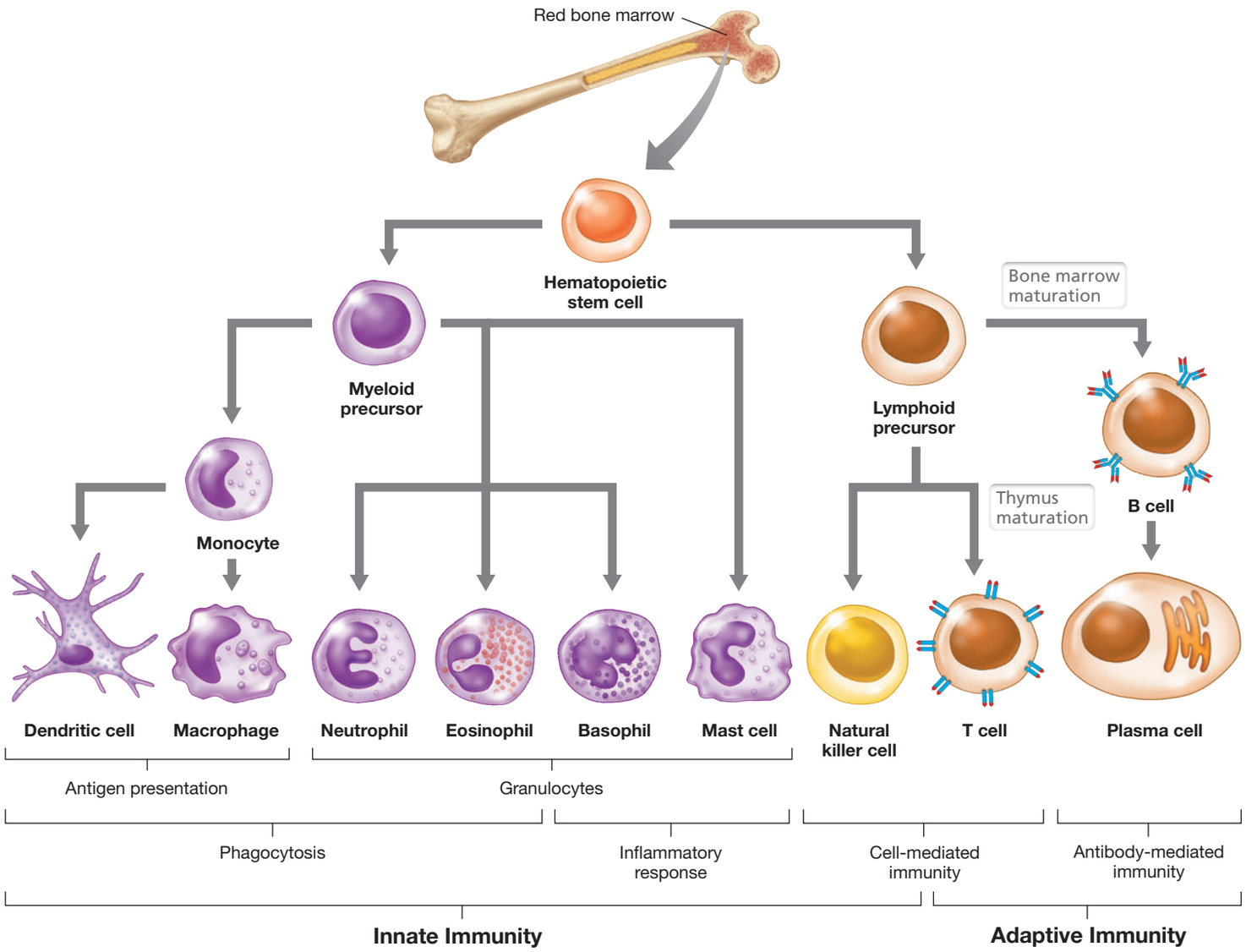


Figure 26.5 Lineage and diversity of immune response cells. Immune cells develop from hematopoietic stem cells in the bone marrow into either myeloid precursors or lymphoid precursors. These precursors, in turn, differentiate into mature cells that have various immune functions. (Erythrocytes also develop from myeloid precursors.)

Lymphocytes

Lymphocytes are specialized leukocytes derived from lymphoid precursor cells (Figure 26.5 and Table 26.2). As we have discussed (Section 26.1), there are two major types of lymphocytes: *B cells* (B lymphocytes) and *T cells* (T lymphocytes) (Figure 26.5). Whereas B cells originate and mature in the bone marrow, T cells begin their development in the bone marrow but migrate to the *thymus* to mature (Figure 26.4*a* and see Figure 27.2). In mammals, the bone marrow and thymus are called **primary lymphoid organs** because they are the sites where lymphoid stem cells differentiate into functional, antigen-specific lymphocytes (Figure 26.4*a*). Naive B and T cells circulate through the blood and lymph system and into the *secondary lymphoid organs* (Section 26.3) where, upon antigen encounter, they become activated and further differentiate into effector and memory cells.

B cell surfaces are covered with **B cell receptors (BCRs)**, membrane-bound immunoglobulins that function to bind free antigen. Following internalization and processing of bound antigen, B cells—like dendritic cells and macrophages—function as specialized APCs, employing class II MHCs to present antigen to T lymphocytes (see Figure 27.6). The T cells, in turn, interact with presented antigens through membrane-bound **T cell receptors (TCRs)**, protein complexes that specifically bind antigens presented on MHCs. We explore the roles of B and T lymphocytes in the adaptive immune response in Chapter 27.

Like B and T cells, natural killer (NK) cells (Figure 26.5) are derived from lymphoid precursors. However, unlike B and T lymphocytes, NK cells function primarily in innate immunity. NK cells rid the body of virus-infected and tumor cells using a mechanism that distinguishes healthy versus compromised cells based on the presence (or absence) of specific surface molecules (Section 26.10).

Check Your Understanding

- How does the development of B, T, and NK cells differ from that of dendritic cells and macrophages?
- Distinguish between the primary lymphoid organs and the secondary lymphoid organs. What is the relationship between the components of these two groups?

III • Phagocyte Response Mechanisms

Phagocytes respond quickly to tissue damage and microbial invasion, whereupon they recognize, engulf, and digest pathogens through various molecular mechanisms. Phagocytic activities are the hallmark of the innate immune response.

Innate immunity is primarily driven by the activities of phagocytes (Section 26.1). These cells recognize common structural features found on and in pathogens. Interactions with pathogens activate genes in the phagocytes that control the transcription, translation, and expression of proteins that eventually lead to the destruction of the pathogens.

Innate immunity responds within minutes of a phagocyte engaging a pathogen, but it is not always effective in controlling infections. To counter these infections, certain phagocytes also activate adaptive immunity by processing and presenting antigens to receptors on T lymphocytes (Chapter 27). However, because an adaptive response to a specific pathogen takes several days to develop, the ability of phagocytes to quickly respond to microbial invasion is an indispensable mechanism for infection containment.

26.5 Pathogen Challenge and Phagocyte Recruitment

When a pathogen breaches the physical and chemical barriers of a host (Figure 26.2) and causes an infection, the immune system is mobilized to protect the host from further damage. Innate immunity is the first line of defense and is critical for host protection for about four days after an infection begins. Phagocytes engulf and destroy pathogens, often initiating complex host-mediated inflammatory reactions when they do (Section 26.8).

Microbial Invasion

The initial inoculum of a pathogen is usually insufficient to cause host damage, even if a pathogen gains access to tissues. For the pathogen to be successful, it must attach, multiply, and colonize the tissue (◀ Figure 25.1), and these events require that the pathogen find appropriate nutrients and environmental conditions for growth.

Following colonization, a pathogen must usually invade tissues to initiate disease. **Invasion** is the ability of a pathogen to enter into host cells or tissues, multiply, spread, and cause disease. Microbial infections often begin at breaks or wounds in the skin (**Figure 26.6*a***) or on the mucous membranes of the respiratory, digestive, or genitourinary tract, surfaces that are normally microbial barriers (Figure 26.2). In some cases, growth may also begin on intact mucosal surfaces, especially if the composition of the normal microbiota has been altered or

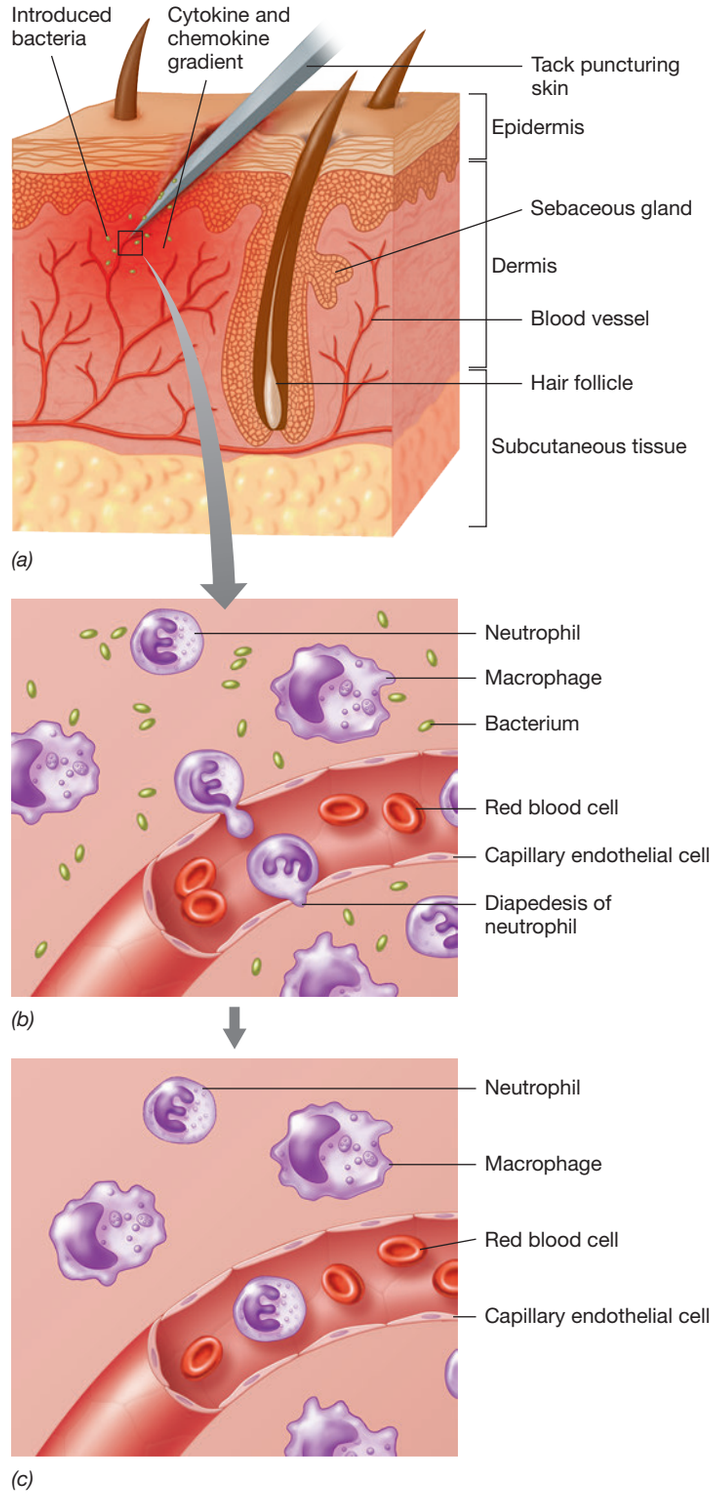


Figure 26.6 Microbial invasion and the innate immune response. *(a)* Tissue damage, such as that caused by a tack puncturing the skin, can lead to invasion by microorganisms and the release of cytokines and chemokines from damaged host cells. *(b)* Phagocytes, especially neutrophils, are recruited to the site of infection by the cytokine–chemokine gradient, squeezing out of dilated capillaries via diapedesis. *(c)* Invading microorganisms are cleared by phagocytosis, and the tissue is restored to health. See Figures 26.1 and 26.5 for functional descriptions of some of the cells and components shown here, and see Table 26.1 for photomicrographs and quantitative data on some of the cells shown here.

eliminated, for example by antibiotic therapy. Tissue damage caused by invasive pathogens or injury triggers the recruitment of large numbers of phagocytes and other immune cells to the site of infection.

Tissue Damage and Chemokine Release

When microorganisms invade and damage host tissues, resident phagocytes (usually tissue macrophages) engage the microbes and become activated. The activated macrophages and damaged host cells release *cytokines* and *chemokines* into the surrounding tissues (Figure 26.6*a*). Cytokines bind specific receptors on cells and induce a particular response from them, usually by activating a signaling pathway that controls transcription and protein synthesis. Chemokines are an important subclass of cytokines that have the specific role of recruiting circulating immune cells, especially neutrophils (Figure 26.5), to the site of injury.

Resident macrophages, which are found in all of the body's organ systems, are stimulated by the presence of invading pathogens to secrete chemokines that establish a gradient of *chemoattractants*. These molecules bind receptors on other immune cells, especially neutrophils and T cells, and recruit them to the site of infection (Figure 26.6*b*). This triggers a neutrophil-mediated inflammatory response and, later, an adaptive immune response facilitated by lymphocytes against the specific invading agent or its toxic products.

Localized inflammation allows neutrophils, the most numerous circulating phagocytes, to migrate quickly along the chemotactic gradient to the site of infection (Figure 26.6*b*). Once there, chemokine-mediated binding reactions promote neutrophil adhesion to the inner wall of the blood capillary, a process called *margination*. The halted neutrophils then undergo *diapedesis*, the movement of leukocytes from the bloodstream to surrounding infected tissues by squeezing between endothelial cells lining the capillaries (Figure 26.6*b*). Higher than normal numbers of neutrophils in the blood or at a site of inflammation indicate an active response to a current infection.

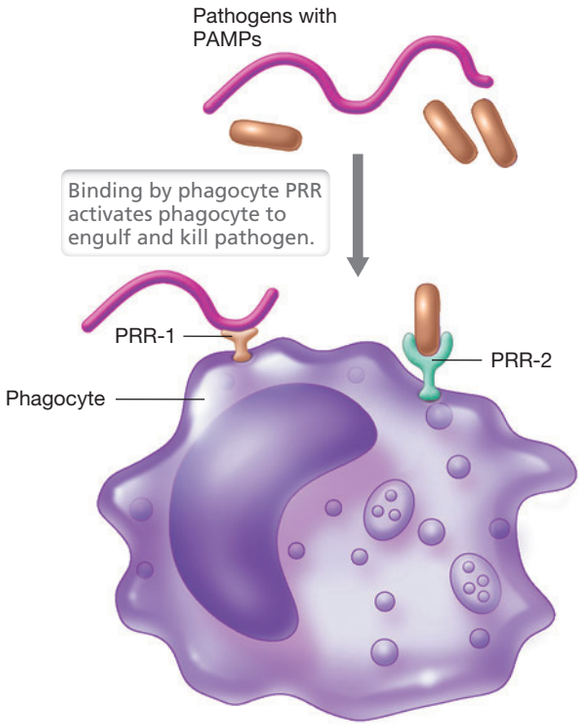
Neutrophils and other phagocytes that encounter pathogens in damaged areas must be able to recognize, capture, and destroy pathogens to clear infections and restore body tissues to a healthy state (Figure 26.6*c*). We explore the molecular mechanisms that facilitate these cellular interactions in the next section.

Check Your Understanding

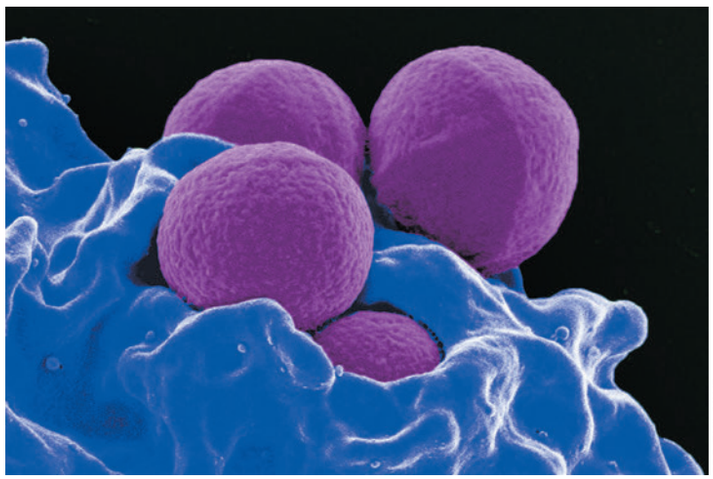
- Describe several mechanisms by which opportunistic microorganisms can invade body tissues. What body sites are often associated with microbial invasion?
- Describe the mechanisms by which circulating phagocytic cells are recruited to a site of infection.

26.6 Pathogen Recognition and Phagocyte Signal Transduction

The first type of immune cell to be activated in the innate response is typically a phagocyte, whose primary function is to engulf and destroy pathogens. Phagocytes include neutrophils, eosinophils, and monocytes, which are found primarily in the blood, and macrophages and dendritic cells, which occur primarily in the body tissues (Figure 26.5). But how do these cells distinguish pathogens and other foreign agents from the myriad other cells in the body?



(a)



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(b)

Figure 26.7 Pathogen recognition by phagocytes. *(a)* Phagocytes interact with pathogens by way of preformed pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs). Binding of a PAMP by a phagocyte PRR stimulates the phagocyte to destroy the pathogen and activate other phagocytes. *(b)* Colorized scanning electron micrograph of a neutrophil (blue) phagocytosing several cells of methicillin-resistant *Staphylococcus aureus* (MRSA; magenta). For more on MRSA, see Explore the Microbial World in Chapter 29 and Section 31.9.

Moreover, once invaders are recognized, how do phagocytes capture and destroy them? We address these topics now.

Pathogen-Associated Molecular Patterns

The macromolecules inside and on the surface of pathogens contain **pathogen-associated molecular patterns (PAMPs)**, repeating

structural subunits common to broadly related groups of infectious agents. PAMPs may include polysaccharides, proteins, nucleic acids, or even lipids. For example, a common PAMP is the lipopolysaccharide (LPS) common to all gram-negative bacterial outer membranes (◀ Section 2.4). Other PAMPs include bacterial flagellin, the double-stranded RNA (dsRNA) of certain viruses, and the lipoteichoic acids of gram-positive bacteria (◀ Section 2.3). Like all PAMPs, these molecules are found on various pathogens but are absent from host cells. Therefore, PAMPs serve as markers by which phagocytes can identify microbes, even if the pathogens have not been encountered previously.

Pattern Recognition Receptors

Phagocytes employ a pathogen-recognition system that triggers a timely and appropriate response, leading to recognition, containment, and destruction of pathogens. Phagocytes produce soluble or membrane-bound proteins called **pattern recognition receptors (PRRs)** that recognize and bind PAMPs, allowing phagocytes to interact quickly and effectively with pathogens (**Figure 26.7*a***). The interaction of a PAMP with a PRR activates the phagocyte to ingest and destroy the targeted pathogen by phagocytosis (Figure 26.7*b* and Section 26.7).

PRRs were first observed in phagocytes of *Drosophila*, the fruit fly, where they are called *Toll receptors*, and they have since been identified as important components of microbial recognition throughout the animal kingdom (see Explore the Microbial World, "Pattern Recognition Receptors of Hydrothermal Vent Tube Worms Facilitate Endosymbiosis"). Structural, functional, and evolutionary homologs of the Toll receptors, called **Toll-like receptors (TLRs)**, are widely expressed on mammalian innate immune cells. TLRs are found associated with membranes on the surface of or in intracellular vesicles of all types of phagocytes. At least nine TLRs in humans interact with a variety of cell surface and soluble PAMPs from viruses, bacteria, and fungi (**Table 26.3**).

Each TLR on a human phagocyte recognizes and interacts with a specific PAMP. For example, TLR-2, a PRR on human phagocytes, interacts with peptidoglycan, a PAMP present in the cell wall of nearly all bacteria (**Figure 26.8**). This PAMP–PRR interaction activates the phagocytes, which then target gram-positive pathogens with exposed peptidoglycan. Access to the peptidoglycan of gram-negative bacterial cell walls is blocked by the surface lipopolysaccharides. However, another PRR found on phagocytes, TLR-4, interacts with the LPS of gram-negative bacteria, such as *Salmonella* spp., *Escherichia coli*, and *Shigella* spp. (Table 26.3 and see Figure 26.9).

Several soluble host molecules function similarly to these phagocyte-associated PRRs. The *NOD-like receptors* (*NLRs*) are a family of soluble PRRs found in the cytoplasm that have a nucleotide-binding domain (NOD). NOD1 and NOD2 interact with

TABLE 26.3 Receptors and targets in the innate immune response

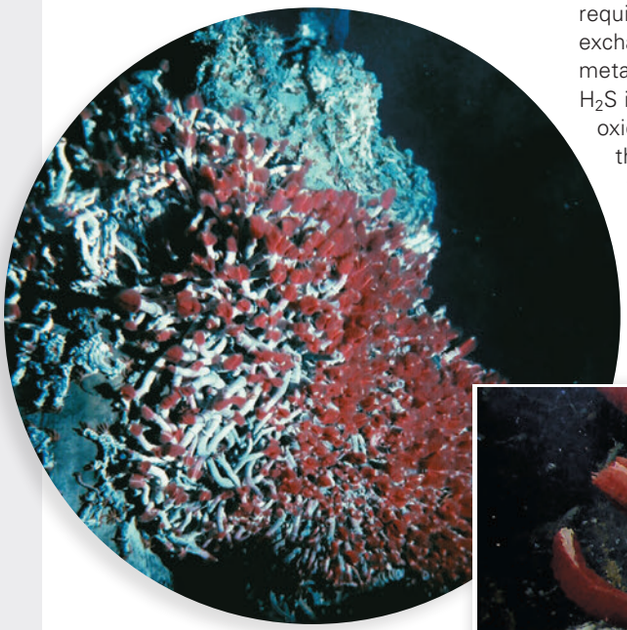
Pattern recognition receptors (PRRs)	Pathogen-associated molecular patterns (PAMPs) and targets	Result of interaction
Soluble extracellular PRRs[a]		
Mannose-binding lectin (soluble)	Mannose-containing components of microbial cell surface, as in gram-negative bacteria	Complement activation
C-reactive protein (soluble)	Components of gram-positive cell walls	
Plasma membrane–associated PRRs		
TLR-1 (Toll-like receptor 1)	Lipoproteins in mycobacteria	Signal transduction, phagocyte activation, and inflammation[b]
TLR-2	Peptidoglycan on gram-positive bacteria; zymosan in fungi	
TLR-4	LPS (lipopolysaccharide) in gram-negative bacteria	
TLR-5	Flagellin in bacteria	
TLR-6	Lipoproteins in mycobacteria; zymosan in fungi	
Endosomal membrane–associated PRRs[c]		
TLR-3	Double-stranded viral RNA[d]	Signal transduction, phagocyte activation, and inflammation
TLR-7, TLR-8	Single-stranded viral RNA	
TLR-9	Unmethylated CpG oligonucleotides in bacteria	
Cytoplasmic PRRs: NLRs (NOD-like receptors)		
NOD1	Peptidoglycan on gram-negative bacteria	Stimulate production of antimicrobial peptides and proinflammatory cytokines
NOD2	Peptidoglycan on gram-positive bacteria	
NLRP3	Inflammasome component	Triggers release of proinflammatory cytokines, increasing inflammation

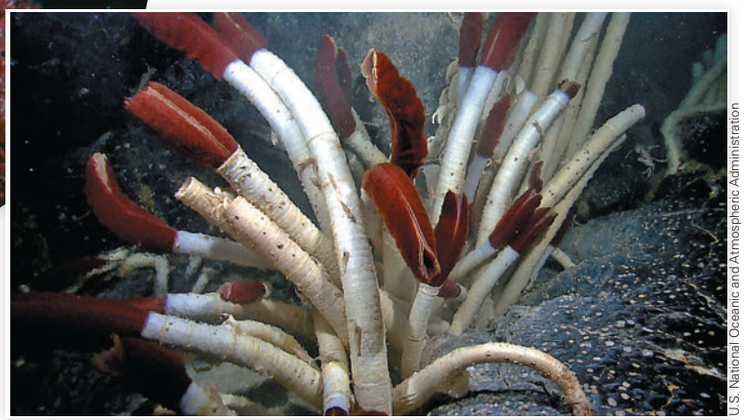
[a]The extracellular soluble PRRs are produced by liver cells in response to inflammatory cytokines.
[b]Toll-like receptors initiate phagocyte activation via signal transduction.
[c]TLR-3, -7, -8, and -9 are found in membranes of intracellular organelles (endosomes) such as lysosomes. A tenth Toll-like receptor, TLR-10, has unknown ligand specificity and function.
[d]Present in Baltimore class III viruses (◀ Figure 11.2*b* and Section 11.10)

Pattern Recognition Receptors of Hydrothermal Vent Tube Worms Facilitate Endosymbiosis

Invertebrates and plants lack adaptive immunity but have a well-developed innate immune response to a wide variety of pathogens. As discussed in Section 26.6, virtually all multicellular organisms respond to pathogen invasion by recognizing signature molecules found on pathogen surfaces. These molecules contain conserved, repetitive structures called pathogen-associated molecular patterns (PAMPs) that include molecules such as the lipopolysaccharide (LPS) and flagellin of gram-negative bacteria, the peptidoglycan of gram-positive bacteria, and the double-stranded RNA of certain viruses. The term *microbe-associated molecular pattern*, or MAMP, is also commonly used to describe signature molecules found on microorganisms. However, "MAMP" is a broader term than "PAMP" because it includes components found on microorganisms that are not pathogenic.

Unlike PAMPs, which are exploited specifically for innate defense against pathogens, MAMPs found on nonpathogenic bacteria can serve an entirely different purpose—that of facilitating, through host pattern recognition receptors (PRRs), a mutualistic partnership rather than a confrontation between a host and the bacteria that colonize it. As we learned in Chapter 23, a wide variety of plants and animals maintain symbiotic relationships with microorganisms. There we discussed the association of tube worms that develop near hydrothermal vents in the deep sea with autotrophic, sulfur-oxidizing bacteria (SOB) that inhabit their *trophosome*, a spongy internal organ that comprises most of the volume of the 1- to 2-m-long worms (**Figure 1**). These SOB form an endosymbiosis with the worms in which the bacteria provide all organic carbon requirements for their animal host in exchange for a steady supply of essential metabolites, in particular H_2S, O_2, and CO_2. H_2S is the energy source for the SOB; they oxidize it to S^0 and then SO_4^{2-} and respire the electrons to generate a proton motive force that drives ATP synthesis. Oxygen (O_2) is required as a terminal acceptor of electrons that have traversed the electron transport chain. CO_2 is the carbon source and is incorporated into bacterial cell material by way of the Calvin cycle, the major means of autotrophy in chemolithotrophic bacteria.





U.S. National Oceanic and Atmospheric Administration

Figure 1 Hydrothermal vent tube worms harboring endosymbiotic sulfur-oxidizing bacteria. Top: A "black smoker" hydrothermal vent community containing several tube worms that obtain organic carbon from sulfur-oxidizing chemolithotrophic bacteria (SOB) living within them. Bottom: A close-up view of tube worms; each worm is 1–2 m long. The red area on the top of each worm, called the plume, is where O_2 and H_2S are taken in to be fed to the worm's SOB endosymbionts residing in the trophosome.

This fascinating association raises the question of how it is established. Specifically, how does the tube worm populate its trophosome with SOB to the exclusion of other, potentially pathogenic, bacteria? The answer appears to be closely linked to MAMPs associated with the endosymbiotic SOB. Although host PRRs are typically used to recognize and eliminate pathogens, the study of tube worms and other animals that harbor endosymbiotic microbes shows a broader functionality for PRRs in that they can also interact beneficially with MAMPs to selectively populate a host with nonpathogenic symbionts.

The tube worm trophosome contains a large number of specialized host immune cells called *bacteriocytes*, and it is within these cells that the bacterial symbionts take up residence. The tube-worm bacteriocytes express high levels of PRRs that recognize MAMPs, such as specific cell surface lipoproteins associated with SOB. This positive interaction locates the bacteria to the trophosome and, with a steady supply of simple nutrients from the hydrothermal vent system delivered by blood circulating in the worm, stimulates colonization and growth of the symbionts in their animal host.

As this example illustrates, in addition to providing a rapid response to pathogen challenge, innate immune mechanisms—specifically, the interaction of PRRs with MAMPs—may also serve the primary role in governing host–symbiont interactions and the establishment of endosymbiotic relationships. Given the critical role innate immune mechanisms play in maintaining the animal–bacterial symbiosis within the tube-worm trophosome, it is likely that similar tightly choreographed molecular mechanisms constitute a host–microbe "dialogue" that helps to maintain balanced communities of beneficial microbiota in virtually all animals, including humans.

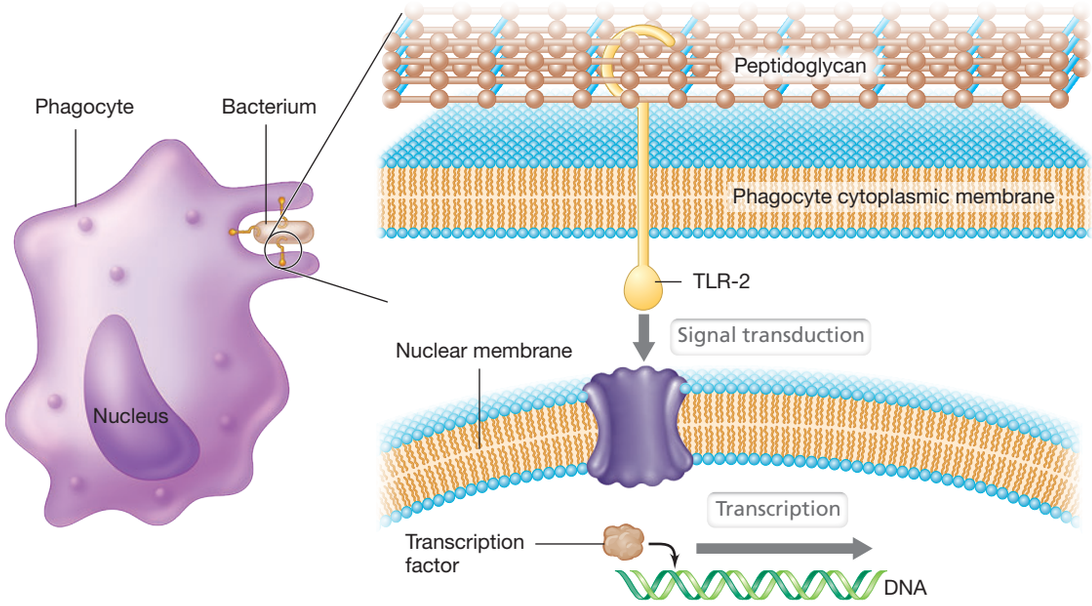


Figure 26.8 A Toll-like receptor. Membrane-spanning TLR-2 interacts with peptidoglycan from gram-positive pathogens. This interaction stimulates signal transduction (see Figure 26.9), activating transcription factors in the nucleus. The result is transcription of genes encoding proteins that induce inflammation and other phagocyte activities. All Toll-like receptors have analogous mechanisms for activating innate immunity.

peptidoglycan components of gram-negative and gram-positive bacterial cell walls, respectively, stimulating production of antimicrobial peptides and inflammatory cytokines (Table 26.3). NOD-like receptor pyrin 3 (NLRP3) interacts with other proteins to form a structure called an **inflammasome**. Activation of the cytoplasmic inflammasome is mediated by cellular stress indicators, such as the loss of potassium ions (K^+) from damaged cells and, especially, the binding of oxidized mitochondrial DNA produced in response to LPS and released into the cytosol; the latter triggers the production of proinflammatory cytokines, initiating inflammation. Later in this chapter we discuss the soluble PRRs in the context of their ability to activate proteins that enhance phagocytosis and destruction of pathogens (Section 26.9).

Signal Transduction in Phagocytes

Interaction of a PAMP with a PRR triggers transmembrane *signal transduction*, initiating gene transcription and translation of host-response proteins in a fashion similar to the two-component regulatory systems previously discussed in *Bacteria* and *Archaea* (◀ Section 7.5). Signal transduction initiated by PAMP–PRR interaction results in enhanced phagocytosis, killing of pathogens, inflammation, and tissue healing.

For example, the binding of LPS (a PAMP derived from the cell wall of degraded gram-negative bacteria) to TLR-4 (a PRR on the surface of phagocytes) typically activates a signal transduction pathway (**Figure 26.9**). TLR-4 then binds proteins in the cytosol of the phagocyte, starting a cascade of reactions that activates transcription factors such as NFκB (nuclear factor kappa B), a protein that binds to specific regulatory sites on DNA, initiating transcription of downstream genes. Many of the NFκB-regulated genes encode host-response proteins, such as the cytokines that activate cells and initiate inflammation (Section 26.5).

TLR-4 is an integral protein having external, transmembrane, and cytoplasmic domains. The external domain of TLR-4 binds LPS complexed with a cell surface protein called CD14 (Figure 26.9). Binding of the CD14–LPS complex by the TLR-4 external domain causes a conformational change in TLR-4 that allows the cytoplasmic domain to interact with an adaptor protein called MyD88, which then binds a protein tyrosine kinase (PTK) called IRAK4. PTKs transfer energy-rich phosphates from ATP to target-protein tyrosines that are exposed when binding alters conformation. Binding of MyD88 by IRAK4 initiates a *kinase cascade* that triggers successive ATP-mediated phosphorylation of TRAF6, IκK (inhibitor of kappa kinase), and IκB (inhibitor of kappa B) proteins. Phosphorylation of IκB causes it to dissociate from, and thereby activate, NFκB. Activated NFκB can then diffuse across the nuclear membrane, bind to NFκB-binding motifs on DNA, and initiate transcription of downstream genes.

As Figure 26.9 shows, signal transduction pathways initiate activation of transcription through ligand–receptor binding on the surface of the phagocytic cell. The ligand–receptor interaction outside the cell induces the binding, recruitment, and concentration of the adaptor proteins and kinase enzymes inside the cell. A single kinase can phosphorylate many signal cascade proteins, thus amplifying the effect of a single ligand–receptor interaction. Signal transduction leading to activation of shared transcription factors and protein synthesis is also the mechanism by which lymphocytes are activated in adaptive immunity, as we discuss in Chapter 27.

Check Your Understanding

- Identify a PAMP shared by a group of microorganisms. Then, identify the cell types that use PRRs to provide innate immunity to pathogens.
- Outline the general features of a signal transduction pathway starting with binding of a PAMP by a membrane-associated PRR.

26.7 Phagocytosis and Phagocyte Inhibition

When phagocytes encounter infectious agents or their harmful products, the activation of signal transduction pathways (Figure 26.9) triggers genetic responses in the phagocyte that direct the containment

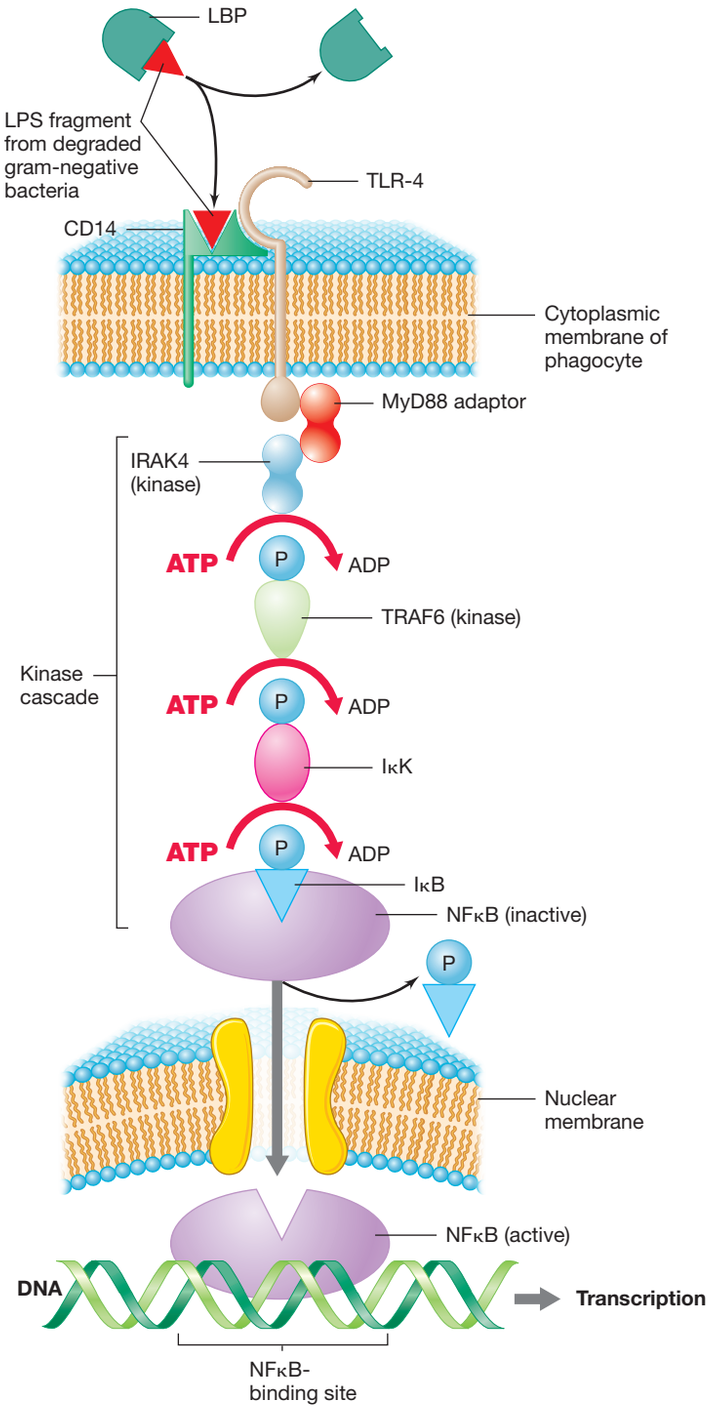


Figure 26.9 Signal transduction in innate immunity. Signal transduction is the transmission of a molecular signal across a cytoplasmic membrane by way of chemical modifications—typically involving a series of proteins in a signaling cascade—that results in a response, such as differential gene expression. In innate immunity, signal transduction is initiated when LPS, a PAMP, is bound by LBP (lipopolysaccharide-binding protein), which then transfers LPS to CD14 on the surface of a phagocyte. The LPS–CD14 complex then binds to the transmembrane TLR-4 receptor. The binding of TLR-4 initiates a series of reactions involving adaptor proteins and kinases, resulting in activation of the transcription factor NFκ B. NFκ B then diffuses across the nuclear membrane, binds to DNA, and initiates transcription of genes encoding proteins essential for innate immunity.

and removal of the threat—**phagocytosis** (Figure 26.10). While these mechanisms effectively protect the body from most infectious exposures, they are not foolproof; many pathogens deploy effective defenses against phagocytes in an attempt to thwart the innate immune response.

Phagocytosis and the Phagolysosome

Most phagocytes contain multiple membrane-bound inclusions called *lysosomes*, cytoplasmic vesicles containing bactericidal substances, such as toxic oxygen compounds, lysozyme, proteases, phosphatases, nucleases, and lipases. Through the molecular mechanisms we have just discussed, phagocytes identify and engage pathogens on surfaces, such as blood vessel walls or fibrin clots, before initiating phagocytosis (Figure 26.10). Activation of the phagocyte through signal transduction causes the phagocyte membrane to envelop and engulf pathogens, eventually pinching off inwardly to form a **phagosome**, a vacuole containing the engulfed pathogen. The phagosome then moves into the cytoplasm and fuses with a lysosome to form a *phagolysosome* (Figure 26.11). The toxic chemicals and enzymes within the phagolysosome kill and digest the engulfed microbial cell.

Genes that control the production of reactive oxygen compounds toxic to pathogens are highly transcribed in activated phagocytes. These reactive oxygen species include hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radicals ($OH\cdot$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), and nitric oxide (NO) (Figure 26.11; ◀ Section 4.16). Phagocytic cells use these toxic oxygen compounds to kill ingested bacterial cells by oxidizing key cellular constituents. The lethal oxidative reactions are contained within the phagolysosome, and this prevents damage to the phagocyte itself.

Inhibiting Phagocytes

Some pathogens have mechanisms for neutralizing toxic phagocyte products, for killing the phagocytes, or for avoiding phagocytosis. For example, several species of *Mycobacterium* produce pigmented compounds called carotenoids that neutralize singlet oxygen and prevent pathogen killing. In addition, *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis, grows and persists within phagocytic cells (▶ Section 31.4). Cells of *M. tuberculosis* use their cell wall glycolipids (◀ Section 16.11) to absorb hydroxyl radicals and superoxide anions, the most lethal toxic oxygen species produced by phagocytes (Figure 26.11).

Some intracellular pathogens produce phagocyte-killing proteins called *leukocidins*. In such cases, the pathogen is ingested as usual, but the leukocidin kills the phagocyte, releasing the pathogen unharmed. Dead phagocytes make up much of the material of *pus*, and *pyogenic* (pus-forming) pathogens such as *Streptococcus pyogenes* (scarlet and rheumatic fevers) and *Staphylococcus aureus* (skin infections) are major leukocidin producers. Localized infections by pyogenic bacteria thus form boils or abscesses.

Another important pathogen defense against phagocytosis is the bacterial capsule (◀ Section 2.6). Because the capsule prevents necessary molecular interactions between the surface of the phagocyte and that of the bacterial cell, encapsulated bacteria are often highly

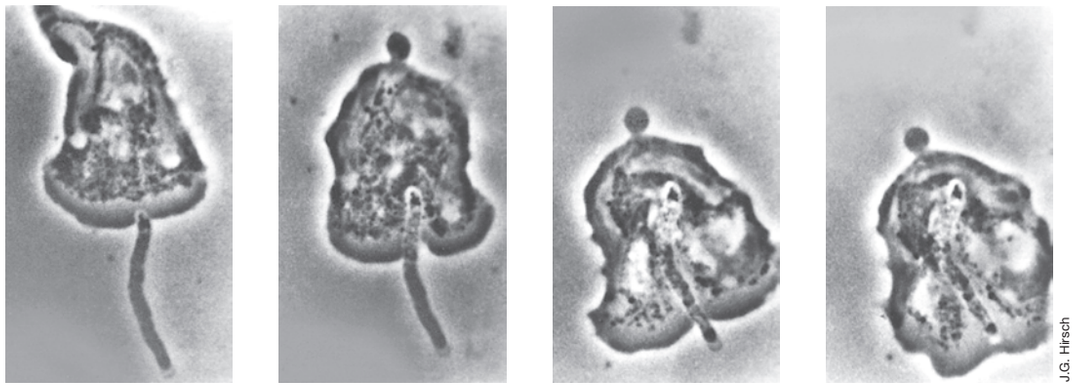


Figure 26.10 Phagocytosis. Time-lapse phase-contrast micrographs of the phagocytosis and digestion of a chain of *Bacillus megaterium* cells by a human phagocyte. The bacterial chain is about 20 μm long.

resistant to phagocytosis. For example, fewer than ten cells of an encapsulated strain of *Streptococcus pneumoniae* can kill a mouse within a few days (◀ Section 25.3 and Figure 25.9), whereas strains lacking a capsule are harmless (◀ Figure 1.38). Surface components other than capsules can also inhibit phagocytosis. For instance, pathogenic *S. pyogenes* produces M protein, a substance that alters the surface of the pathogen and inhibits phagocytosis.

Soluble PRRs and other host molecules such as antibodies (Chapter 27) can interact with capsules and other pathogen surface molecules, thereby reversing the protective effect of bacterial defense mechanisms and enhancing phagocytosis. As an example, the vaccine directed against *Streptococcus pneumoniae*, the agent of bacterial pneumonia, uses capsule polysaccharides to induce protective antibodies (▶ Section 28.3). Thus, the battle between pathogen and the host innate immune system is a dynamic one, where both sides deploy multiple weapons in attempts to thwart the success of the other.

Check Your Understanding

- Identify the mechanism used by phagocytes to induce pathogen killing.
- Describe several reasons why phagocytes are not always effective at removing pathogens from the body.

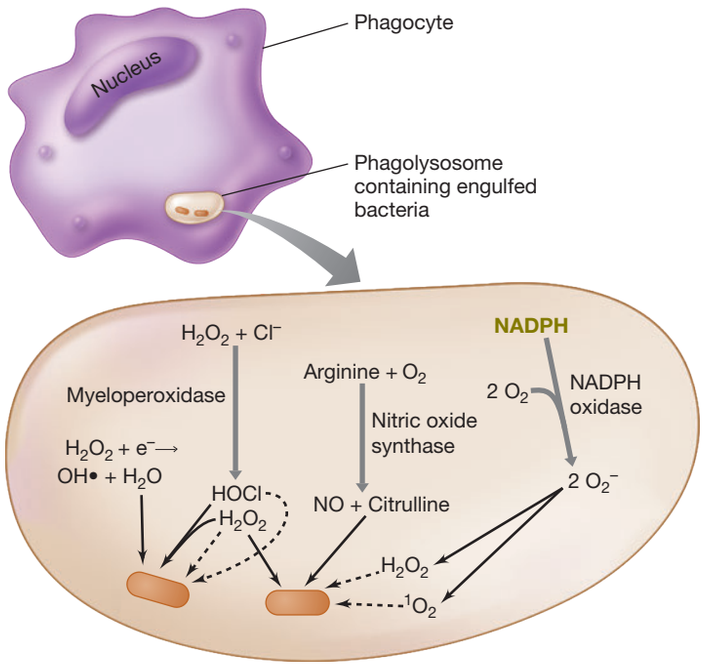


Figure 26.11 Activity of phagocyte enzymes in generating toxic oxygen compounds. These compounds include hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), hypochlorous acid (HOCl), the superoxide anion (O_2^-), singlet oxygen ($^1O_2$), and nitric oxide (NO). Formation of these toxic compounds requires a substantial increase in the uptake and utilization of molecular oxygen, O_2. This increase in oxygen uptake and consumption by activated phagocytes is called the *respiratory burst*.

IV • Other Innate Host Defenses

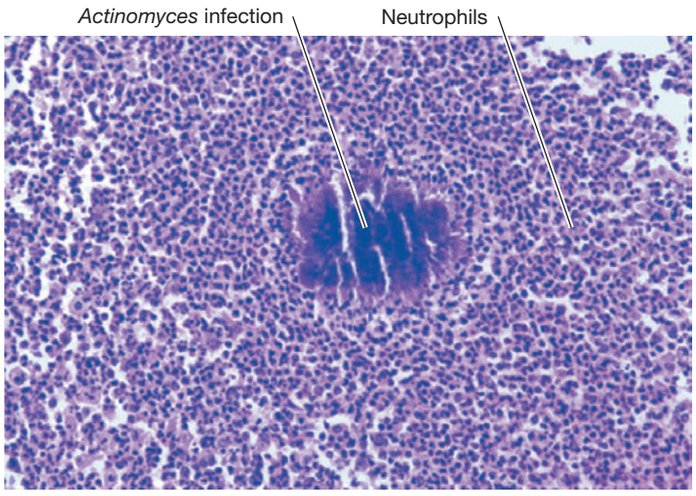
In addition to phagocytes, weapons and defenses of the innate immune system include generalized responses, such as inflammation and fever, antibacterial proteins of the complement system, and antiviral natural killer cells and interferons.

In addition to the physical and chemical barriers to pathogen invasion and the direct destruction of pathogens by activated phagocytes, mammalian immune systems have other inborn mechanisms that help counter infection by pathogens. Although unpleasant for the host, inflammation and fever can be effective host defense strategies for controlling microbial growth in the body and eliminating pathogens. These mechanisms, along with a consideration of the complement system and the activities of cytotoxic *natural killer cells*, round out our discussion of the innate immune response.

26.8 Inflammation and Fever

Inflammation is a complex biological response to noxious stimuli, such as physical injury, toxins, and pathogens. Inflammation is characterized by redness (erythema), swelling (edema), pain, and heat, usually localized at the site of infection or injury (**Figure 26.12*a***). The mediators of inflammation are a group of cell activator and chemoattractant molecules, including cytokines and chemokines. Various cells, including those damaged by injury, produce these

(a)



(b)

Figure 26.12 Inflammation. *(a)* Photograph of a child's foot showing swelling due to infection with vaccinia virus; fluid accumulation results from the inflammatory response. *(b)* Photomicrograph showing infection by *Actinomyces*, a filamentous bacterium. The stained cells surrounding the dark mass of bacteria in the center are neutrophils (Figure 26.5), indicating acute inflammation.

activators. The most important chemokines and cytokines are called *proinflammatory* because of their inflammation-inducing capacity, and they are produced in high concentrations by phagocytes and lymphocytes during pathogen challenge.

Both innate and adaptive immune responses to infection can cause inflammation, and both responses trigger the release of molecules that recruit and activate effector cells, such as neutrophils. Although it is a generalized immune response, inflammation plays a crucial role to isolate and limit tissue damage by initiating the destruction of pathogen invaders and removing damaged cells as tissues are repaired. As we will soon discuss, however, the inflammatory response may also inadvertently result in considerable damage to healthy host tissue.

Inflammatory Cells and Local Inflammation

Immune-mediated inflammation is an acute response that begins at the site of pathogen entry into the body. The PRRs on macrophages and other tissue cells at the site of infection engage the pathogen PAMPs (Figure 26.7). This activates local cells to produce and release mediators including cytokines and chemokines that interact with receptors on other cells, such as neutrophils (Figure 26.5). For example, local tissue macrophages that are activated by PAMP–PRR interaction secrete a chemokine called CXCL8. This molecule activates neutrophils to migrate along the chemokine gradient toward the source of the CXCL8, where they begin to ingest and kill the pathogen. The neutrophils, in turn, secrete even more CXCL8, attracting more neutrophils and amplifying the response, eventually destroying the pathogens (Figure 26.12*b*).

The chemokine and cytokine mediators released by injured cells and phagocytes contribute to inflammation. For example, macrophages and other cells at the site of infection produce proinflammatory cytokines including interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNF-α). These cytokines increase vascular permeability, causing the edema, erythema, and local heating associated with inflammation (**Figure 26.13*a***). Although edema stimulates local neurons, causing pain, the pressure associated with swelling also serves to force fluids away from blood vessels and into the lymphatic system, simultaneously helping to strengthen the immune response and prevent the spread of pathogens to the bloodstream. If pathogens do enter the bloodstream, a condition called *bacteremia*, this could trigger the much more serious and potentially life-threatening *septicemia* (◀ Section 25.2).

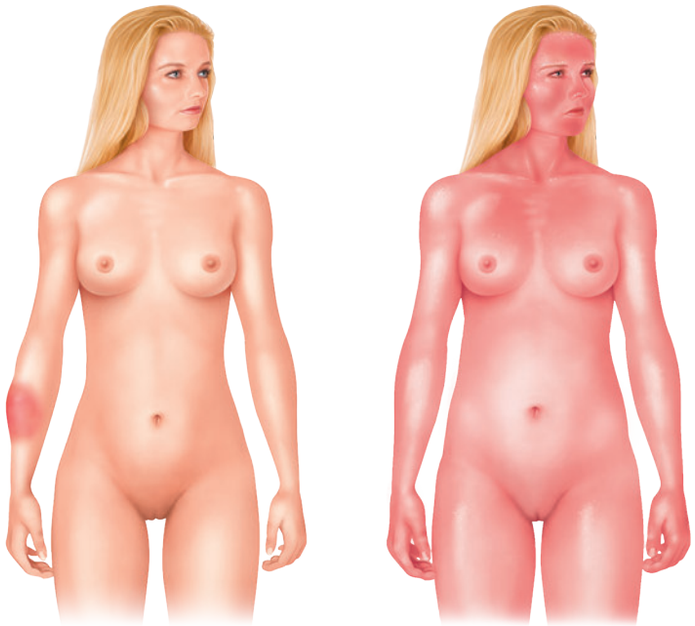
The usual outcome of the inflammatory response is a rapid localization and destruction of the pathogen by macrophages and recruited neutrophils. As the pathogens are destroyed, inflammatory cells are no longer stimulated, and as a result, their numbers at the infection site are diminished. As cytokine production decreases, the attraction of phagocytes to affected tissues lessens, and inflammation subsides.

Fever

Certain cytokines released during an inflammatory response induce **fever**, a condition of elevated body temperature. For example, the proinflammatory cytokines IL-1, IL-6, and TNF-α are *endogenous pyrogens*. These substances stimulate the hypothalamus, the temperature-controlling center of the brain, to produce *prostaglandins*, chemical signals that raise body temperature and cause fever. These same cytokines released in small amounts at local sites of infection induce localized heating, which increases blood flow and promotes healing. The release of endogenous pyrogens is a physiological response to the presence of *exogenous pyrogens*, components of pathogens that induce fever, such as the lipid A endotoxin of gram-negative bacteria LPS (◀ Section 2.4).

Although uncomfortable for the host, fever is an important component of the innate immune response to some infections. The rise from normal human body temperature, about 37°C (98.6°F), to fever temperature, usually 38–40°C (100.4–104°F), is a beneficial response to infection because higher body temperatures inhibit growth of most pathogens. Slower growth limits multiplication of the invading pathogen, thereby minimizing tissue damage and easing the workload on immune cells, especially phagocytes. Elevated body temperatures also increase the production of *transferrins*, molecules that bind and sequester iron in blood and lymph, thus depriving pathogens of this important nutrient (◀ Sections 3.1 and 25.4).

While low to moderate fever is an important component of the innate immune response, a continuous or uncontrolled rise in body temperature (>40°C) is a rare but life-threatening condition that



(a) Local infection leads to inflammation in a small part of the body, followed by healing.

(b) Systemic infection leads to inflammation and disease throughout the body.

Figure 26.13 Local and systemic inflammation. *(a)* Local infection, mediated by proinflammatory cytokines from local macrophages, results in inflammation that subsides as the infection is cleared. *(b)* Systemic infection causes systemic release of proinflammatory cytokines, resulting in widespread systemic inflammatory symptoms including severe edema, fever, and septic shock, even if the infection is controlled.

may accompany certain disease conditions and requires immediate medical intervention. This usually includes the administration of antipyretic (fever-reducing) medications, such as acetaminophen or ibuprofen, which counteract the effect of endogenous pyrogens on the hypothalamus.

Systemic Inflammation and Septic Shock

In some cases, the inflammatory response fails to localize the pathogens, and the reaction spreads throughout the body. Uncontrolled *systemic inflammation* can be more dangerous than the original infection, with inflammatory cells and mediators contributing to body-wide inflammation. An inflammatory response that spreads inflammatory cells and mediators through the entire circulatory and lymphatic systems can lead to *septic shock*, a life-threatening condition.

Although there are potentially many causes of pathogen-induced septic shock, one example is systemic infection by enteric bacteria, such as *Salmonella* species or *Escherichia coli*, which can be introduced into the peritoneal cavity or the bloodstream by a ruptured or leaking bowel. The primary infection is often cleared by the activity of phagocytes and antibiotic treatment. However, the endotoxic outer membrane LPS from these gram-negative bacteria interacts with a PRR on phagocytes, stimulating production of proinflammatory cytokines that are released into the circulation. These cytokines induce systemic responses that parallel the localized inflammatory response but on a much larger scale that affects many organ systems, ultimately leading to a body-wide inflammatory event (Figure 26.13*b*). The result is a massive efflux of fluids from the central vascular tissue, causing a loss of systemic blood pressure and the influx of fluids from vascular tissues into extravascular spaces. Septic shock causes death in up to 30% of affected individuals.

Check Your Understanding

- Identify the molecular mediators of inflammation and fever and define their individual roles.
- Identify the major symptoms of localized inflammation and of septic shock.

26.9 The Complement System

The **complement system**, or simply *complement*, is a group of sequentially interacting plasma proteins, many with enzymatic activity, that functions to boost the efficiency of both innate and adaptive immune responses for the destruction of pathogens. Found throughout the body, complement proteins are produced in the liver and can be activated by the presence of a pathogen. Upon pathogen recognition, complement proteins interact with one another to trigger a cascade of events in a process called *complement activation*, the major outcomes of which are enhanced phagocytosis, inflammation, and lysis of invading cells.

The individual proteins of complement are designated as C (for complement) followed by a number or letter for the subunits, as C1, C2, C3, and so on. Complement proteins exist in an inactive conformation until they are enzymatically split to assume their active forms. The cleaving of C3 to its products, C3a and C3b, is the key event in complement activation. Three different mechanisms (pathways) are known that lead to complement activation, and we consider each of these now.

Classical Complement Activation

The *classical pathway* of complement activation is initiated when complement proteins interact with antibodies bound to pathogen surfaces. The antibodies are said to *fix* (bind) the ever-present complement proteins, and thus, classical complement activation depends upon the adaptive immune response. The complement proteins react in a defined sequence, or *cascade*, with activation of one complement protein leading to activation of the next, and so on.

The key steps of classical activation of complement, shown in **Figure 26.14*a***, start with binding of antibody to antigen (initiation), followed by binding of the C1 complex (C1q, C1r, and C1s) to the antibody–antigen complex. This complex recruits and cleaves C2 into its fragments, C2a and C2b, and C4 into its fragments, C4a and C4b. C2a and C4b interact and are deposited on the pathogen surface. The resulting C2a-C4b complex functions as a *C3 convertase*, an enzyme that cleaves C3 to yield C3a and C3b. C3b then binds to the convertase, forming a *C5 convertase* that cleaves C5 into C5a and C5b. The liberated C5b then binds C6 and C7, forming a complex that inserts into the membrane of the pathogen. The C5b–7 complex recruits C8 and several copies of C9, forming a large C5b–9 unit called the *membrane attack complex* (*MAC*). The MAC forms a pore through the cytoplasmic membrane of the pathogenic cell, allowing extracellular fluids to rush in and lyse the cell (Figure 26.14*a*; see

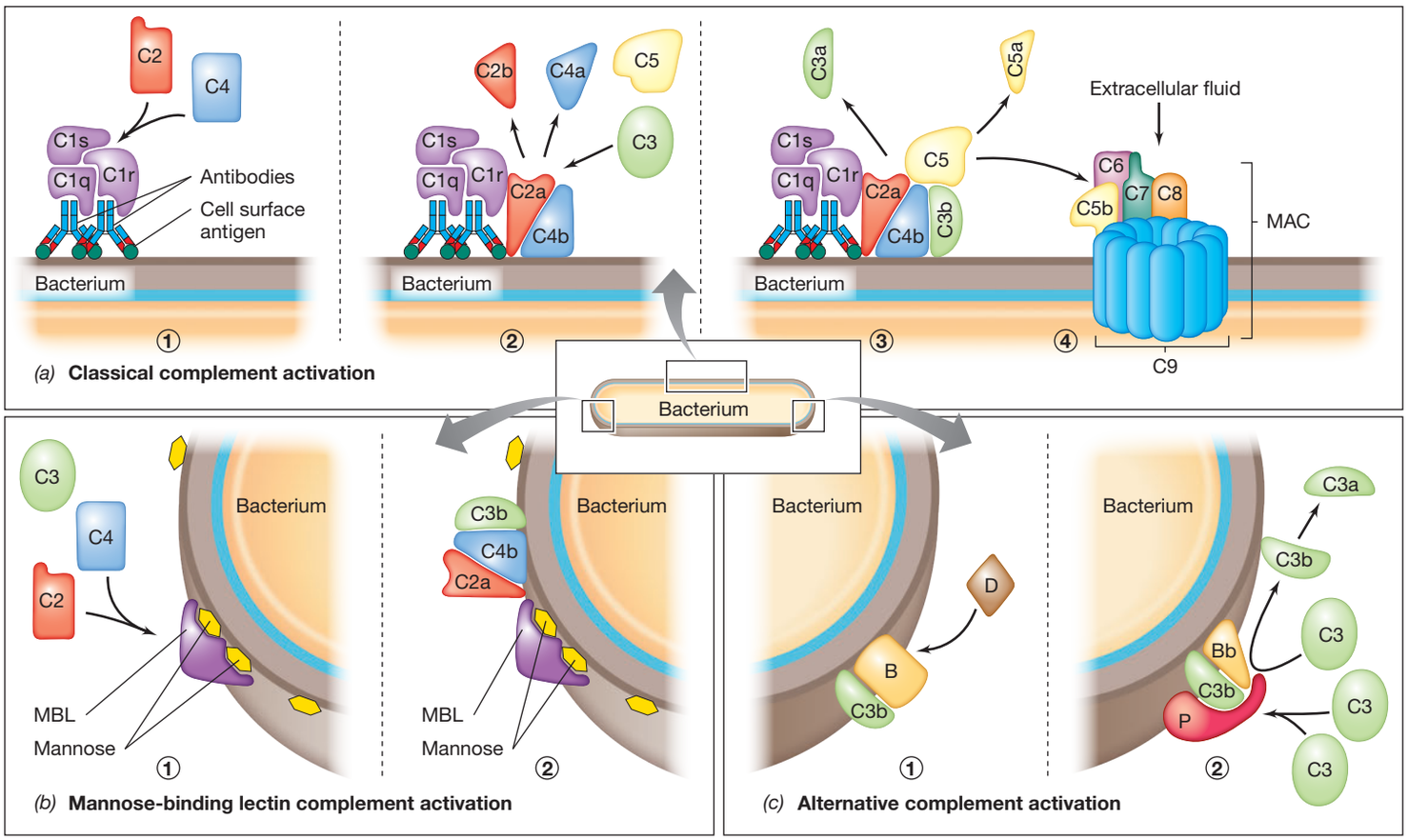


Figure 26.14 Complement proteins and complement activation in the immune response. *(a)* The sequence, orientation, and activity of the components of the classical complement pathway as they interact to lyse a cell. ① Binding of the antibody and the C1 protein complex (C1q, C1r, and C1s). ② The C2a-C4b complex is a C3 convertase that splits C3. ③ The C2a-C4b-C3b complex cleaves C5, and C5b then binds an adjacent membrane site. ④ Sequential binding of C6, C7, C8, and C9 to C5b produces a pore, the membrane attack complex (MAC), in the membrane. *(b)* The mannose-binding lectin (MBL) pathway. ① MBL binds to mannose on the bacterial surface and recruits C2 and C4. ② MBL anchors formation of C2a-C4b-C3b. This complex activates C5, as in step 3 of part *a*, and initiates formation of the MAC (step 4 in *a*). *(c)* The alternative pathway. ① C3b bound to the cell binds protein B, which is cleaved by protein D. ② The resulting C3b-Bb complex is stabilized on the membrane by factor P (properdin) and then acts on C3 in the blood, causing more C3b to bind to the membrane. Bound C3b-Bb-P then activates C5, as in step 3 of the classical activation pathway above, and initiates formation of the MAC (step 4 in *a*).

also Figure 26.15*b*). Dozens to hundreds of MACs may perforate a single bacterial cell at the point of lysis.

When activated by specific antibody, MAC formation lyses many gram-negative bacteria. However, gram-positive bacteria, such as *Streptococcus* species, are not usually lysed by complement because their thick cell walls make the cytoplasmic membrane less accessible to MAC proteins. Gram-positive bacteria can, however, be destroyed through *opsonization*.

Opsonization is the coating of pathogens with host immune proteins, such as antibodies or C3b, resulting in enhanced phagocytosis of target cells (**Figure 26.15*a***). Opsonization neutralizes pathogens and makes them much more likely to be identified, engulfed, and destroyed by phagocytes. This is because most phagocytes, including neutrophils and macrophages, have antibody receptors (FcR) and C3b receptors (C3R) on their surfaces, which bind antibody and C3b complement protein, respectively. Normal phagocytic processes are enhanced about 10-fold by antibody–FcR interactions and amplified another 10-fold by C3b–C3R interactions.

By-products of complement activation include chemoattractants called *anaphylatoxins*; these molecules cause inflammatory reactions at the site of complement deposition. For example, when C3 is cleaved to C3a and C3b, C3b opsonizes the target cell as described above; meanwhile, release of soluble C3a attracts and activates phagocytes, increasing phagocytosis. In addition, both C3a and the C5a cleavage product are able to bind receptors on the surface of mast cells, causing them to degranulate and release large amounts of proinflammatory histamine (Figure 26.15*c*).

Mannose-Binding Lectin and Alternative Pathways

The *mannose-binding lectin pathway* and *alternative pathway* of complement activation (Figure 26.14*b* and *c*) depend on recognition of shared pathogen components by complement proteins and are an important part of the innate immune response. The mannose-binding lectin (MBL) pathway depends on the activity of a serum MBL protein. MBL is a soluble PRR (Section 26.6) that binds to mannose-containing polysaccharides found only on bacterial cell surfaces (Figure 26.14*b*). The MBL–polysaccharide complex is similar to the C1 complex of the classical pathway (Figure 26.14*a*) in that it fixes C2a and C4b, again producing C3 convertase and binding

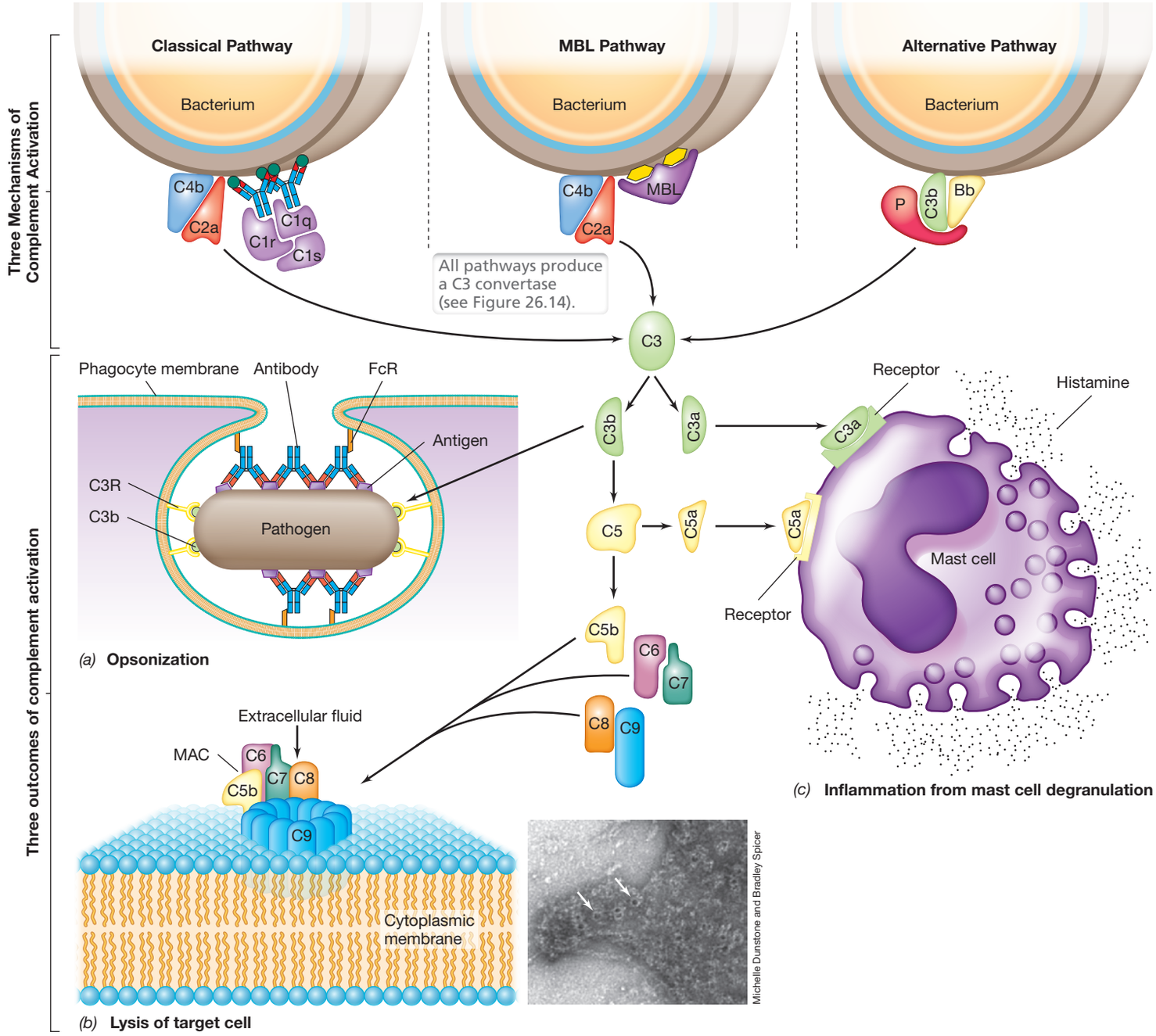


Figure 26.15 **Complement proteins and the outcomes of complement activation.** Each complement activation pathway leads to the production of a C3 convertase that cleaves C3 to its active products, C3a and C3b. These initiate three possible outcomes: opsonization, lysis by membrane attack complex (MAC) formation, and inflammation. *(a)* Pathogens coated with C3b (green) or with specific antibodies become opsonized, allowing for enhanced recognition by phagocytes through C3R and FcR proteins. *(b)* Transmembrane pores (white arrows) formed by MAC components C5b through C9 cause lysis of target cells. The transmission electron micrograph is a negative stain image showing human MACs attacking a foreign (rabbit) erythrocyte. *(c)* During complement-activated inflammation, liberated C3a and C5a bind receptor proteins on mast cells, causing the mast cells to degranulate and release proinflammatory histamine.

C3b to C2a-C4b. As before, this complex catalyzes formation of the C5–9 MAC and leads to lysis or opsonization of the bacterial cell.

The *alternative pathway* is a nonspecific complement activation mechanism that uses many of the classical complement pathway components, as well as several unique serum proteins. Together they induce pathogen opsonization and phagocytosis and activate the C5–9 MAC. C3b is produced by spontaneous hydrolysis of C3 into C3a and C3b, which occurs at low levels in the blood and tissues at all times. If pathogens such as bacteria are present, C3b binds to the bacterial cell surface (opsonization). C3b can then bind the alternative pathway serum protein factor B, which is cleaved by factor D to yield soluble Ba and Bb (Figure 26.14*c*). The C3b-Bb complex may then be bound by factor P (properdin) to form C3b-Bb-P, another C3 convertase. C3b-Bb-P then attracts and cleaves additional C3, depositing more C3b on the membrane and amplifying the remaining steps of the complement cascade (Figure 26.15).

Both the alternative pathway and the MBL pathway nonspecifically target bacterial invaders and lead to activation of opsonization (Figure 26.15*a*) and MACs (Figure 26.15*b*) via formation of stable C3 convertases. MBL, factors B, D, and P, and classical complement proteins are part of the innate immune response, but unlike the classical pathway, neither the alternative pathway nor the MBL pathway requires prior antigen exposure or the presence of antibodies for activation.

We now move on to consider the immune system's response to viruses rather than bacterial pathogens. Many pathogens are viruses, and because of their small size and unique properties, special immune defense mechanisms are required to deal with them.

Check Your Understanding

- In what ways does the classical pathway of complement activation differ from the mannose-binding lectin and alternative pathways?
- What is opsonization, and how does opsonization help fight bacterial infection?
- Why are the mannose-binding lectin and alternative pathways considered part of the innate immune system?

26.10 Innate Defenses Against Viruses

In addition to opsonization and the activity of phagocytes, the immune system has other innate defenses that are especially important for controlling and eliminating viral infections. These include *natural killer cells* (Figure 26.5) and *interferons*. Natural killer cells are lymphocyte-like cells that recognize and kill compromised (unhealthy) cells, such as those infected with intracellular pathogens, in particular viruses. Whereas natural killer cells help rid the body of already infected cells, interferons, small proteins of the cytokine family, help healthy cells ward off viral infection. We consider both of these innate defenses here.

Natural Killer Cells

Natural killer cells (NK cells) are cytotoxic innate-immunity lymphoid cells that lack BCRs and TCRs and, thus, are distinct from T cells and B cells (Figure 26.5). The role of NK cells is to seek out and destroy compromised cells, such as cells infected with intracellular pathogens (such as viruses) or cancer cells (**Figure 26.16**). When an NK cell engages an infected or otherwise diseased cell, granules in the cytoplasm of the NK cell migrate to the contact site and release their contents against the target cell. These granules contain *perforin* and proteases called *granzymes*. Perforin binds the membrane of the target cell and forms a pore through which granzymes enter the target cell (Figure 26.16*b*). Granzymes are cytotoxins that cause *apoptosis*, or programmed cell death, characterized by noninflammatory DNA degradation and membrane blebbing. During the killing process, NK cells remain unaffected and their membranes are not damaged by perforin. Also during this process, NK cell numbers do not increase nor do NK cells exhibit immune memory after interaction with target cells. With this in mind, how do NK cells recognize compromised host cells that should be destroyed? The key to this mechanism is the heterodimeric membrane protein found on the surface of most cells: the major histocompatibility complex (MHC) protein.

Mastering Microbiology
Art Activity: Figure 26.15 Natural killer cells

There are two classes of MHC proteins—*class I* and *class II*—and their primary function is to present antigen to various immune cells to trigger an immune response (Chapter 27). MHC II proteins are expressed on antigen-presenting cells (APCs) only, which include B cells, macrophages, and dendritic cells (Figure 26.5). By contrast, MHC I proteins are found on the surfaces of all nucleated cells. In uninfected cells, which contain no pathogens or foreign antigens, MHC I proteins bind and present *self peptides*, protein fragments derived from the normal degradation of self proteins, to the external environment (the term "self" here means "native to the cell"). In cells that have been infected by viruses or other intracellular

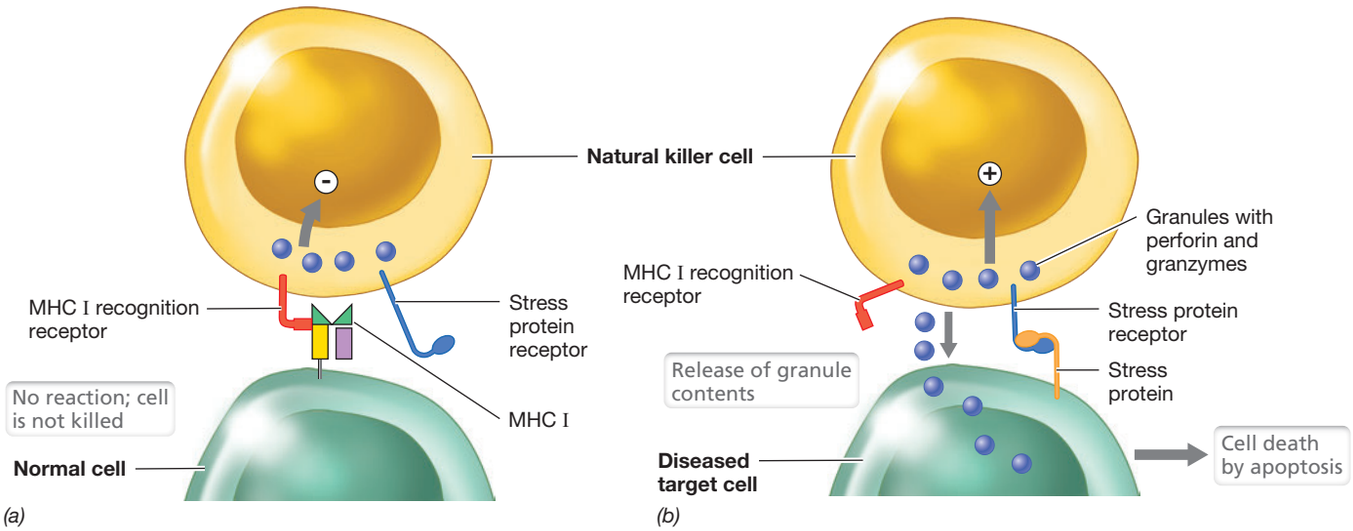


Figure 26.16 Natural killer cells. Natural killer (NK) cells have two receptors: One interacts with MHC I on healthy cells; the second receptor interacts with cell stress proteins, found only on tumor cells or pathogen-infected cells. *(a)* Recognition of MHC I on healthy cells prevents the NK cell from releasing its toxic contents. *(b)* Pathogen-infected cells or tumor cells express stress proteins and often reduce MHC I expression. Especially in the absence of MHC I recognition, the NK cell interacts with the stress protein and releases perforin and granzymes, inducing apoptosis and killing the diseased cell.

pathogens, MHC I proteins display peptides derived from the infectious agent. This serves as a signal to a special type of T lymphocyte called a *T-cytotoxic cell* to destroy the infected cell.

Because MHC I proteins facilitate the destruction of cells infected with an intracellular pathogen, such as a virus, many viral genomes encode proteins that suppress expression of MHC I in host cells. Without MHC I proteins on their surfaces—a condition that is also common in cancer cells—T-cytotoxic cells cannot identify and eliminate diseased host cells. This, then, is where NK cells play a major role; NK cells recognize and destroy compromised host cells that have reduced expression of MHC I on their surfaces.

NK cells recognize and destroy pathogen-infected or tumor cells by using a two-receptor system. The molecular targets of NK cells are MHC I proteins on the surface of other cells (Figure 26.16*a*). As NK cells circulate and interact with other cells in the body, they use special receptors on their surfaces to recognize MHC I proteins on normal, healthy cells. Binding of the MHC I recognition receptors on NK cells to MHC I on other cells deactivates the NK cell, turning off the perforin and granzyme killing mechanisms. In addition to a deficiency of MHC I proteins, pathogen-infected or tumor cells frequently express *stress proteins* on their surfaces; NK cells have complementary receptors for many of these stress proteins. Especially in the absence of the MHC I interactions, the stress receptors on NK cells engage stress proteins on target cells. This interaction triggers the release of perforin and granzymes from the NK cell (Figure 26.16*b*). In this way, pathogen-infected or tumor cells that exhibit stress proteins and no longer express the MHC I proteins of healthy cells are removed from the body.

Mastering Microbiology
Art Activity: Figure 26.16 Antiviral activity of interferons

In addition to these functions, NK cells are the primary effector cells of **antibody-dependent cell-mediated cytotoxicity (ADCC)**, a process by which cells coated (opsonized) with a class of antibody called *immunoglobulin G* (*IgG*; ▶ Section 27.3) are lysed by cytotoxic immune cells. Using special receptors on their surfaces, NK cells engage IgG antibodies that have previously bound a target cell. This interaction activates the NK cell, causing it to release its cytotoxic contents and kill the opsonized target cell.

Interferons

Interferons, in particular IFN-α and IFN-β, are small proteins in the cytokine family that prevent viral replication by stimulating the production of antiviral proteins in uninfected cells (**Figure 26.17**). Host cells produce and secrete interferons in response to certain viral infections or exposure to inactivated viruses or viral nucleic acids. Interferons are produced in large amounts by cells infected with viruses of low virulence, but highly virulent viruses inhibit host protein synthesis before interferon can be produced, significantly reducing interferon production. The presence of double-stranded RNA (dsRNA) also induces interferon synthesis. In nature, dsRNA exists only in certain RNA viruses, such as rhinoviruses (one of many common cold viruses, ▶ Section 31.7); the viral dsRNA stimulates the animal cell to synthesize and release interferon.

Antiviral interferon activities are *host*-specific rather than *virus*-specific. That is, interferon produced by a species activates receptors only on cells from the same species. As a result, interferon produced by cells of an animal in response to, for example, a rhinovirus, could also inhibit multiplication of, for example, influenza viruses in cells of the same species. However, the interferon would have no effect on the multiplication of viruses, including the original rhinovirus, in other species. We examine the potential use of antiviral interferons as chemotherapeutic or prophylactic treatments in Chapter 28.

A third type of interferon, IFN-γ, stimulates phagocytes and is produced by NK cells in response to cytokine signaling. For example, macrophages that have ingested microorganisms produce the cytokine interleukin-12 (IL-12). NK cells receive the IL-12 signal and become activated, causing them to secrete IFN-γ. The resulting exposure to IFN-γ activates the macrophages and causes them to more efficiently kill ingested microbes. As we will see in the next chapter, similar cooperation occurs between antigen-presenting phagocytes and a subset of T lymphocyte called a *T-helper cell* (▶ Section 27.8), and this interaction serves as a key link between innate and adaptive immunity.

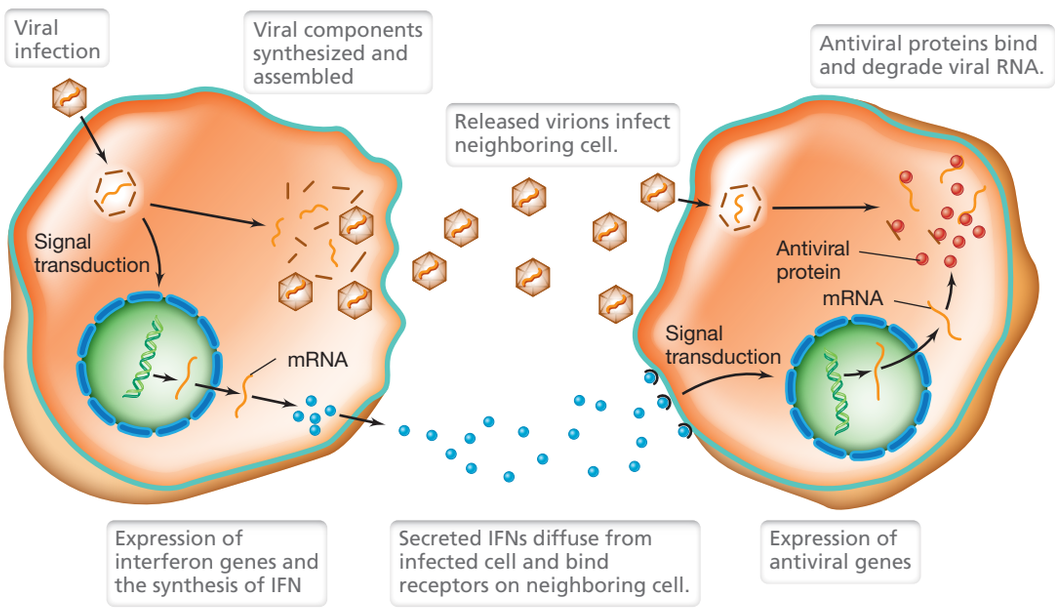


Figure 26.17 Antiviral activity of interferons. Host cells secrete interferons, a type of cytokine, in response to viral infection. The interferons bind to uninfected cells, triggering a signal transduction pathway that leads to the synthesis of proteins that bind viral nucleic acids and interfere with viral replication.

Check Your Understanding

- Identify and compare the targets and the recognition mechanisms used by T-cytotoxic cells and NK cells.
- Under what conditions are interferons produced, and how do they limit the transmission of viruses from one host cell to another?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Fundamentals of Host Defense

26.1 Innate immunity is an inborn protective response to infection characterized in part by recognition and elimination of common pathogens, primarily through the activity of phagocytes. Adaptive immunity is the acquired ability of the immune system to eliminate specific pathogens from the body via lymphocyte-mediated responses, including the production of antibodies that bind foreign antigens on pathogens or their products.

Q List two different types of phagocytes. How do T cells and B cells differ in their functions? From where in the human body do all of these cells originate, and which of these require maturation before they are functional?

26.2 The human body possesses numerous protective defenses against infectious agents. Natural host resistance to infection includes physical barriers posed by the skin and mucosa, as well as chemical barriers including acidic secretions, defensins, and lysozyme. The specificity of pathogens for particular tissues limits which hosts and tissues might be susceptible to infection.

Q How does the human normal microbiota play a role in preventing disease?

II • Cells and Organs of the Immune System

26.3 Cells involved in innate and adaptive immunity originate from hematopoietic stem cells in bone marrow. The blood and lymph systems circulate cells and proteins that are important components of the immune response. Diverse leukocytes participate in immune responses in all parts of the body.

Q Describe the significance of bone marrow, blood, and lymph to cells and proteins associated with the immune system.

26.4 Leukocytes are differentiated white blood cells derived from either myeloid or lymphoid precursor cells. Cells of myeloid lineage include monocytes and granulocytes. Monocytes include macrophages and dendritic cells, specialized phagocytes that function as antigen-presenting cells (APCs) to initiate an adaptive immune response. Granulocytes include neutrophils and eosinophils, which are phagocytes but not APCs, and basophils and mast cells, which are important inducers of the inflammatory response but may also cause allergic reactions. Lymphocytes include B and T cells, which facilitate adaptive immunity. Natural killer (NK) cells, which play a key role in destroying virus-infected and cancerous host cells, are also derived from a lymphoid precursor.

Q What is the origin of the phagocytes and lymphocytes active in the immune response?

III • Phagocyte Response Mechanisms

26.5 Pathogens may colonize host tissues when appropriate nutrients and growth conditions are present, such as on mucosal surfaces, especially where the composition of the normal microbiota has been altered. Innate responses to microbial invasion and tissue damage are initiated by the release of chemokines, which recruit phagocytes and other immune cells to sites of infection.

Q Describe a scenario in which microorganisms invade body tissues. What factors allow for the migration of phagocytes to sites of infection?

26.6 Common pathogens carry pathogen-associated molecular patterns (PAMPs) on their surfaces and intracellularly. Phagocytes recognize PAMPs through preformed (innate) pattern recognition receptors (PRRs). The recognition and interaction process stimulates phagocytes to destroy the pathogens through a signal transduction mechanism that induces phagocytosis of the infectious agent.

Q Identify some PAMPs that are recognized by PRRs. Which cells express PRRs? How do PRRs associate with PAMPs to promote innate immunity?

26.7 Phagocytosis is the engulfing of infectious particles by phagocytes. Engulfed pathogens are bathed in toxic oxygen compounds and enzymes inside the phagolysosome, killing and degrading them. However, some pathogens have developed various defense mechanisms to avoid or inhibit phagocytes, including secretion of leukocidins, the presence of a capsule, and biosynthesis of carotenoid pigments, which combat oxidative stress.

Q Explain how phagocytes kill microorganisms, with particular attention to oxygen-dependent mechanisms. Then identify at least three properties of pathogens that inhibit the effectiveness of phagocytes.

IV • Other Innate Host Defenses

26.8 Fever and inflammation, characterized by pain, swelling (edema), redness (erythema), and heat, are normal and generally beneficial outcomes that result from activation of nonspecific immune response effectors. However, uncontrolled systemic inflammation—called septic shock—or excessively high fever can lead to serious illness or death.

Q Identify the cells that initiate inflammation and the cells that are activated by inflammatory signals.

26.9 The complement system is composed of soluble proteins that catalyze bacterial opsonization and cell lysis. Complement is triggered by antibody interactions or by interactions with nonspecific activators, such as mannose-binding lectin. Complement activation may occur through either innate or antibody-dependent mechanisms. Complement may enhance phagocytosis, cause target cell lysis, or induce an inflammatory response.

Q Describe the complement system. Is the order of protein interactions important? Why or why not? Identify the components of the mannose-binding lectin pathway for complement activation. Identify the components of the alternative pathway for complement activation. How do these complement activation pathways differ from the classical pathway?

26.10 Through an antigen-independent mechanism, natural killer cells respond to both the presence of stress proteins and the absence of major histocompatibility complex (MHC) I on the surface of virus-infected cells or tumor cells, using perforin and granzymes to kill the compromised target cells. Interferons are cytokines produced by virus-infected cells that limit the spread of infection by stimulating the expression of antiviral proteins in uninfected cells. Other interferons activate phagocytes to enhance phagocytosis.

Q What is the activation signal for NK cells? How does this differ from the activity of T-cytotoxic lymphocytes?

APPLICATION QUESTIONS

1. Describe the relative importance of innate immunity compared to adaptive immunity. Is one more important than the other? Could humans survive in a normal environment without an immune system?
2. Describe the potential problems that would arise if a person had an acquired inability to phagocytose pathogens. Could the person survive in a normal environment such as a college campus? What defects in the phagocyte might cause lack of phagocytosis? Explain.
3. Inflammation is the hallmark of an active immune response. Explain how inflammation is triggered by innate immune mechanisms. Why does inflammation subside as an infection is controlled?
4. Do you agree with the following statement? Complement is a critical component of antibody-mediated defense. Explain your answer. What might happen to persons who lack complement component C3? C5? Factor B (alternative pathway)? Mannose-binding lectin (MBL)?

CHAPTER GLOSSARY

Adaptive immunity the acquired ability to recognize and destroy a particular pathogen or its products, dependent on previous exposure to the pathogen or its products

Antibody a soluble protein produced by B cells and plasma cells that interacts with antigen; also called immunoglobulin

Antibody-dependent cell-mediated cytotoxicity (ADCC) the process by which NK cells bind IgG-coated target cells and kill them by secreting cytolytic toxins

Antigen a molecule capable of interacting with specific components of the immune system and that often functions as an immunogen to elicit an adaptive immune response

Antigen-presenting cell (APC) a macrophage, dendritic cell, or B cell that takes up and processes antigen and presents it to T-helper cells

Basophil a circulating granulocyte that contains granules of inflammatory mediators that, in some cases, contribute to allergic reactions

B cell a lymphocyte that has immunoglobulin surface receptors, produces immunoglobulin, and may present antigens to T cells

B cell receptor (BCR) a cell surface immunoglobulin that acts as an antigen receptor on a B cell

Chemokine a soluble protein that recruits immune cells to an injury site; a type of cytokine

Complement system a series of proteins that react sequentially with antibody–antigen complexes, mannose-binding lectin, or alternative activation pathway proteins to amplify or potentiate target cell destruction

Cytokine a soluble protein produced by a leukocyte or damaged host cell; modulates an immune response

Dendritic cell a phagocytic antigen-presenting cell found in various body tissues; transports antigen to secondary lymphoid organs

Epitope the portion of an antigen that reacts with a specific antibody or T cell receptor

Eosinophil a phagocytic granulocyte especially active against extracellular parasites, such as helminths

Fever an increase in body temperature resulting from infection or the presence of toxins in the body

Granulocyte a leukocyte derived from a myeloid precursor that contains cytoplasmic granules consisting of toxins or enzymes that are released to destroy target cells

Hematopoiesis the process by which precursor stem cells in the bone marrow or gut differentiate into any type of blood cell

Hematopoietic stem cell a progenitor cell found primarily in the bone marrow but also in the gut that can differentiate into any of a variety of blood cells

Immune memory (memory) the ability to rapidly produce large quantities of specific immune cells or antibodies after subsequent exposure to a previously encountered antigen

Immunity the ability of an organism to resist infection

Immunoglobulin a soluble protein produced by B cells and plasma cells that interacts with antigen; also called antibody

Inflammasome a cytosolic signaling complex that contains NOD-like receptor pyrin 3 (NLRP3) and mediates the activation of potent proinflammatory cytokines

Inflammation a nonspecific reaction to noxious stimuli such as toxins and pathogens, characterized by redness (erythema), swelling (edema), pain, and heat, usually localized at the site of infection

Innate immunity the inherent ability to recognize and destroy an individual pathogen or its products that does not rely on previous exposure to a pathogen or its products

Interferons cytokine proteins produced by virus-infected cells that induce signal transduction in nearby cells, resulting in transcription of antiviral genes and expression of antiviral proteins

Invasion the ability of a pathogen to enter into host cells or tissues, spread, and cause disease

Leukocyte a nucleated cell in blood; also called a white blood cell

Lymph nodes organs that contain lymphocytes and phagocytes arranged to encounter microorganisms and antigens as they travel through the lymphatic circulation

Lymphocytes a subset of nucleated cells in blood involved in the adaptive immune response; B cells and T cells

Macrophage a large leukocyte found in tissues that has phagocytic and antigen-presenting capabilities

Major histocompatibility complex (MHC) a genetic region that encodes several proteins important for antigen processing and presentation; MHC I proteins are expressed on all nucleated cells, whereas MHC II proteins are expressed only on antigen-presenting cells

Mast cell tissue granulocyte similar in function to basophils that contains intracytoplasmic granules with inflammatory mediators

Monocyte Circulating phagocyte that contains many lysosomes and can differentiate into a macrophage or dendritic cell

Mucosa-associated lymphoid tissue (MALT) a part of the lymphatic system that interacts with antigens and microorganisms that enter the body through mucous membranes, including those of the gut, the genitourinary tract, and the respiratory tract

Natural killer (NK) cell a specialized lymphocyte that recognizes and destroys infected host cells or cancer cells in a nonspecific manner

Neutrophil a leukocyte exhibiting phagocytic properties, a granular cytoplasm (granulocyte), and a multilobed nucleus; also called polymorphonuclear leukocyte or PMN

Opsonization the deposition of antibody or complement protein on the surface of a pathogen or other antigen that results in enhanced phagocytosis

Pathogen-associated molecular pattern (PAMP) a repeating structural component of a microorganism or virus recognized by a pattern recognition receptor (PRR)

Pattern recognition receptor (PRR) a protein that is soluble or bound to a phagocyte membrane and that recognizes a pathogen-associated molecular pattern (PAMP)

Phagocyte a cell that engulfs foreign particles, and can ingest, kill, and digest most pathogens

Phagocytosis a mechanism for ingesting particulate material in which a portion of the cytoplasmic membrane surrounds the particle and brings it into the cell; for phagocytes of the immune system, the process of engulfing and killing foreign particles and cells

Phagosome an intracytoplasmic vacuole containing engulfed materials, especially pathogens or foreign particles

Plasma the liquid portion of the blood containing proteins and other solutes

Plasma cell a differentiated B cell that produces soluble antibodies

Primary lymphoid organ an organ in which antigen-reactive lymphocytes develop and become functional; the bone marrow is the primary lymphoid organ for B cells; the thymus is the primary lymphoid organ for T cells

Secondary lymphoid organ an organ at which antigens interact with antigen-presenting phagocytes and lymphocytes to generate an adaptive immune response; these include lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT)

Serum the liquid (noncellular) portion of the blood with clotting proteins removed

Specificity the ability of cells of the adaptive immune response to interact with particular antigens

T cell a lymphocyte that interacts with antigens through a T cell receptor for antigen; T cells are divided into functional subsets including Tc (T-cytotoxic) cells and Th (T-helper) cells. Th cells are further subdivided into Th1 (inflammatory) cells and Th2 cells, which aid B cells in antibody formation

T cell receptor (TCR) an antigen-specific receptor protein on the surface of T cells

Toll-like receptor (TLR) one of a family of pattern recognition receptors (PRRs) found on phagocytes, structurally and functionally related to Toll receptors in *Drosophila*, that recognize a pathogen-associated molecular pattern (PAMP)

27 Adaptive Immunity: Highly Specific Host Defenses

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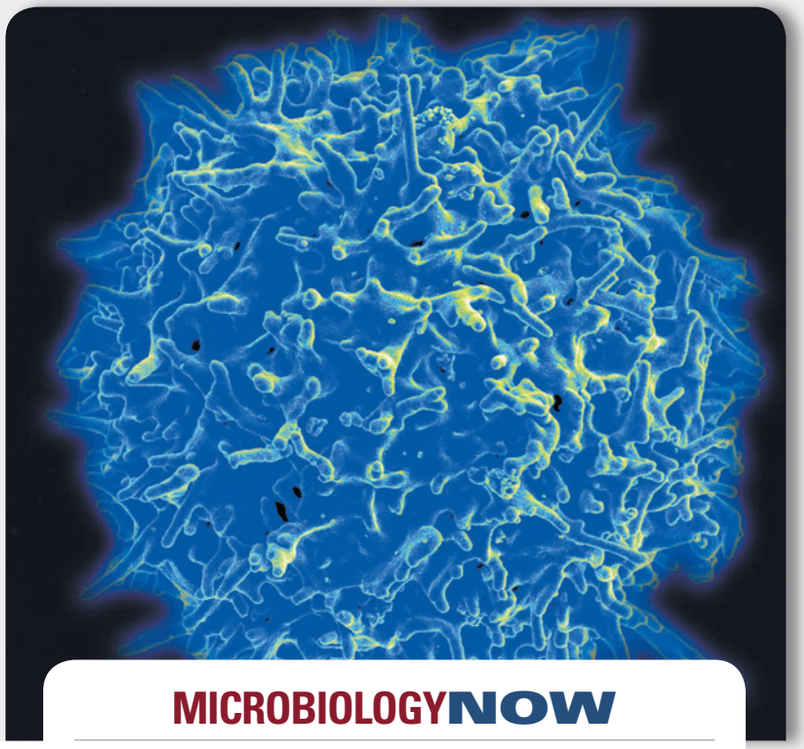

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Controlling HIV through "Public" T Cell Receptors on CD4 T Cells

Control of human immunodeficiency virus (HIV) proliferation in infected individuals typically requires surveillance of viral activity and *highly active anti-retroviral therapy* (*HAART*; see Section 31.15). HAART requires administration of combination anti-retroviral drugs to reduce the viral load and prevent HIV mutation into drug-resistant strains. However, in a small fraction (<1%) of cases, HIV-positive individuals exhibit natural resistance to HIV and maintain an undetectable viral load even in the absence of HAART. These rare individuals, termed "HIV controllers," maintain high numbers of CD4 T cells that produce a robust immune response against HIV.

As discussed in this chapter, CD4 T cells (see scanning electron micrograph) are "helper" T cells that secrete cytokines to coordinate the adaptive immune response. This is initiated through the interaction of T cell receptors (TCRs), found on the surface of T cells, with antigens presented on major histocompatibility complex (MHC) proteins expressed on the surface of antigen-presenting cells. MHC proteins are encoded by human leukocyte antigen (HLA) genes, and because of a phenomenon called *polymorphism*, many different HLA alleles occur in the human population.

Research has shown that HIV controllers produce CD4 T cells with high-affinity "public" TCRs that recognize a highly conserved HIV capsid epitope called *Gag293* when presented by a broader than usual array of distinct HLA class II molecules. By generating an especially efficient "immunological synapse"—the highly organized interaction that occurs when a TCR engages antigen presented by MHC proteins—the public TCRs conferred unusual cytotoxic activity on the CD4 T cells, allowing them to selectively destroy HIV-infected host cells. This property appears to be a key factor in suppressing HIV replication in HIV controllers and suggests that high-affinity public TCRs are linked to spontaneous control of HIV infection, a finding that ultimately may prove to be of enormous clinical significance.

Source: Galperin, M., et al. 2018. $CD4^+$ T cell-mediated HLA class II cross-restriction in HIV controllers. *Sci. Immunol. 3, eaat0687* doi:10.1126/sciimmunol.aat0687.

In the previous chapter we discussed the key features of innate immunity and how this system protects against infection and disease from a broad range of pathogens. Here, we build on this foundation with a focus on the powerful and highly specific immune mechanisms of adaptive immunity, mechanisms that depend on both cellular and molecular components and that complement the innate response.

I • Principles of Adaptive Immunity

Adaptive immune mechanisms are highly specific and exhibit immune memory. B and T lymphocytes are the primary effector cells activated in response to immunogen exposure.

Innate and adaptive immunity can be viewed as two arms of the immune response that work together to protect the host from attack by foreign substances. Whereas innate immunity is characterized by broadly targeted responses triggered by *common* structural features found on microorganisms, adaptive immunity is directed toward *specific* molecular components of the microbes (their antigens). Thus, it is the individual antigenic properties of different pathogens that orchestrate the adaptive immune response.

We begin this part of the chapter by considering the major characteristics of the adaptive immune response and explore how adaptive immune responses are tailored to address invasions from various pathogens, including viruses, bacteria, and fungi.

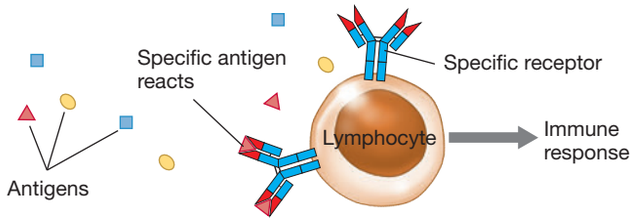
27.1 Specificity, Memory, Selection Processes, and Tolerance

Adaptive immunity is primarily a function of *B* and *T lymphocytes*. B lymphocytes (B cells) specialize in the production of antibodies that interact with and protect against extracellular antigens, thus conferring *antibody-mediated* (*humoral*) *immunity* on the host. T lymphocytes (T cells) express antigen-specific receptors called **T cell receptors (TCRs)** on their surfaces that defend against intracellular pathogens, such as viruses and certain bacteria, thus conferring *cell-mediated* (*cellular*) *immunity* on the host. The combination of antibody-mediated and cell-mediated defenses comprises adaptive immunity, a system that is characterized by three major features: *specificity, memory*, and *tolerance*. None of these features is characteristic of the innate immune response (Chapter 26).

Immune Specificity and Memory

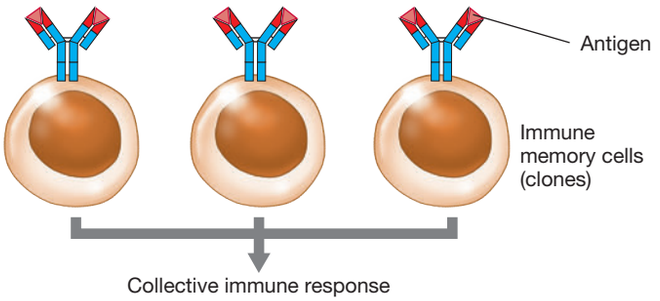
Overall, the immune response is highly specific, but the innate and the adaptive systems differ in their degree of specificity. Innate immunity is activated upon recognition of features common to a broad diversity of microorganisms, such as the peptidoglycan of all gram-positive bacteria or the lipopolysaccharide (LPS) of all gram-negative bacteria. By contrast, adaptive immune mechanisms are directed against pathogen-specific antigens, such as a specific protein associated with a single strain of a particular pathogen.

Each B cell or T cell expresses protein receptors that exhibit **specificity** for a single type of antigen. Just as T cells have antigen-binding TCRs on their surfaces, the antigen-binding proteins of B cells are membrane-bound immunoglobulins called **B cell receptors (BCRs)**. The specificity of the antigen–BCR or antigen–TCR interaction is dependent on the capacity of the lymphocyte cell receptor to interact with a particular antigen but not with other antigens (**Figure 27.1*a***). A pathogen that invades the body introduces multiple antigens that are specific to that pathogen, and each of these antigens will be



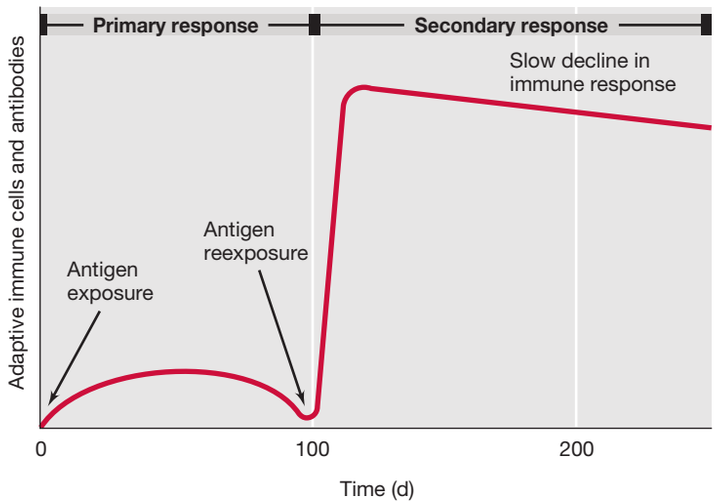
Specificity: Lymphocytes have surface receptors that interact with individual antigens.

(a)



Memory: The first antigen exposure induces multiplication of antigen-reactive cells, resulting in multiple copies, or *clones*. After a subsequent exposure to the same antigen, the immune response is faster and stronger due to the large number of responding cells.

(b)



Immune responses: As a result of immune memory, antigen reexposure triggers a much stronger secondary response.

(c)

Figure 27.1 Specificity and memory in the adaptive immune response. Key features of antibody-mediated and cell-mediated immunity are *(a)* specificity and *(b)* memory. *(c)* The primary response induces both immune cells and antibodies. The antigens given at day 0 and day 100 must be identical to induce a secondary response. The secondary response may generate more than 10-fold increases in immune cells and antibody concentrations.

recognized by distinct lymphocytes bearing receptors that specifically bind them.

In addition to specificity, the adaptive immune system exhibits **memory (immune memory)** (Figure 27.1*b*). Lymphocytes must encounter antigen to proliferate in the body and, in the case of B cells, stimulate production of detectable and effective antigen-activated antibodies. The first exposure to an antigen generates a **primary immune response** in which antigen recognition by specific B or T lymphocytes leads to B and T cell activation, proliferation, and differentiation, thereby resulting in large numbers of antigen-specific B and T cell **clones**. Some of these clones differentiate into long-lived **memory cells**, cells which persist in the body for years and confer long-term antigen-specific immunity. Subsequent exposure to the same antigen activates these clones and generates a faster and stronger **secondary immune response** that peaks within a few days (Figure 27.1*c*). This capacity to respond more quickly and vigorously to subsequent exposures to the eliciting antigen provides the host with immediate resistance to previously encountered pathogens, a topic we develop in more detail in Section 27.3. Immune memory is one of the most important outcomes of an immune response because vaccinating humans or other animals with killed or weakened pathogens (or their products) is a major and relatively inexpensive means of conferring immunity to dangerous pathogens; this in turn promotes long-term public health (see Section 27.2).

T Cell Selection and Tolerance

The adaptive immune system must be able to discriminate between harmless *host* antigens ("self") and potentially dangerous *foreign* antigens ("nonself") and function to destroy only the latter. T cells undergo immune selection *for* potential antigen-reactive cells and selection *against* those cells that react strongly with self antigens. Selection against self-reactive cells results in the development of another key characteristic of the adaptive immune response: **tolerance**. Tolerance is a key component of the immune response and ensures that adaptive immunity is directed against agents that pose genuine threats to the host and not against the host's own proteins. The failure to develop tolerance may result in dangerous reactions to self antigens, a condition called *autoimmunity* (▶ Section 28.1).

T lymphocyte precursors leave the bone marrow via the bloodstream and enter the thymus (**Figure 27.2**); the thymus and bone marrow are the *primary lymphoid organs* (◀ Section 26.4). During the process of T cell maturation in the thymus, immature T cells undergo a two-step selection process to (1) select potential antigen-reactive cells (positive selection) and (2) eliminate cells that react strongly with self antigens (negative selection). **Positive selection** requires the interaction of immature T cells in the thymus with peptide antigens that are actually of self origin. As we discussed in Chapter 26, T cells interact only with antigens that are presented on *major histocompatibility complex proteins* (*MHCs*) expressed on the surface of host cells. Using their TCRs, some immature T cells bind to MHCs that present self peptides on the thymic epithelial tissue. The T cells that do not bind MHC–peptide complexes will be of no use in the immune response and are therefore permanently eliminated via *apoptosis* (programmed cell death). By contrast, those T cells that bind thymic MHC proteins receive survival signals and therefore remain viable. Positive selection retains T cells that recognize self-peptide:self-MHC and deletes T cells that do not recognize self-peptide:self-MHC and would therefore be unable to recognize MHC–peptide outside the thymus.

The second aspect of T cell maturation is **negative selection**. In this process, the positively selected T cells continue to interact with thymic MHC–peptide. Precursor T cells that recognize self-peptide:self-MHC *too strongly* could potentially mount a harmful immune response against host tissues, and therefore these cells are eliminated by apoptosis. The removal of self-reactive precursor T cells through the processes of positive and negative selection is called **clonal deletion**, and this important mechanism prevents the propagation of precursor T cells that could potentially cause autoimmune complications. Precursor T cells having TCRs that react less strongly with self-peptide:self-MHC survive negative selection and continue further differentiation (Figure 27.2).

This two-stage T cell selection in the thymus ensures the generation of self-tolerant T cells capable of reacting strongly to foreign antigens only. Precursor T cells that are either useless (do not bind) or harmful (bind too tightly) are deleted in the thymus. Approximately 95–98% of all immature T cells do not survive the thymic selection process. The remaining selected T cells are destined to interact very strongly with nonself antigens. They are not destroyed in the thymus because their weak binding interactions with thymic self antigens signal them to proliferate. The selected and growing T cells leave the thymus and migrate to the spleen, mucosa-associated lymphoid tissue (MALT), and lymph nodes—the *secondary lymphoid organs or tissues*—where they can engage foreign antigens presented by B cells and other antigen-presenting cells (APCs).

B Cell Selection and Tolerance

To respond to the nearly infinite variety of environmental antigens, the immune system must have the capacity to generate an essentially limitless variety of antigen-specific lymphocytes. To this end, and as we shall see in Section 27.4, the body produces an enormous diversity of B cells, each expressing on its surface BCRs that are specific for a single antigen. B cell selection occurs in the *bone marrow*—the primary lymphoid organ responsible for B cell maturation in mammals—when each immature B cell is exposed to self antigen. As with T cells, the development of tolerance in B cells is necessary because antibodies produced by self-reactive B cells (autoantibodies) may cause autoimmunity and damage to host tissues (▶ Section 28.1). Therefore, B cells must undergo their own clonal deletion process. Immature B cells whose BCRs bind to self-cell-surface antigens undergo apoptosis and are removed from the B cell repertoire. B cells whose BCRs do not react with self antigens complete development and migrate to peripheral tissues.

In the periphery (secondary lymphoid tissues), any B cell that recognizes (binds to) its specific foreign antigen becomes activated and can then proliferate and differentiate in a process called **clonal expansion** (**Figure 27.3**). This produces a pool of B cells, each of which expresses identical antigen-specific receptors. B cells that have not interacted with antigen are not activated and, therefore, do not proliferate. B cell activation and clonal expansion may occur from encountering either T-independent or T-dependent antigens. *T-independent antigens* are typically common microbial components composed of repeating subunits, such as lipopolysaccharides and bacterial capsules. Cross-linking of BCRs by T-independent antigens activates the B cell and triggers clonal expansion in the absence of

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Art Activity: Figure 27.2 T cell selection and clonal deletion

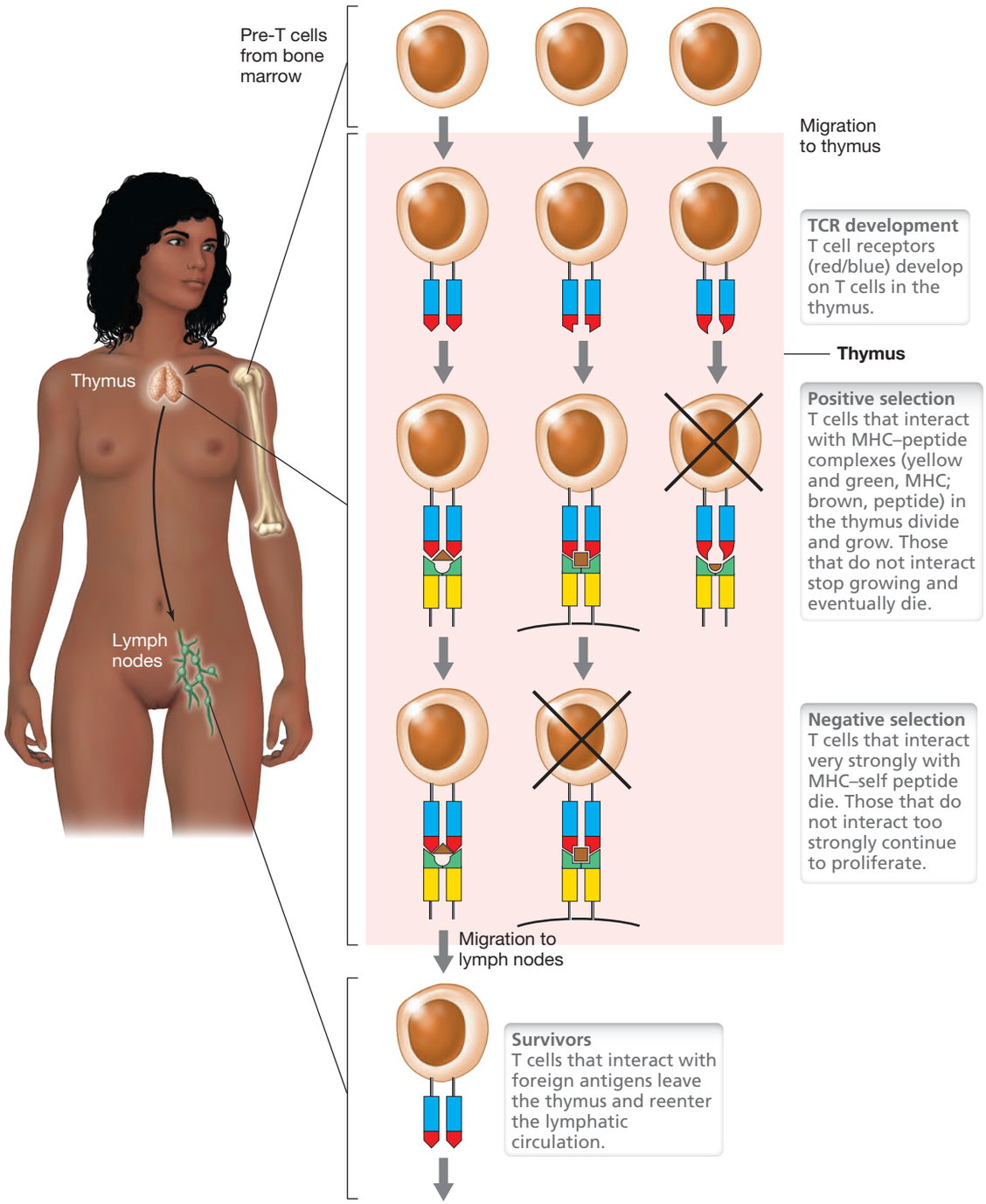


Figure 27.2 T cell selection and clonal deletion. T cells undergo positive selection for functionality and negative selection for dangerously strong recognition of self antigens in the thymus.

T cell help. Although relatively rapid, immune responses to T-independent antigens are comparatively weak, producing antibody-secreting *plasma cells* but no long-lived memory cells.

B cells that interact with *T-dependent antigens*, which are typically pathogen-specific peptides, must engage a corresponding *T-helper* (*Th*) *cell* (Section 27.8) to become activated and generate a clone of antigen-specific B cells. Upon activation, the newly produced B cells differentiate primarily into antibody-producing plasma cells but also a smaller population of antigen-specific memory cells (Figure 27.3). It is the production of memory cells that confers long-term immunity to the eliciting antigen. Upon subsequent exposure to the same antigen, memory B cells do not require T cell help for activation and, therefore, are able to rapidly undergo clonal expansion and trigger an adaptive response. We explore these concepts further in Section 27.3.

In addition to clonal deletion, **clonal anergy** (clonal unresponsiveness) also plays a role in final selection of the B cell pool. Some immature B cells are reactive to self antigens but are not activated by them. This is because in addition to antigen recognition, B cell

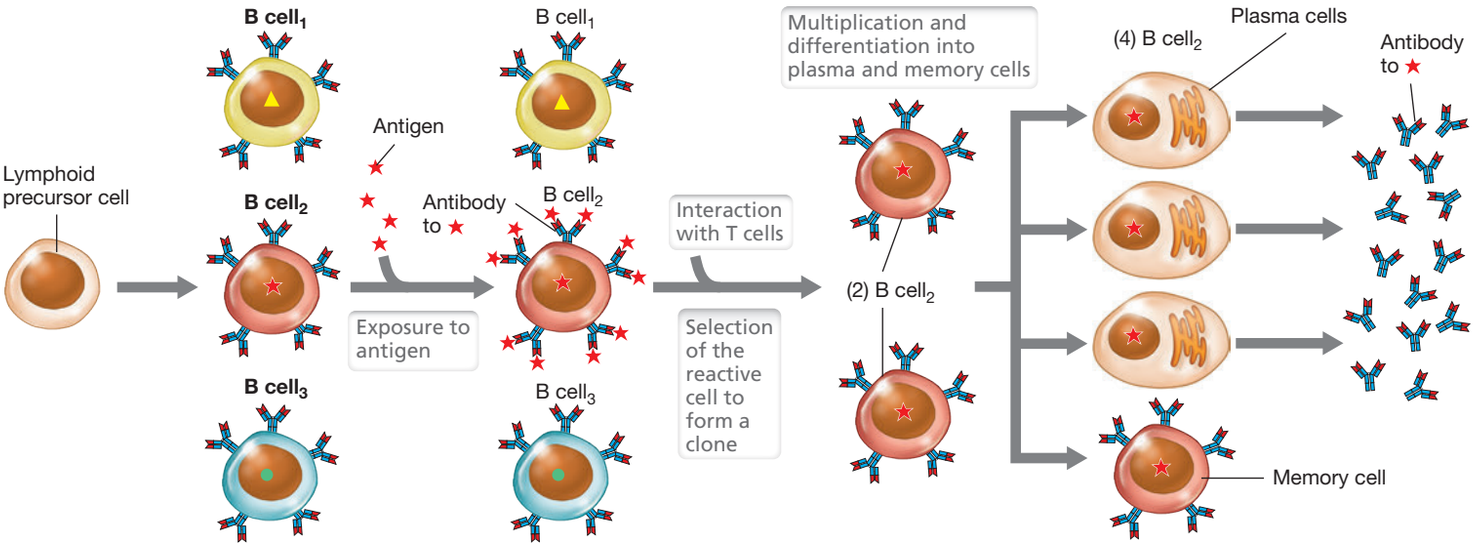


Figure 27.3 B cell clonal selection and expansion. Among the repertoire of individual B cells (represented by B cell$_1$, B cell$_2$, and B cell$_3$), only B cell$_2$ specifically interacts with the introduced antigen (shown as a star). The antigen drives selection and, following interaction with a Th cell, proliferation of B cell$_2$ to form a clone of antigen-specific B cells. Clonal copies of the original antigen-reactive B cell have the same antigen-specific surface antibody. Continued exposure to antigen results in continued expansion of the clone. Clones differentiate further into many antibody-producing plasma cells and comparatively few long-lived memory B cells. Compare this figure with Figure 27.6.

activation requires a *second signal* from a T-helper cell. The nature of this second signal is a positive interaction between proteins on the surface of B cells and Th cells that triggers the release of cytokines, especially IL-4 (*inter*leukin-4), from the Th cell that activates the B cell. If no second signal is generated because the available Th cells have been rendered tolerant to the antigen in the thymus, the B cell remains unresponsive. Using a similar mechanism, a second signal is also required to activate T cells that are interacting with antigens presented on MHCs (Section 27.7). The requirement for a second activation signal is the key to establishing and maintaining clonal anergy in potentially dangerous self-reactive B and T lymphocytes.

With their key properties of specificity, memory, and tolerance, lymphocytes stand ready to deploy the adaptive immune response. The selection and control processes just described ensure that this response will be directed only against foreign antigens. We now examine the nature of antigens in more detail and consider the basic classes of adaptive immunity.

Check Your Understanding

- Distinguish between immune specificity, memory, and tolerance.
- Distinguish between positive and negative T cell selection. How do positive selection and negative selection contribute to the development of tolerance in T cells?
- Distinguish between clonal deletion and clonal anergy in B cells.

27.2 Immunogens and Classes of Immunity

The adaptive immune response can be induced by a vast range of foreign antigens. As we have discussed, **antigens** are substances that interact with antibodies or TCRs. Most, but not all, foreign antigens are **immunogens**, substances that induce an immune response. Here we examine the features of effective immunogens, define the features of antigens that promote interactions with antibodies and TCRs, and discuss the classes of immunity.

Immunogens and Antigen Binding

Immunogens share several intrinsic properties that enable them to induce an adaptive immune response. First, *molecular size* is an important property of immunogenicity; for a molecule to be immunogenic, it must be sufficiently large. Certain low-molecular-weight compounds called *haptens*—such as the disaccharide lactose—do not induce immune responses themselves. However, antibodies can still bind haptens, and they may induce an immune response if attached to a larger *carrier* molecule. Because antibodies can bind them, haptens are considered antigens, but they are not themselves immunogens. Proteins and complex carbohydrates are effective immunogens, whereas nucleic acids, simple polysaccharides with repeating subunits, and lipids are typically not. Thus, *sufficient molecular complexity* is another key property of immunogenicity. Finally, unlike soluble molecules, large, insoluble macromolecules are usually excellent immunogens because phagocytes readily engulf and process them. Thus, *appropriate physical form* is another intrinsic property of immunogenicity.

Extrinsic factors, such as the immunogen dose and the route of administration, also influence immunogenicity. The administered dose of an immunogen can be important for an effective immune response, and in general, doses of 10 µg to 1 g are effective in most mammals. Immunogens administered parenterally (outside of the gastrointestinal tract), usually by injection, are normally more effective than those given topically or orally. When administered by oral or topical routes, antigens may be significantly degraded before coming in contact with immune cells and, as a result, may exhibit diminished or no immunogenicity.

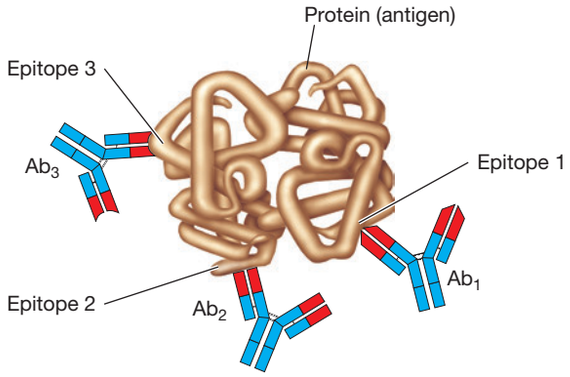


Figure 27.4 Antigens and epitopes for antibodies. Antigens may contain several different epitopes, each capable of reacting with a different antibody (Ab). In some cases, epitopes may be formed from nonlinear parts of a folded polypeptide, as is the case for epitope 1, recognized here by Ab_1. The folding brings two distant portions of the polypeptide together to make a single epitope.

As discussed in Chapter 26, an antibody or TCR does not interact with an immunogen as a whole but only with a distinct portion of the molecule called an **epitope** (also called an *antigenic determinant*) (**Figure 27.4**). Epitopes may include sugars, short peptides of four to six amino acids, and other organic molecules that are components of a larger immunogen. Antibodies are able to recognize epitopes on proteins or polysaccharides in their native conformations. By contrast, TCRs recognize epitopes only after the antigens have been partially degraded or *processed* into peptides by antigen-presenting cells or target cells. T cells recognize peptides shorter than about 20 amino acids presented to them in association with MHC molecules on the surface of host cells.

Antibodies and TCRs can discriminate between closely related epitopes. However, specificity is not absolute, and an individual antibody or TCR may interact to some extent with several different but structurally similar epitopes. The antigen that induces the production of an antibody and, in turn, interacts with that antibody is called the *homologous* antigen, whereas any noninducing antigens that interact with the antibody are called *heterologous* antigens. An interaction between an antibody or a TCR and a heterologous antigen is called a *cross-reaction*.

The engagement of BCRs and TCRs on lymphocytes with naturally encountered immunogens induces an adaptive immune response that often leads to long-term immunity. But as we now discuss, immunity may also be conferred either by purposely introducing an eliciting antigen (vaccination) or through passive transfer mechanisms, administered either naturally or artificially.

Classes of Adaptive Immunity: Active and Passive

There are two broad classes of adaptive immunity. *Active* adaptive immunity may develop following a natural or intentional exposure to an immunogen. By contrast, *passive* adaptive immunity is generated from the transfer of antibodies or immune cells from an immune individual to a nonimmune one. Active and passive immunity are illustrated in **Figure 27.5** and contrasted in **Table 27.1**.

Active and passive immunity can be further classified as being either natural or artificial. *Natural active immunity* is the outcome of exposure to antigens through infection and is typically mediated by both B and T cells. By contrast, *natural passive immunity* occurs when a nonimmune person acquires immune cells or antibodies through natural transfer of cells or antibodies from an immune person, such as from a mother to her fetus before birth or from a mother to a newborn through breastfeeding (Figure 27.5).

Artificial active immunity is conferred by **vaccination (immunization)** and is a major weapon for the prevention and treatment of many infectious diseases. The introduction of antigen into the host triggers B and T cell activation, resulting in antibody production in a primary immune response and, more importantly, production of a population of immune memory cells. A second dose, often called a "booster," of the same antigen results in a secondary immune response in which existing memory cells are quickly activated, producing much higher levels of antibodies and a larger

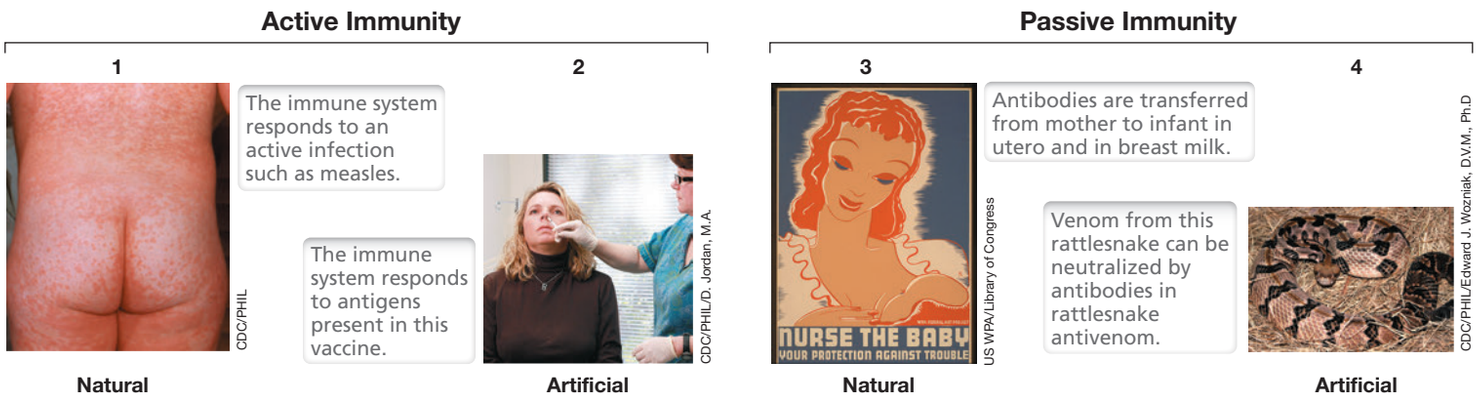


Figure 27.5 Active and passive immunity. Photos, left to right: (1) Childhood measles showing typical systemic measles rash. Natural active immunity requires infection with a pathogen to activate the adaptive immune response. (2) Vaccination by nasal inhalation of antigen. Artificial active immunity occurs from exposure to particular antigens in a vaccine. (3) A 1934 United States government poster promoting breastfeeding. Natural passive immunity occurs when immunity is transferred from one individual to another by natural means, such as the transfer of maternal antibodies in breast milk. (4) Timber rattlesnakes produce highly toxic venom. An antivenom consisting of purified antibodies to timber rattlesnake venom can be prepared in horses and artificial passive immunity conferred on a snakebite victim by injecting the victim with the antivenom.

TABLE 27.1 A comparison of active and passive immunity

Active immunity	Passive immunity
Exposure to antigen; immunity achieved by purposely administering antigen or through infection	No exposure to antigen; immunity achieved by injecting antibodies or antigen-reactive T cells
Specific immune response made by individual achieving immunity	Specific immune response made by the donor of antibodies or T cells
Activated by antigen; immune memory in effect	No immune system activation; no immune memory
Maintained via stimulation of memory cells (i.e., booster immunization)	Cannot be maintained and decays rapidly
Develops over a period of weeks	Develops immediately

population of immune memory cells (Figure 27.1*c*). Active immunity may persist throughout life as a result of immune memory.

Artificial passive immunity is conferred when an individual receives antibodies from an immune individual through injection of an *antiserum*. In passively immunized individuals, transferred antibodies gradually disappear from the body, no immune memory is conferred, and a later exposure to the antigen does not elicit a secondary response. Artificial passive immunity is often used as postexposure therapy for acute infectious diseases, such as tetanus or rabies, for individuals not already protected against such diseases by previous vaccination. This approach is also used for treating individuals following bites from venomous animals (antivenom, Figure 27.5). Antisera containing a concentrated suspension of antibodies specific to the venom (toxin) are generated in large immunized animals, such as horses, or obtained from humans following a natural or artificial active immune response against the antigen and stored for use when needed to treat a human or other animal from a potentially fatal toxemia.

Check Your Understanding

- Identify the intrinsic and extrinsic properties of an immunogen.
- Describe an epitope recognized by an antibody and compare it to an epitope recognized by a TCR.
- Give an example for each: natural and artificial active immunity and natural and artificial passive immunity.

II • Antibodies

In response to the presence of immunogens, B cells produce antibodies (immunoglobulins) to specifically bind, neutralize, and—with the help of other immune cells—eliminate foreign substances from the body.

Antibodies provide antigen-specific immunity that protects against pathogens and dangerous soluble proteins, such as toxins. Here we look at the production, structural diversity, and antigen-binding function of antibodies. We also consider the organization and recombination of genes that encode antibodies, which underlie the nearly unlimited potential for the adaptive immune response to react to foreign molecules.

27.3 Antibody Production and Structural Diversity

An antibody, or *immunoglobulin* (*Ig*), is a soluble protein made by a B lymphocyte or a plasma cell (◀ Figure 26.5) in response to antigen exposure. Each antibody binds to a specific antigen. Antibodies help control the spread of infections by recognizing and binding antigens from pathogens and their products in extracellular environments, such as blood and mucus secretions, and in so doing facilitate the removal of these foreign substances from the body by a variety of mechanisms.

B Cells, Antibodies and Their Activities, and Memory

Each B cell has about 100,000 immunoglobulins of identical antigen specificity that function as B cell receptors (BCRs) on the cell's surface. To make antibodies, a B cell must first bind antigen by way of its BCR (**Figure 27.6**). The BCR–antigen interaction causes the B cell to ingest the bound antigen by endocytosis. The B cell then digests the antigen and generates from it a suite of pathogen-derived antigenic peptides that are affixed to proteins of the *class II major histocompatibility complex* (*MHC II*) (Section 27.5) and displayed (*presented*) on the surface of the B cell. The B cell now functions as an antigen-presenting cell and presents the MHC II–peptide complex to a *T-helper* (*Th*) *cell*, which in turn stimulates the B cell to proliferate and differentiate into antibody-secreting plasma cells and memory B cells (Figure 27.6). We consider MHCs and T cells in more detail later in this chapter.

Th cells do not interact directly with pathogens but instead stimulate, or "help," other immune cells, such as B cells and macrophages, to become activated to carry out an immune response. In this case, the cell being "helped" is the antigen-presenting B cell on which the Th cell recognized the MHC II–peptide complex. The recognition of the MHC II–peptide complex on the B cell surface by a cognate Th cell (the Th cell with a TCR specific for that peptide) stimulates the Th cell to produce cytokines and express cell surface receptors that, in turn, stimulate antigen-specific B cells to grow and divide. This results in the expansion of B cell clones having specificity to the original antigen. Activated B cell clones also differentiate into plasma cell clones, each with the ability to produce and secrete large amounts of antibodies of identical antigenic specificity (Figure 27.6). This primary antibody response (Figure 27.1*c*) is detectable within about five days after antigen exposure, and serum levels of antibodies reach their peak within a few weeks.

Some of the activated B cell clones remain in circulation in the immune system as long-lived *memory B cells* (Figure 27.6). Subsequent exposure to the same antigen, for example by reinfection with the same pathogen, stimulates the antigen-reactive memory B cells, producing a secondary antibody response characterized by more efficient production of higher quantities of high-affinity antibodies (Figures 27.1*c* and 27.6). Recall that the secondary response, conferred by immune memory, is the basis for vaccination (Section 27.2).

Antibodies released from plasma cells interact with antigen, which is often located directly on a pathogen. Antibody binding may have multiple effects on a pathogen, but most antibody interactions do not directly kill pathogens; instead they block interactions between pathogens (or their products) and host cells. For example, antibodies present on mucous membranes may specifically interact

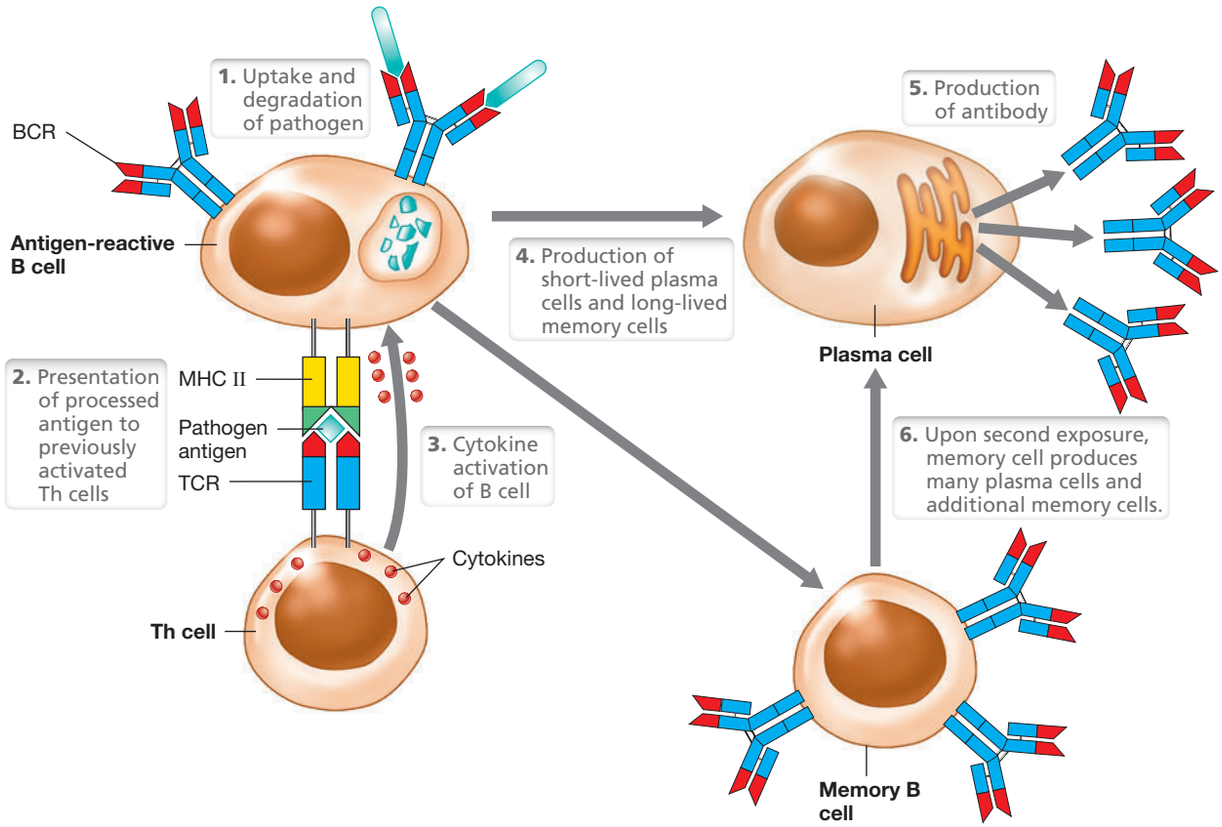


Figure 27.6 B cell–T cell interaction and antibody-mediated immunity. B cells interact with antigen and Th cells to produce antibodies. B cells initially function as antigen-presenting cells. First, their antigen-specific Ig receptor traps antigen. Following endocytosis, antigens are processed into peptide fragments, which are bound by MHC II and transported to the B cell surface. The MHC II–peptide complex is bound by the TCR on the Th cell, causing the Th cell to secrete IL-4 and IL-5. These cytokines activate the B cell to produce clones that differentiate into many antibody-producing plasma cells and a smaller number of memory cells. The latter are long-lived and can differentiate into antibody-producing plasma cells upon secondary exposure to the same antigen.

with influenza virus antigens able to bind to host cells, thereby blocking attachment of the influenza virus to host cells on the mucosal surface. In addition, circulating antibodies in blood and lymph serum can *neutralize* toxins by binding them and thereby prevent their deleterious effects on host cells (**Figure 27.7**). In other cases, antibodies coat pathogens by binding to their surface antigens, a process called *opsonization*, thereby marking them for destruction by receptor-mediated phagocytosis (◀ Section 26.9). Phagocytes have antibody receptors called *Fc receptors* (*FcR*) that bind to any antibody attached to an antigen, resulting in enhanced phagocytosis of the antibody-coated pathogens (◀ Figure 26.15*a*).

Immunoglobulin G Structure and Function

There are five classes of immunoglobulins—*IgG*, *IgM*, *IgA*, *IgD*, and *IgE*—distinguished from one another by their different physical, chemical, and immunological properties. Based on these distinctions, each antibody class has a defined distribution and general function (**Table 27.2**). IgG is the most common circulating antibody, comprising up to 80% of serum immunoglobulins, and it can activate complement through the classical pathway (◀ Section 26.9). IgG is composed of four polypeptide chains that are interconnected by disulfide (S—S) bonds (**Figure 27.8**). In each IgG molecule, two identical short chains of about 25 kilodaltons (kDa) (called *light*

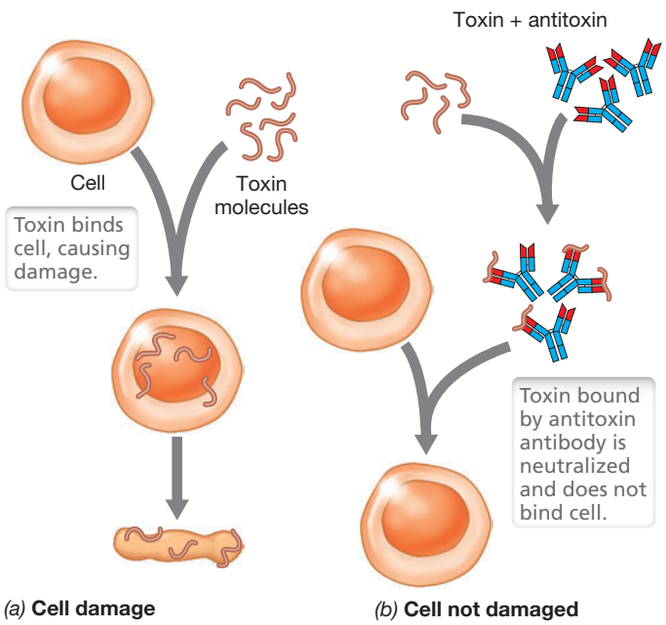


Figure 27.7 Neutralization of an exotoxin by an antitoxin antibody. *(a)* Toxin results in cell destruction. *(b)* Antitoxin prevents cell destruction. Examples of exotoxins include botulism and tetanus toxins and are discussed in Section 25.6.

TABLE 27.2 Properties of human immunoglobulins

Class/H chain[a]	Structural conformation/ Antigen-binding sites	Serum concentration (mg/ml)/Percent of total circulating antibody	Properties	Distribution
IgG/γ	Monomer/2	13.5/70–80%	Major circulating antibody; four subclasses: IgG1, IgG2, IgG3, IgG4; IgG1 and IgG3 activate complement	Extracellular fluid; blood and lymph; intestine; crosses placenta
IgM/μ	Pentamer/10 Monomer/2	1.5/6–10% 0	First antibody to appear in primary response to extracellular pathogens or after immunization; pentamer especially effective in agglutinating antigens; strong complement activator	Blood and lymph; monomer is B cell surface receptor (BCR)
IgA/α	Monomer/2 Dimer/4	3.5/10–20% 0.05/0.2–0.3%	Important circulating antibody Major secretory antibody	Monomer conformation is present in blood and lymph; dimer conformation is present in secretions, such as mucus, saliva, and breastmilk colostrum
IgD/δ	Monomer/2	0.03/0.2–0.3%	Minor circulating antibody; mostly associated with mature B cells	B cell surface receptor (BCR); blood and lymph (trace)
IgE/ε	Monomer/2	0.00005/0.0003%	Facilitates parasite immunity but also triggers allergic reactions	Blood and lymph; binds to mast cells and eosinophils

[a]All immunoglobulins may have either λ or κ light-chain types, but not both.

chains) are paired with two identical longer chains of about 50 kDa (called *heavy chains*), yielding a Y-shaped, symmetrical molecule. Each heavy chain interacts with a light chain to form a functional *antigen-binding site*. Therefore, an IgG antibody molecule is *bivalent* because it has two identical sites that bind two identical antigenic determinants.

Heavy and light chains consist of variable and constant protein domains. A heavy-chain *variable domain* is connected to three heavy-chain *constant domains* (Figure 27.8*a*). The variable domain (V_H) and the first constant domain (C_H1) compose part of the *Fab* (*f*ragment, *a*ntigen-*b*inding) portion of the immunoglobulin, so named because it contains the antigen-binding site. The two constant domains located most distal to the variable domain (C_H2 and C_H3) compose the *Fc* (*f*ragment, *c*rystalline) region of the antibody, named for its tendency to crystallize in solution. As previously mentioned, it is the Fc region of the antibody that binds FcR molecules on the surface of phagocytes to facilitate phagocytosis. The light chains of the antibody consist of one variable (V_L) and one constant (C_L) domain each and contribute to the two Fab regions only (Figure 27.8*a*).

Because the variable domains of a given antibody bind a specific antigenic determinant, their amino acid sequences vary in each different antibody. The variable domain of a light chain (V_L) interacts with the variable domain of a heavy chain (V_H) to bind antigen. By contrast, the constant domains of each antibody are identical in amino acid sequence for all Ig molecules of a given class.

Other Immunoglobulin Classes and Their Functions

Immunoglobulins of the other classes differ from IgG in structure and function. Because the amino acid sequence of heavy-chain constant domains determines antibody class, the heavy chain called *gamma* (γ) defines the IgG class. Likewise, *alpha* (α) defines IgA; *mu* (μ) defines IgM; *delta* (δ) defines IgD; and *epsilon* (ε) defines IgE (Table 27.2). The heavy chains of IgM and IgE contain four constant domains, whereas the heavy chains of IgG, IgA, and IgD contain only three (**Figure 27.9*a, b***).

The monomeric and pentameric structures of IgM are shown in Figure 27.9*b* and *c*, respectively. Circulating IgM forms an aggregate of five or six immunoglobulin molecules attached by at least one J (joining) peptide (Figure 27.9*c*). Pentameric

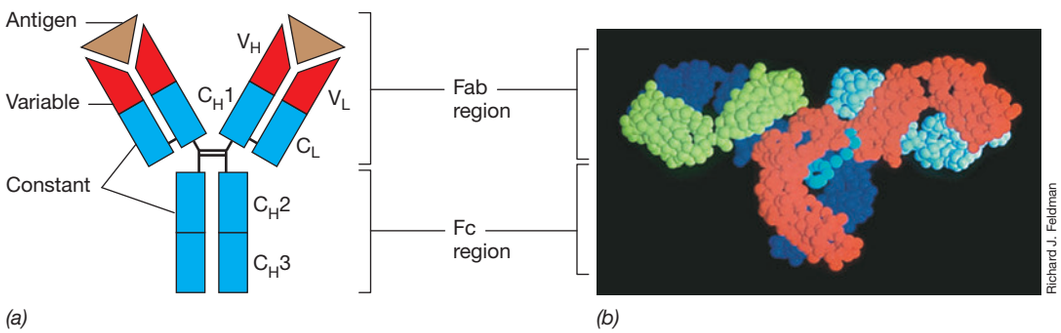


Figure 27.8 Immunoglobulin G structure. *(a)* IgG consists of two heavy chains (50,000 molecular weight) and two light chains (25,000 molecular weight), with a total molecular weight of 150,000. One heavy and one light chain interact to form an antigen-binding unit. The variable domains of the heavy (V_H) and light chains (V_L) bind antigen. The constant domains (C_H1, C_H2, C_H3, C_L) are identical in all IgG proteins. The chains are covalently joined with disulfide bonds, shown here as connecting black lines. The Fab (*f*ragment, *a*ntigen-*b*inding) region contains the antigen-binding site. The Fc (*f*ragment, *c*rystalline) stem of the antibody binds receptor molecules on phagocytes to facilitate phagocytosis of opsonized pathogens. *(b)* Space-filling model of an IgG molecule. The heavy chains are red and dark blue. The light chains are green and light blue.

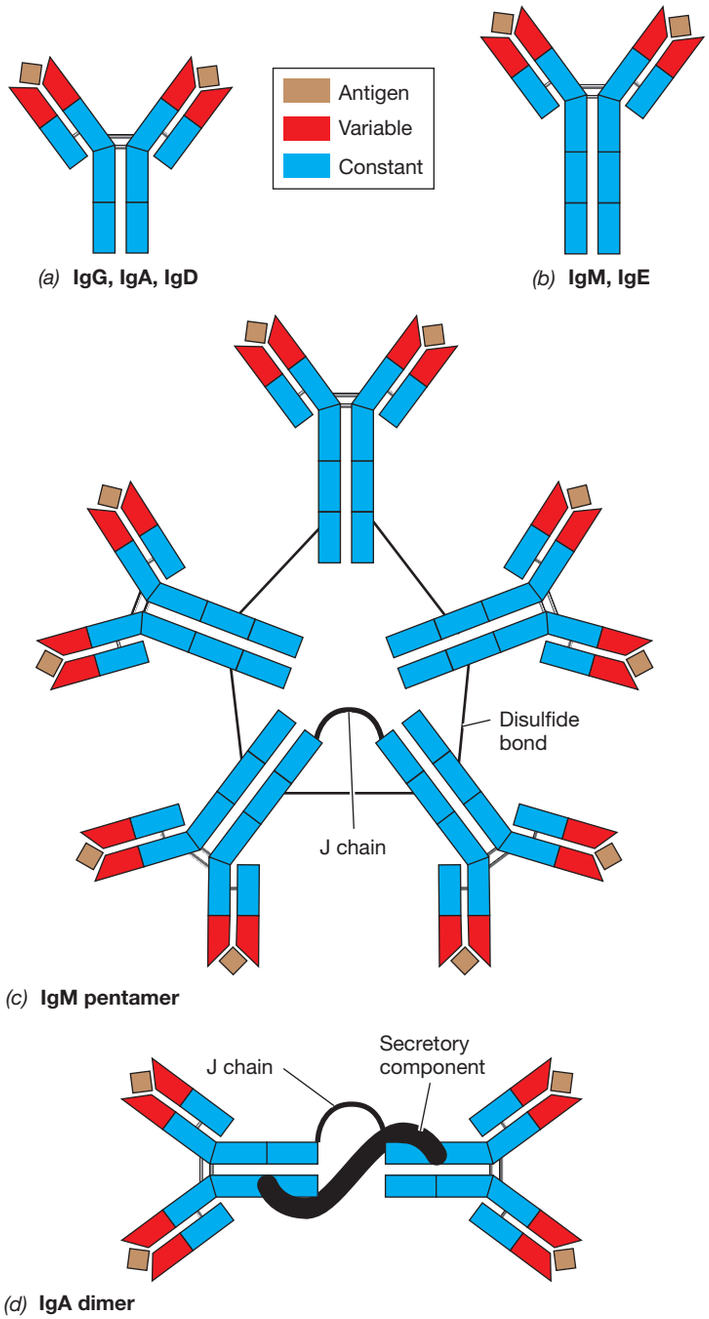


Figure 27.9 Immunoglobulin classes. All classes of Igs have V_H and V_L that bind antigen. *(a)* IgG, IgA, and IgD have three constant domains. *(b)* The heavy chains of IgM and IgE have a fourth constant domain. *(c)* IgM is found in serum as a pentameric protein consisting of five IgM proteins covalently linked to one another via disulfide bonds and a joining (J) chain protein. Because it is a bivalent pentamer, IgM can bind up to ten antigens, as shown. *(d)* Secretory IgA is often found in body secretions as a dimer consisting of two IgA proteins covalently linked to one another by a J chain protein. The secretory component aids in transport of IgA across mucosal membranes.

IgM is the first class of antibody produced in a typical immune response to a bacterial infection, but each individual antigen-binding site of an IgM pentamer generally has low *affinity* (binding strength) for antigen. However, the *avidity* (combined antigen-binding strength of multiple epitopes) of IgM antibodies is enhanced because each IgM pentamer has ten binding sites available for interaction with antigen (Table 27.2 and Figure 27.9*c*). The ten antigen-binding sites make IgM especially effective at agglutinating (clumping) antigens, thereby increasing phagocytic efficiency. Like IgG, IgM is a strong activator of complement through the classical pathway. Whereas up to 10% of serum antibodies are pentameric IgM, IgM monomers do not circulate in the blood but rather are one of the classes of antibodies that function as BCRs.

Dimers of IgA are present in high numbers in secreted body fluids, including saliva, tears, breast milk colostrum, and mucosal secretions from the gastrointestinal, respiratory, and genitourinary tracts. The mucosal surfaces in an average adult, which total about 400 m^2 (skin has about 6 m^2), contain MALT and produce large amounts (about 10 g) of secretory IgA every day. By comparison, an individual produces only about 5 g of serum IgG per day. Whereas *serum* IgA typically occurs in monomeric form (Table 27.2), *secretory* IgA consists of two IgA molecules covalently linked by a J chain peptide and complexed with a protein called the *secretory component* that aids in transport of IgA across membranes (Figure 27.9*d*). The high concentration of secretory IgA in breast milk colostrum likely plays a key role in preventing gastrointestinal disease in newborns.

Although IgE is present in small amounts in serum, most IgE is bound to cells. For example, through its Fc region IgE binds eosinophils, arming these granulocytes to target parasitic pathogens such as schistosomes and other helminths (▶ Section 34.7). IgE also binds to tissue mast cells, where subsequent binding of antigen to antigen-binding sites of IgE causes degranulation of the mast cells. The ensuing release of chemical mediators, such as histamine and serotonin, triggers immediate-type hypersensitivities (a type of allergic response; ▶ Section 28.1). Like IgM, IgE has a fourth heavy-chain constant domain (Figure 27.9*b*), and this allows for binding of IgE to eosinophils and mast cells, the key step for activating the protective and allergic reactions, respectively, associated with these cell types.

IgD (Figure 27.9*a*), present in serum in low concentrations, has no known unique immune protective function. However, IgD, like IgM, is abundant on the surfaces of mature B cells, especially memory B cells, where it functions as a BCR.

Antigen Exposure, Immune Memory, and the Primary and Secondary Responses

We have seen that immune memory is a major outcome of the adaptive immune response (Figure 27.1*c*). Starting with a B cell, an antibody-mediated immune response begins with antigen exposure and culminates with the production and secretion of antigen-specific antibodies, and the type and route of antigen exposure influences the class of the antibodies produced. Blood and lymph, as well as the spleen and MALT (Chapter 26), are key sites for engagement of antigens by immune components. Bloodborne pathogens/antigens are captured in the spleen, where IgM, IgG, and serum IgA antibodies are produced. Antigen introduced subcutaneously, intradermally, topically, or intraperitoneally is carried through the lymphatic system to the nearest lymph nodes, again stimulating production of IgM, IgG, and serum IgA. Antigen introduced to mucosal surfaces is delivered to the nearest MALT. For example, antigen delivered orally is delivered to the MALT in the intestinal tract, stimulating production of secretory IgA in the gut.

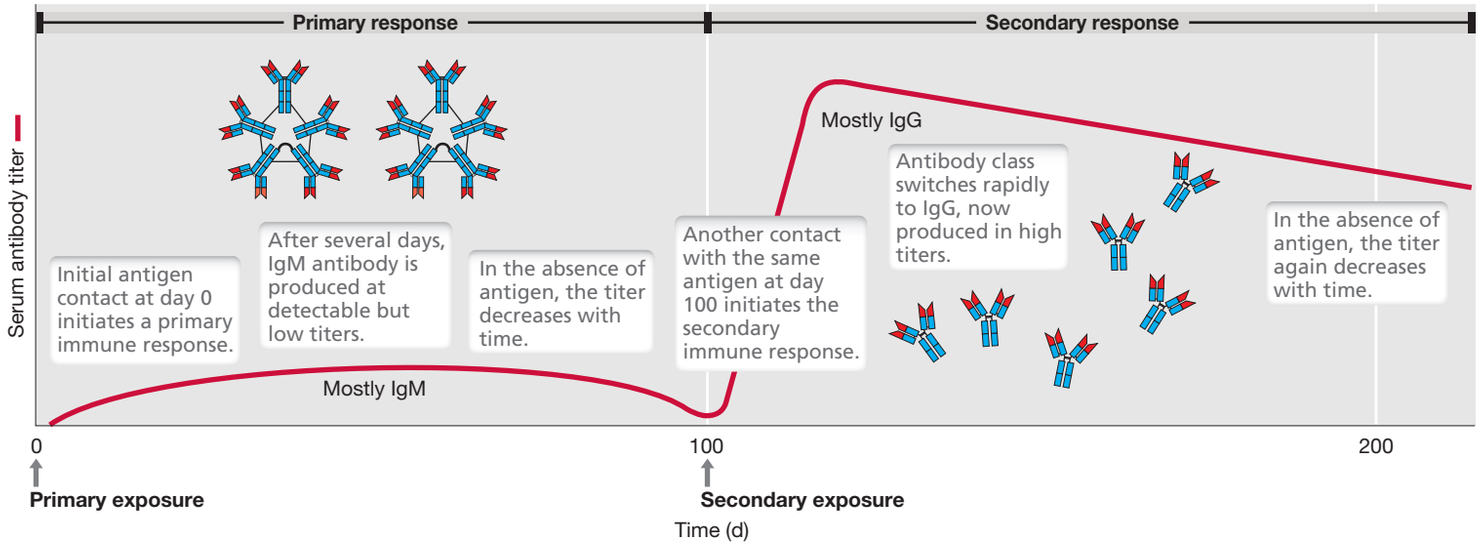


Figure 27.10 Primary and secondary antibody responses in serum. The antigen exposures at day 0 and day 100 must be to identical antigen to induce a secondary response. The secondary response, also called a booster response, may be more than 10-fold greater than the primary response. Note the class switch from IgM production in the primary response to IgG production in the secondary response.

Following initial BCR–antigen contact, each B cell stimulated by T-dependent antigen multiplies and differentiates to form antibody-secreting plasma cells and memory B cells (Figure 27.6). Plasma cells in this *primary antibody response* are relatively short-lived (less than one week) and secrete large amounts of mostly IgM antibody (**Figure 27.10**). After a latent period of several days, antibody appears in the blood and a gradual increase in *antibody titer* (antibody quantity) occurs. As antigen disappears, the primary antibody response slowly diminishes.

Mastering Microbiology
Art Activity: Figure 27.10 Primary and secondary antibody responses in serum

Memory B cells generated by the initial exposure to antigen may circulate in the host for years. A second exposure to the same antigen stimulates memory B cells to rapidly differentiate into plasma cells and produce antibody without input from helper T cells. The second and each subsequent exposure to antigen causes the antibody titer to rise rapidly to a level often 10–100 times greater than the titer following the first exposure (Figure 27.10). This rise in antibody titer is called the *secondary antibody response.* The secondary response is a function of immune memory and typically results in a switch from the production of IgM to another antibody class, a transition called *class switching* (Figure 27.10). This switch is induced by a cytokine signal produced in response to the specific type of pathogen. The signal prompts a recombination event that switches the heavy-chain constant region, resulting in the production of the antibody class that can most efficiently address the threat. For example, in serum, the most common antibody class switch is from IgM to IgG, whereas in mucosal tissues, switches to IgA predominate. It is important to note that class switching does not cause a change in antigen specificity; the new class of antibody generated remains specific to the original antigen.

Serum antibody titer slowly decreases over time, but subsequent exposure(s) to the same antigen can trigger another secondary response. This results in the maintenance of high levels of memory cells and circulating antibody specific for the antigen, providing long-term active immunity against individual infectious diseases.

Check Your Understanding

- Compare and contrast B cell activation from T-dependent and T-independent antigens (Section 27.1).
- Differentiate among antibody classes using structural characteristics, distribution patterns, and functional roles.
- Explain the rationale for periodic revaccination in children and adults.

27.4 Antigen Binding and the Genetics of Antibody Diversity

We have seen that antibodies consist of four polypeptides, two heavy (H) chains and two light (L) chains (Figure 27.8), and each chain is composed of constant (C) and variable (V) domains. The V domains of one H and one L chain interact to form the antigen-binding site. Here we examine the structural features of the V domains that define the antigen-binding site and then explore the genetics behind the enormous diversity of antibodies that, collectively, B cells are capable of producing.

Variable Domains and Antigen–Antibody Interaction

The V domains of an H and an L chain bind antigen strongly but not covalently. To accommodate all possible antigens that a host might encounter in a lifetime, the synthesis of billions of different antibodies, each with its own unique antigen-binding site, is necessary. The V domains define these unique antigen-binding sites.

Amino acid sequences differ in the V domains of Igs that bind different antigens. Amino acid variability is especially apparent in several **complementarity-determining regions (CDRs)**. Three CDRs in each of the V domains provide most of the molecular contacts with antigen (**Figure 27.11**). The amino acid sequences of CDR1 and CDR2 differ in minor ways between different Igs, while CDR3s differ dramatically in sequence from one another. Three distinct gene segments encode CDR3 of the H chain: the carboxy-terminal portion of

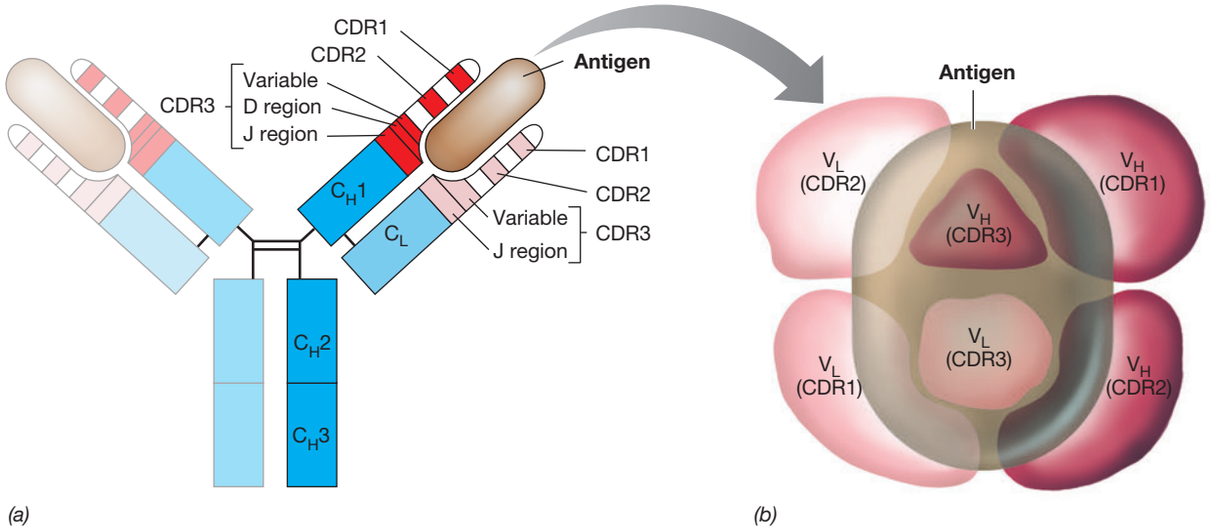


Figure 27.11 Antigen binding by immunoglobulin light and heavy chains. *(a)* An Ig is shown schematically, with bound antigen (light brown). The V domains on the H and L chains are shown in red (heavy chain) and pink (light chain) with their antigen-binding complementarity-determining regions (CDR1, CDR2, and CDR3). C_H1, C_H2, and C_H3 (dark blue) are constant domains in the H chain, and C_L (light blue) is the constant domain in the L chain. *(b)* CDRs from both H (red) and L (pink) chains, shown from above, are conformed to make a single antigen-binding site on the Ig. The highly variable CDR3s from both H and L chains cooperate at the center of the site. Antigen (light brown) may contact all CDRs. The shape of the site may be a shallow groove or a deep pocket, depending on the antibody–antigen pair involved.

the V domain, followed by a short "diversity" (D) segment of about three amino acids, and a longer "joining" (J) segment about 13–15 amino acids long. The light-chain CDR3 is similar except that it lacks the D segment. The heavy- and light-chain CDRs, six in total, confer antigen specificity on the antibody molecule (Figure 27.11).

The Ig three-dimensional structure was shown in Figure 27.8*b*. Each antigen–antibody interaction requires the specific binding of an antigenic *epitope* with the CDR domains of the H and L chains. A single antigen-binding site of an antibody molecule measures about 2 × 3 nm, creating a space large enough to accommodate an epitope of 10 to 15 amino acids. Antigen binding is ultimately a function of the Ig folding pattern of the H and L polypeptide chains. The Ig folds of the V region bring all six CDRs together, resulting in the formation of a unique and specific antigen-binding site (Figure 27.11).

Because antigen diversity is enormous, the immune system must be capable of producing antibodies to recognize a virtually unlimited variety of antigens. In order to accomplish this from a relatively small number of immunoglobulin genes, several unusual genetic mechanisms come into play. **Table 27.3** summarizes these diversity-generating mechanisms, and we explore each below.

Organization of Immunoglobulin Genes

The gene encoding each immunoglobulin H or L chain is constructed from several gene segments. In each B cell, Ig gene segments undergo a series of random, somatic rearrangements characterized by genetic recombination followed by deletion of intervening sequences. These events produce a single, functional antibody gene derived from a relatively small pool of Ig gene segments. As a B cell matures, V, D, and J gene segments are enzymatically recombined to form a single Ig heavy-chain gene (**Figure 27.12*a***). The single V gene segment encodes CDR1 and CDR2, whereas CDR3 is encoded by a mosaic of the 3′ end of the V region, followed by the D and J gene segments.

In each B cell, only one productive Ig-encoding rearrangement each occurs in the heavy- and light-chain genes. Called *allelic exclusion*, this mechanism causes each B cell to express Ig genes from only one of the two inherited parental alleles, thus ensuring that all Igs from a given clone of B cells have identical antigen specificity. Finally, separate C gene segments encode the class-defining constant domains of Igs. Therefore, four different gene segments—V, D, J, and C—recombine to form one functional heavy-chain gene (Figure 27.12*a*). Light chains, which lack D segments, are encoded in a similar way by recombination of light-chain V, J, and C segments (Figure 27.12*b*). The gene segments required for all Igs exist in all nucleated cells of the body but undergo recombination only in developing B lymphocytes.

V, D, J, and C gene segments are separated by noncoding DNA sequences typical of gene arrangements in eukaryotes (◀ Section 6.6).

TABLE 27.3 Generation of antigen-binding receptor diversity in B cells and T cells

Diversity-generating mechanism	B cell Ig receptors, heavy and light chains	T cell receptors, α and β chains
Somatic recombination of tandem genes	Yes	Yes
Random reassortment	Yes	Yes
Imprecise V-D-J or V-J joining	Yes	Yes
Nucleotide additions at V-D-J or V-J junctions	Yes	Yes
D gene segments read in all 3 frames	No	Yes
Somatic hypermutation	Yes	No

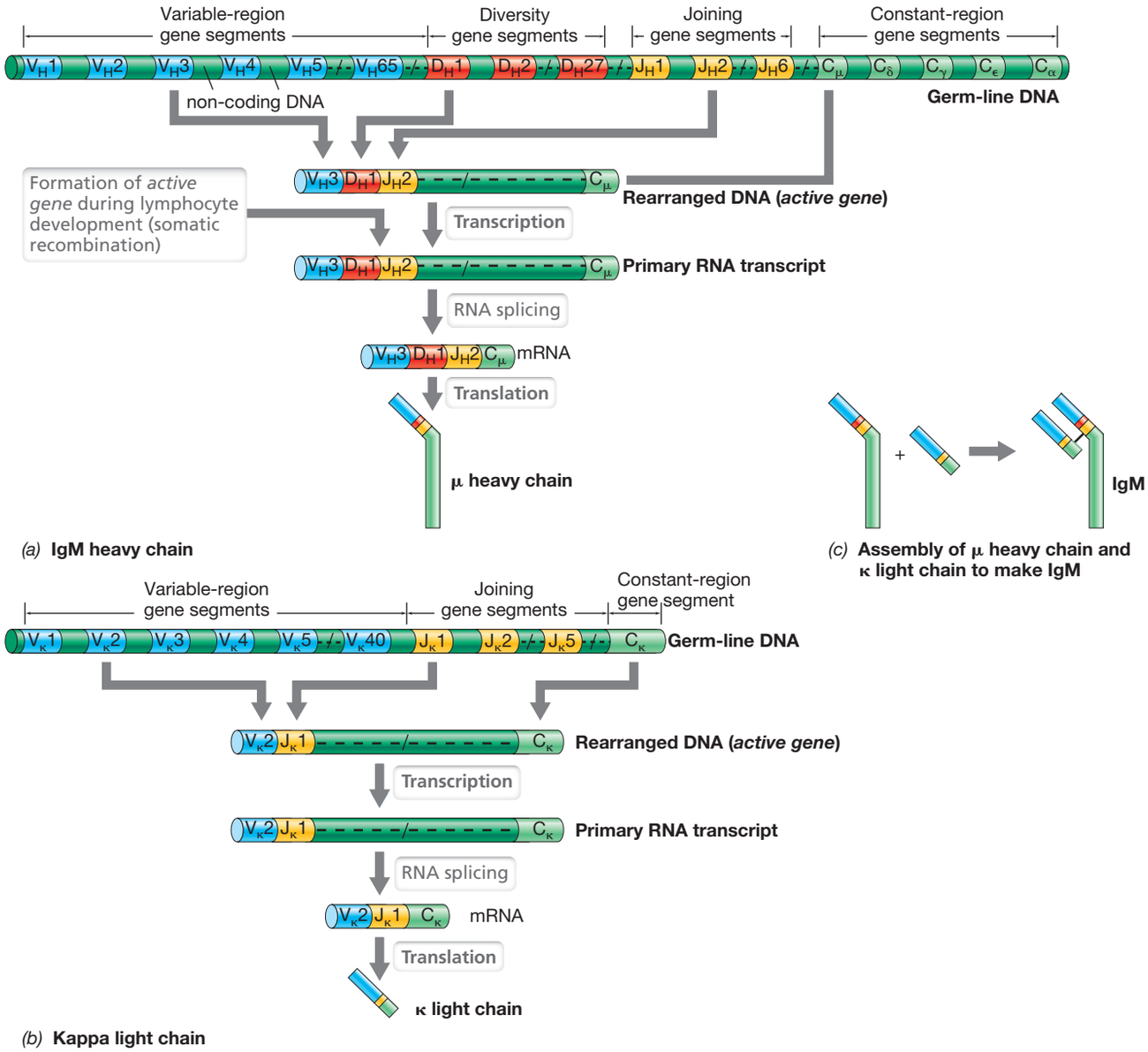


Figure 27.12 Immunoglobulin gene rearrangement in human B cells. Ig genes are arranged in tandem on three different chromosomes. *(a)* The H chain gene complex on human chromosome 14. The filled boxes represent Ig coding gene segments. The dashed lines indicate intervening sequences and are not shown to scale. *(b)* The κ light-chain complex on human chromosome 2. The λ light-chain genes are in a similar complex on human chromosome 22. *(c)* Assembly of one-half of an antibody molecule.

Genetic recombination occurs in each B cell during its development. One each of the V, D, and J segments is recombined at random to form a functional heavy-chain gene, while randomly recombined V and J segments form a complete light-chain gene. The active gene, still containing an intervening sequence between the VDJ or VJ gene segments and the C gene segments, is transcribed, and the resulting primary RNA transcript is spliced to yield the final messenger RNA (mRNA). The mRNA is then translated to make the heavy and light chains of the Ig molecule.

VDJ Recombination and Somatic Hypermutation

If one considers all of the random reassortments possible in genes encoding immunoglobulin heavy and light chains and from these calculates the number of unique molecules that could be encoded, the total diversity is astonishing. In humans, for example, based on the numbers of kappa (κ) light-chain gene segments, there are about 40 V × 5 J possible rearrangements, or 200 possible κ light chains. For the alternative lambda (λ) light chain, the type produced instead of κ by some B cells in every individual, there are about 30 V × 4 J = 120 possible combinations. About 10,500 possible heavy chains can be formed by the rearrangement of about 65 V × 27 D × 6 J genes. Assuming that each heavy chain and light chain has an equal chance to be expressed, there are 10,500 × 200 = 2,100,000 possible immunoglobulins with κ light chains and 10,500 × 120 = 1,260,000 possible immunoglobulins with λ chains. Therefore, at least 3,360,000 possible antibodies can be expressed based on random reassortment alone!

On top of this impressive diversity, additional diversity is generated in the CDR3 regions of both heavy and light chains by several unique mechanisms. First, the DNA-joining mechanism that constructs V-D or D-J gene segments in the heavy chain or the V-J gene segments in the light chain is an imprecise process. The final nucleotide sequence at these regions occasionally varies by a few nucleotides from the original genomic sequence, and when this occurs, it will alter the amino acid sequence in this region. Even more diversity is generated at V-D and D-J coding joints in the heavy-chain genes and at V-J coding joints in light-chain genes by either random (N) or template-specific (P) nucleotide additions. Because these coding joints are contained within the sequences that encode CDR3 on both heavy and light chains, N and P diversity at V-domain coding joints changes or adds amino acids in the CDR3 of both heavy and light chains.

VDJ recombination events are antigen-independent and occur as B cells develop in the bone marrow. In mature B cells that have migrated to lymphoid tissues, immunoglobulin diversity is further expanded upon antigen exposure by a process called **somatic hypermutation**—the mutation of B cell Ig genes at much higher rates than those observed in other genes. Somatic hypermutation of Ig genes is typically evident after a second exposure to an immunizing antigen and occurs only in the V regions of rearranged heavy- and light-chain genes, creating B cells with slightly altered Ig cell surface receptors. These mutated B cells compete for available antigen, and B cells whose receptors have a higher affinity for antigen than the original B cell receptor are selected. This process, called *affinity maturation*, is one of the factors responsible for a dramatically stronger secondary immune response (Figures 27.1*c* and 27.10). Affinity maturation also adds more diversity to the pool of antibody specificity in the adaptive immune response, thus making the potential antibody repertoire essentially infinite.

Check Your Understanding

- Draw a complete Ig molecule and identify antigen-binding sites on the antibody.
- Describe antigen binding to the CDR1, 2, and 3 regions of the heavy-chain and light-chain variable domains.
- Describe the recombination events that produce a mature heavy-chain gene and other somatic events that further enhance antibody diversity.

III • The Major Histocompatibility Complex (MHC)

The major histocompatibility gene complex (MHC) encodes cell surface proteins that present antigens to T cells, providing an important link between innate and adaptive immune mechanisms.

The *major histocompatibility complex* (*MHC*) is a series of genes found in all vertebrates that encodes a group of proteins important in antigen presentation. The MHC proteins in humans are called **human leukocyte antigens (HLAs)** and were first identified as the major antigens responsible for immune-mediated tissue transplant rejection. We now know that MHC proteins function primarily as antigen-presenting molecules, binding pathogen-derived peptides and displaying these peptides for interaction with T cell receptors.

27.5 MHC Proteins and Their Functions

The MHC proteins consist of two protein classes encoded by about 4 megabase pairs (Mbp) of DNA (**Figure 27.13*a***). **MHC class I proteins** are expressed on the surfaces of all nucleated cells and function to present peptide antigens to *T-cytotoxic* (*Tc*) *cells*. If a peptide antigen presented by MHC class I is recognized by the TCR of a Tc cell, the antigen-containing cell is quickly destroyed (Section 27.8). **MHC class II proteins** are found only on the surface of B lymphocytes, macrophages, and dendritic cells—the antigen-presenting cells (APCs; ◀ Section 26.4). Through their MHC class II proteins, APCs present antigens to *T-helper* (*Th*) *cells*, stimulating cytokine production that leads to antibody-mediated immunity or inflammatory responses (Section 27.8).

Class I and Class II MHC Proteins

Class I MHC proteins consist of the membrane-embedded *alpha* (α) *chain*, encoded by a gene located in the MHC gene region, and the *beta-2 microglobulin* (β_2m), a smaller, noncovalently associated protein encoded by a gene not included in the MHC gene cluster (Figure 27.13*b* and *d*). The α chain folds to form a peptide-binding groove that accommodates peptides of 8 to 11 amino acids (Figure 27.13*d* and *e*). The peptides in infected cells are derived from *endogenous* (intracellular) antigens, for example, from viral proteins produced inside the cell. Following translocation to the cell surface, the MHC–virus-peptide complexes are recognized by TCRs on activated Tc cells, and the Tc cells then kill the infected target cells.

Class II MHC proteins consist of two membrane-integrated polypeptides, α and β, expressed primarily by APCs. Together, one α and one β polypeptide form a functional heterodimer (Figure 27.13*c*, *f*). The α1 and β1 domains of the MHC class II protein interact to form a binding site for TCR–peptide similar to the MHC class I binding site for TCR–peptide. However, the peptide-binding groove in the class II MHC is able to bind and display peptides that may be significantly longer than 8–11 amino acids. Peptides presented by class II MHCs are typically 10 to 20 amino acids long and are proteolytic fragments derived from *exogenous* (extracellular) antigens from pathogens that have been phagocytosed and digested by APCs. The APCs use the MHC class II–peptide complex to interact with TCRs on Th cells, leading to Th activation and an adaptive immune response (Section 27.8).

Antigen Processing and Presentation to T Cells

MHC proteins are expressed on cell surfaces only when they are complexed with a peptide, either a self peptide or a foreign peptide. In essence, then, *MHC–peptide complexes reveal to the immune system the protein composition of the cell* and, in this capacity, function to alert the immune system when a cell contains foreign antigens. T cells, through their TCRs, scan cell surfaces to identify any cells expressing foreign antigens as an indication of infection. When the latter are encountered, the TCR interacts with the foreign antigen presented on the MHC I protein, and this interaction targets the cell for destruction. T cells do not destroy cells presenting MHC–self peptide complexes because self-reactive T cells that escape clonal deletion in the thymus typically become anergic upon recognition of self antigens (Section 27.7).

UNIT 6

Mastering Microbiology
Activities: Microbiology Animation: Antigen Processing and Presentation: Steps

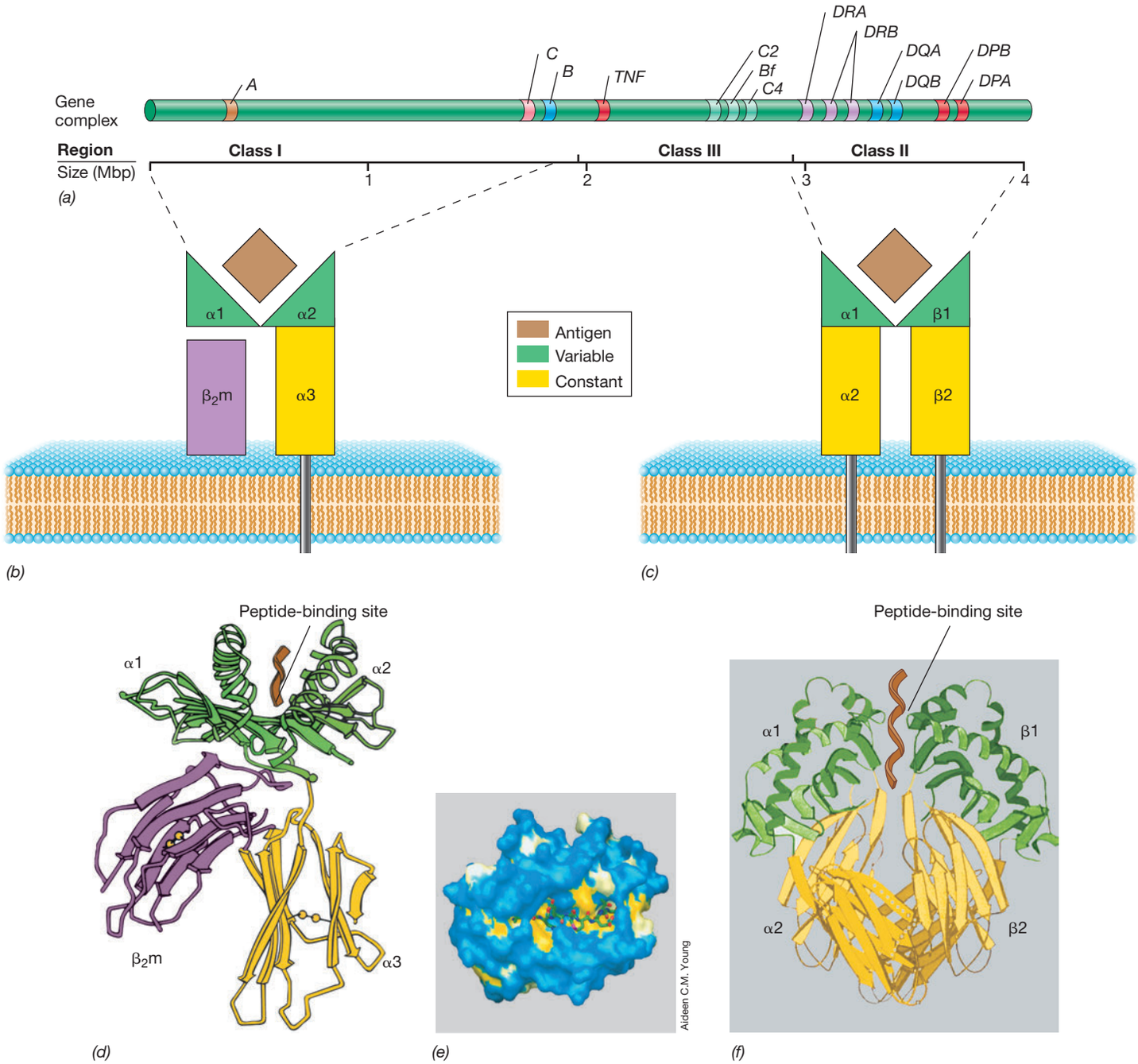


Figure 27.13 Human leukocyte antigen (HLA) genes and MHC proteins. *(a)* The HLA complex, located on human chromosome 6, contains more than 4 megabase pairs (Mbp). Class II genes *DPA* and *DPB* encode class II proteins DPα and DPβ; *DQA* and *DQB* encode DQα and DQβ; *DRA* and two *DRB* loci encode DRα and DRβ proteins. The class I MHC proteins HLA-A, HLA-C, and HLA-B are encoded by genes *A, C*, and *B*. The class II and class I loci are highly polymorphic and encode peptide-binding proteins. Class III MHC genes encode proteins associated with immune-related functions, such as complement proteins C4 and C2 and the cytokine TNF (tumor necrosis factor). *(b)* Schematic of MHC class I protein. The $\alpha 1$ and $\alpha 2$ domains interact to form the peptide antigen–binding site. *(c)* Schematic of MHC class II protein. The $\alpha 1$ and $\beta 1$ domains combine to form the peptide antigen–binding site. *(d)* MHC class I protein structure. Beta-2 microglobulin (β_2m) binds noncovalently to the α chain. The antigen peptide (brown) is bound cooperatively by the $\alpha 1$ and $\alpha 2$ domains. *(e)* An MHC I protein with a bound peptide, as seen from above. A peptide of nine amino acids is shown as a molecular backbone structure embedded in a space-filling model of a mouse MHC I protein. *(f)* Structure of MHC class II protein dimer. The antigen peptide (brown) is shown embedded in the peptide-binding site of the MHC II proteins.

Two distinct antigen-processing schemes are at work to initiate adaptive immune responses: one for MHC I antigen presentation and another for MHC II antigen presentation. MHC I proteins present peptide from viruses or other intracellular pathogens; such infected cells are called *target cells* (**Figure 27.14*a***). Proteins derived from infecting viruses, for example, are taken up and digested in the cytoplasm in a structure called the *proteasome*. Peptides about ten amino acids long are transported into the endoplasmic reticulum (ER) through a pore called the *transporter for antigen processing* (*TAP*). Once the foreign peptides have entered the ER they are bound by MHC I, and the MHC I–peptide complex moves to the cell surface where it integrates into the cell membrane. When the TCR on the surface of a Tc cell interacts with both the foreign peptide (recognized as "nonself") and the MHC I protein (recognized as "self") on the surface of the target cell, the Tc cell releases *perforin* and *granzyme*, cytotoxic proteins that kill the virus-infected target cell.

MHC II proteins are expressed on APCs, where they function to present peptide antigens from engulfed pathogens (Figure 27.14*b*). Similar to MHC I, MHC II proteins are assembled in the endoplasmic reticulum. In the ER, the MHC II peptide-binding groove is blocked by a protein called invariant chain (Ii) until the MHC II complex is situated in a lysosome (Figure 27.14*b*). After ingestion of a pathogen or a pathogen product by an APC, the phagosome containing the foreign antigen fuses with a lysosome to form a phagolysosome (◀ Section 26.7). Within the phagolysosome, enzymes digest both the foreign antigens and the Ii peptide but not MHC II. The foreign peptides are then loaded onto the newly freed MHC II peptide-binding groove, and the complex is inserted into the cell surface for presentation to T-helper cells. The latter, through their TCRs, recognize the MHC II–peptide complex and secrete cytokines that activate APCs, such as macrophages for enhanced phagocytosis or B cells for antibody production.

In addition to the TCR, each T cell expresses a unique cell surface protein that functions as a *coreceptor*. Tc cells express the **CD8 coreceptor**, and Th cells express the **CD4 coreceptor** (Figure 27.14). When the TCR binds to the peptide–MHC complex, the coreceptor on the T cell also binds to the MHC protein on the antigen-presenting cell, strengthening the molecular interactions between the cells and enhancing activation of the T cell. CD4 binds only to MHC II, strengthening Th cell interaction with APCs that express MHC II protein. Likewise, CD8 binds only to MHC I, enhancing the binding of Tc cells to MHC I–bearing target cells. In clinical medicine, the CD4 and CD8 proteins are used as T cell markers to differentiate Th (CD4) cells from Tc (CD8) cells in diagnostic tests, for example, in assessing the course of disease in an AIDS patient (▶ Section 31.15 and Figure 31.48).

We now consider the fact that human MHC proteins allow for some sequence variation from individual to individual and how this variation affects the immune response.

Check Your Understanding

- Identify the cells that display MHC class I and MHC class II proteins on their surfaces.
- Compare the MHC I and MHC II protein structures and peptide-binding sites. How do they differ? How are they similar?
- Define the sequence of events for processing and presenting antigens from both intracellular (endogenous) and extracellular (exogenous) pathogens.

27.6 MHC Polymorphism, Polygeny, and Peptide Binding

Although MHC class I and class II proteins theoretically can bind all possible antigen peptides for presentation to T cells, MHC proteins in different individuals of the same species are not identical. Different individuals typically have subtle differences in the amino acid sequence of homologous MHC proteins. These genetically encoded MHC variants, called *polymorphisms*, are the major immunological barriers for successful tissue transplantation from one individual to another.

Mastering Microbiology
Art Activity: Figure 27.14 Antigen presentation by MHC I and MHC II proteins

Polymorphism, Polygeny, and the Immune Barrier to Tissue Transplantation

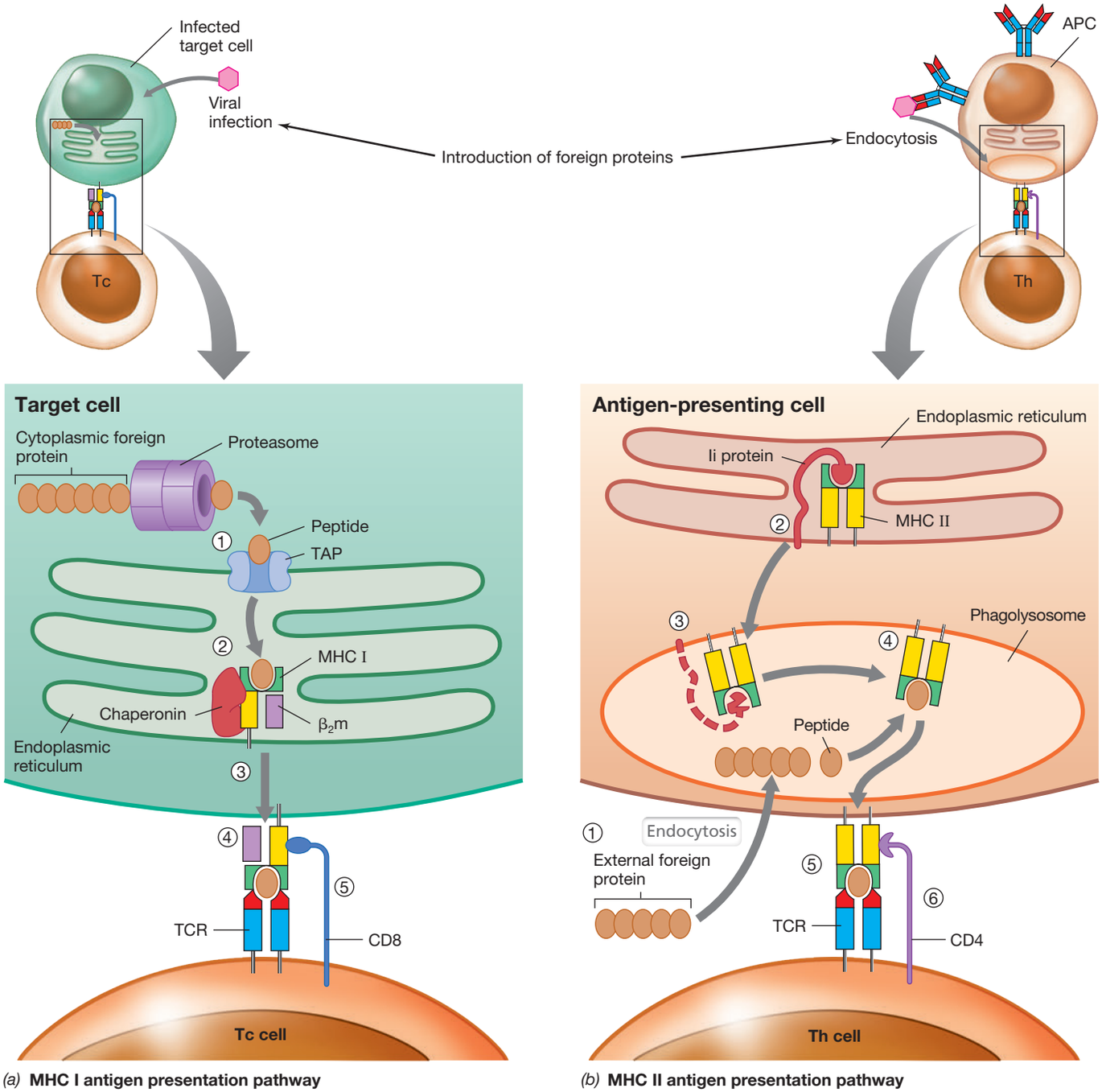
Polymorphism is the occurrence, within a population, of multiple alleles (alternate forms of a gene) at a specific *locus* (the location of the gene on the chromosome). For example, the MHC class I locus *HLA-A* (Figure 27.13*a*) has over 2000 known alleles, each of which encodes a distinct HLA-A protein that occurs within the human population. The genome of each person, however, contains only two of the *HLA-A* alleles; one allele is of paternal origin and one is of maternal origin. The two allelic protein products are expressed equally (**Figure 27.15*a***).

Additional MHC class I protein diversity is a result of **polygeny**, in which evolutionary gene duplication events have led to the occurrence of two additional genetically, structurally, and functionally related loci, *HLA-B* and *HLA-C*. These gene loci are also polymorphic, having more than 2600 and 1500 allelic variants, respectively. Thus, an individual typically displays six structurally distinct proteins derived from the three polymorphic class I loci (three products derived from maternal origin and three products derived from paternal origin) (Figure 27.15*b*). Likewise, highly polymorphic and equally expressed alleles encode MHC class II proteins at the *HLA-DR*, *HLA-DP*, and *HLA-DQ* alpha- and beta-chain loci (Figure 27.13*a*).

As a result of polymorphism and polygeny, most individuals have unique MHC profiles. Only closely related family members are likely to have all of the same MHC genes and proteins, and this property can be used to prove (or disprove) paternity and has also been used to trace ancestral lineages and pathways of early human migration (**Figure 27.16**). These highly polymorphic variations in MHC proteins are major barriers to successful tissue transplants because the MHC proteins on the donor tissue (graft) are recognized as foreign by the recipient's immune system. An immune response directed against the graft MHC proteins thus causes rejection and death of the graft tissue. However, matching MHC alleles between donors and recipients minimizes tissue graft rejection. Control of tissue rejection may also be accomplished by administering drugs that suppress the immune system.

Peptide Antigen Binding

Most of the allelic variations in MHC proteins occur as amino acid changes concentrated in the peptide-binding groove (Figure 27.13*d–f*), and each polymorphic variation of the MHC protein binds a different set of peptide antigens. The peptides bound by a single MHC protein share a common structural pattern—a peptide **motif**—and each different MHC protein binds a different motif. For example, for a certain MHC class I protein, the bound peptides contain eight



(a) **MHC I antigen presentation pathway** *(b)* **MHC II antigen presentation pathway**

Figure 27.14 Antigen presentation by MHC I and MHC II proteins. *(a)* ① Protein antigens, such as virus components manufactured within the cell, are degraded by the proteasome in the cytoplasm. The peptide fragments are transported into the endoplasmic reticulum (ER) through a pore formed by the TAP proteins. ② MHC I proteins in the ER are stabilized by chaperonins until peptide fragments are bound. ③ When peptide fragments are bound by MHC I, the complex is transported to the cell surface. ④ The MHC I–peptide complex interacts with T cell receptors (TCRs) on the surface of Tc cells. ⑤ The CD8 coreceptor on the Tc cell engages MHC I, resulting in a stronger complex. The Tc cell is activated by the binding events, causing it to release cytokines and cytolytic toxins and kill the target cell. *(b)* ① External foreign proteins are imported into the cell and digested into peptide fragments in phagolysosomes. ② MHC II proteins in the ER are assembled with Ii, a blocking protein that prevents MHC II from binding with peptides in the ER. ③ The MHC II–Ii assembly is transported to the lysosome, where it remains until the lysosome fuses with the phagosome, forming a phagolysosome where Ii is degraded, ④ freeing the MHC II protein to bind the foreign peptide fragments. ⑤ The MHC II–peptide complex is transported to the cell surface, where it interacts with TCRs and ⑥ the CD4 coreceptor on Th cells. The Th cells then release cytokines that interact with other cells to promote an immune response. Note that the APC in part *b* may be either a B cell, which ingests antigen by endocytosis (shown), or a macrophage or dendritic cell, which engulf antigens through phagocytosis.

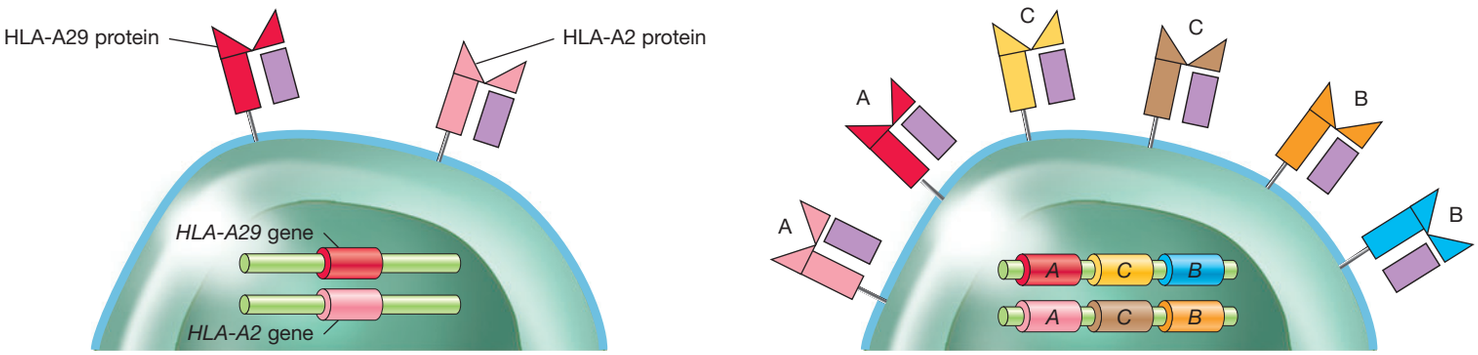


Figure 27.15 Polymorphism and polygeny in MHC genes and proteins. *(a)* Polymorphism in *HLA-A* loci results in equal expression of proteins encoded by both alleles. There are over 2000 HLA-A alleles in the human population, but only two (one at each locus) are found in each individual. *HLA-B* and *HLA-C* exhibit similar levels of polymorphism. *(b)* Polygeny in MHC results in duplicated polymorphic *HLA-A, HLA-B*, and *HLA-C* genes that potentially encode three pairs of different MHC proteins. The colors represent alternate alleles of each gene and their respective protein.

amino acids with a phenylalanine (F) at position 5 and a leucine (L) at position 8 (◀ Figure 6.27 for the structures of amino acids). All other positions in the peptide can be occupied by any amino acid (designated by an X). Thus, all peptides sharing the sequence X-X-X-X-**F**-X-X-**L** would bind to that MHC protein. Another MHC class I protein encoded by a different MHC allele binds a peptide motif of nine amino acids with a tyrosine (Y) at position 2 and an isoleucine (I) at position 9 (X-**Y**-X-X-X-X-X-X-**I**).

The invariant amino acids in each motif are called *anchor residues*—they bind directly and specifically within an individual MHC peptide-binding groove. Thus, an individual MHC protein can bind and present many different peptide antigens if the peptides contain the same anchor residues. Because each MHC protein binds a different motif with different anchor residues, the six possible MHC I proteins encoded in an individual's genome bind six different motifs. In this way, each individual can present a large number of different peptide antigens using the limited number of MHC I molecules available. MHC II proteins bind peptides in a similar manner.

Because of polymorphisms and polygeny within the human species, at least a few peptide antigens from virtually any pathogen will display a motif that can be bound and presented by the MHC proteins. This system of generating antigen-binding diversity is therefore quite different from the genetic mechanisms used to synthesize Igs (Section 27.4) and TCRs (Section 27.7), in which each receptor interacts specifically with only a *single* antigen or antigenic determinant.

Figure 27.16 Polymorphic *HLA* genes and tracing ancestral lineages. The image shows what a Neanderthal man might have looked like. By analyzing the distribution of specific *HLA* alleles within different populations, researchers are increasingly able to reconstruct migration and interbreeding patterns of ancient humans and other hominids, including as far back as the Neanderthal era. The intermixing of *HLA* genes between disparate ancient populations increased genetic diversity and likely enhanced survival by helping to strengthen and shape the modern immune response.

Check Your Understanding

- Define polymorphism and polygeny as they apply to MHC genes.
- How does a single MHC protein present many different peptides to T cells?

UNIT 6

IV • T Cells and Their Receptors

There are many different classes of T cells, and each class carries out specific functions. Using their T cell receptors, T cells of various types interact with antigens presented on MHCs to initiate and implement an adaptive immune response to pathogen invasion.

Adaptive immunity is ultimately initiated by the recognition of foreign peptides on infected cells by T lymphocytes. The infected cells that are first recognized by T cells may include the same phagocytes that participate in the innate immune response (Chapter 26). Antigen presentation activates *naive* (uncommitted) T lymphocytes to differentiate into effector T cells that carry

out cell-mediated immunity. In the absence of antigen-activated T cells, there is little antigen-specific immunity and no immune memory.

27.7 T Cell Receptors: Proteins, Genes, and Diversity

APCs ingest bacteria, viruses, and other antigenic material by phagocytosis (in the case of macrophages and dendritic cells) or through internalization of a molecular antigen bound to a BCR. Phagosomal or cytoplasmic antigens are then digested, complexed with MHC proteins, and moved to the cell surface for antigen presentation to T cells. The TCRs of T cells can recognize (bind) antigens only when the peptides are complexed with MHC proteins on host cell surfaces. For example, a macrophage or dendritic cell that has encountered a virus will display MHC I and MHC II proteins embedded with viral peptides (**Figure 27.17**). These viral peptide–MHC complexes are the sites for T cell activation and interaction.

Mastering Microbiology
Art Activity: Figure 27.17 T cell immunity

Each T cell expresses a TCR that is specific for a single peptide–MHC complex. Antigen-specific T cells are found closely associated with APCs in the spleen, lymph nodes, and MALT. T cells constantly sample surrounding APCs for peptide–MHC complexes. Peptide–MHC complexes that interact with the TCR signal the T cell to grow and divide, producing antigen-reactive clones that coordinate cell-mediated killing, induce inflammation, and activate antibody-producing B cells. Here we examine how the TCR interacts with antigens presented on an APC or an infected target cell.

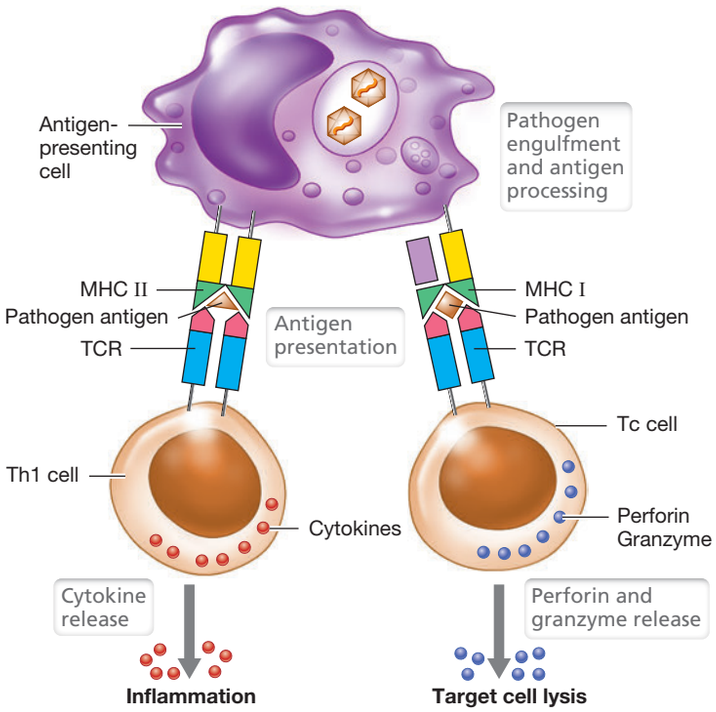


Figure 27.17 T cell–mediated immunity. Antigen-presenting cells, such as the phagocytes in innate immunity, ingest, degrade, and process antigens. They then present antigens to T cells that secrete protein cytokines that activate the adaptive immune response. T-helper 1 (Th1) cells produce cytokines that activate other cells and induce inflammation. T-cytotoxic (Tc) cells produce perforin and granzyme, proteins that destroy nearby target cells.

TCR Structure and Diversity

The TCR is a membrane-spanning protein that extends from the T cell surface into the extracellular environment. Similar to B cells with their BCRs, each T cell has thousands of copies of its specific TCR on its surface. A functional TCR consists of an α and a β polypeptide chain, and like Igs, each TCR chain has a variable (V) domain and a constant (C) domain (**Figure 27.18**). The V_α and V_β domains interact cooperatively to form an antigen-binding site that contains complementarity-determining region (CDR1, CDR2, and CDR3) segments. However, unlike Igs, which can bind antigens in a native conformation, *TCRs recognize only peptides bound to self MHC*; other antigens, such as complex polysaccharides, are not recognized by TCRs.

The three-dimensional structure of the TCR bound to MHC I–peptide is shown in Figure 27.18*a*. Both TCR and MHC proteins bind directly to peptide antigen. The MHC protein binds one face of the peptide—the MHC motif—and the TCR binds the other peptide face—the T cell epitope (Figure 27.18*b*). The CDR regions of the TCR bind directly to the MHC–peptide complex, and each CDR has a specific binding function. The CDR3 regions of the TCR α and β chains bind the antigen epitope; the CDR1 and CDR2 regions of the TCR α and β chains bind mainly to the MHC protein.

The adaptive immune response can generate TCR diversity sufficient to bind nearly every possible peptide antigen generated by APCs or target cells. T cells generate receptor diversity in ways similar to the generation of Ig diversity in B cells, and Table 27.3 compares the receptor diversity–generating mechanisms for each cell type. Analogous to the H and L chains of Igs, the TCR α and β chains are encoded by distinct constant- and variable-domain gene segments. TCR V-region gene segments are grouped together sequentially; those encoding the α chain include 80 V and 61 J segments, whereas those encoding the β chain include 52 V, 2 D, and 13 J gene segments (**Figure 27.19**). As we have seen for Igs (Section 27.4), antigen-binding diversity in TCRs is generated by somatic recombination, imprecise V-J (α-chain) or V-D-J (β-chain) joining, and random reassortment. Additional TCR diversity is generated because the D region of the β chain can be transcribed in all three reading frames (◀ Section 6.9 and Figure 6.34), leading to production of three separate transcripts from each D-region gene segment.

Similar to the assembly of Ig H and L chains, individual α and β chains are produced by each T cell at random and assembled to form the α:β heterodimer. The somatic hypermutation mechanisms responsible for increased receptor diversity in Ig genes do not operate in T cells, and thus additional TCR diversity from these events is not possible. Despite this, potential TCR diversity is still enormous; an estimated 10^{15} different TCRs can be generated! It is important to note that not all TCR interactions with MHC–peptide result in activation of the T cell. As we now consider, many T cells that escape deletion in the thymus never receive the full set of activation signals necessary to become effector cells.

T Cell Activation and Anergy

Like B cells, T cells require a second signal for activation in addition to interactions with MHC–peptide. As we described in Section 27.1, T cells that react strongly with self antigens are deleted in the thymus to prevent autoimmunity. However, many self antigens are not

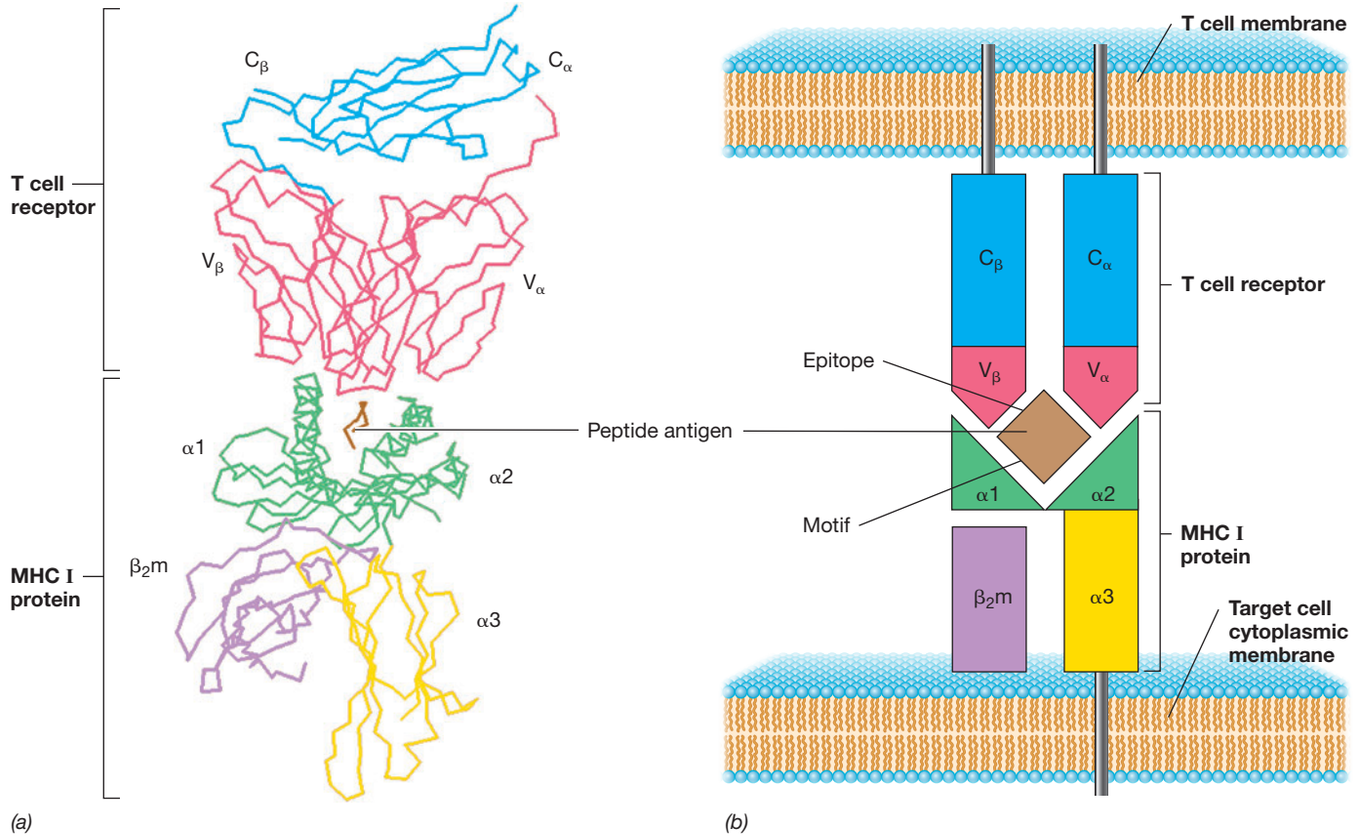


Figure 27.18 **The TCR:MHC I–peptide complex.** *(a)* A three-dimensional structure showing the orientation of TCR, peptide (brown), and MHC. This structure was derived from data deposited in the Protein Data Bank. *(b)* A diagram of the TCR:MHC–peptide structure. Note that the peptide is bound by both MHC and TCR proteins and has a distinct surface structure that interacts with each (a motif interacts with the MHC and an epitope with the TCR, as discussed in the text).

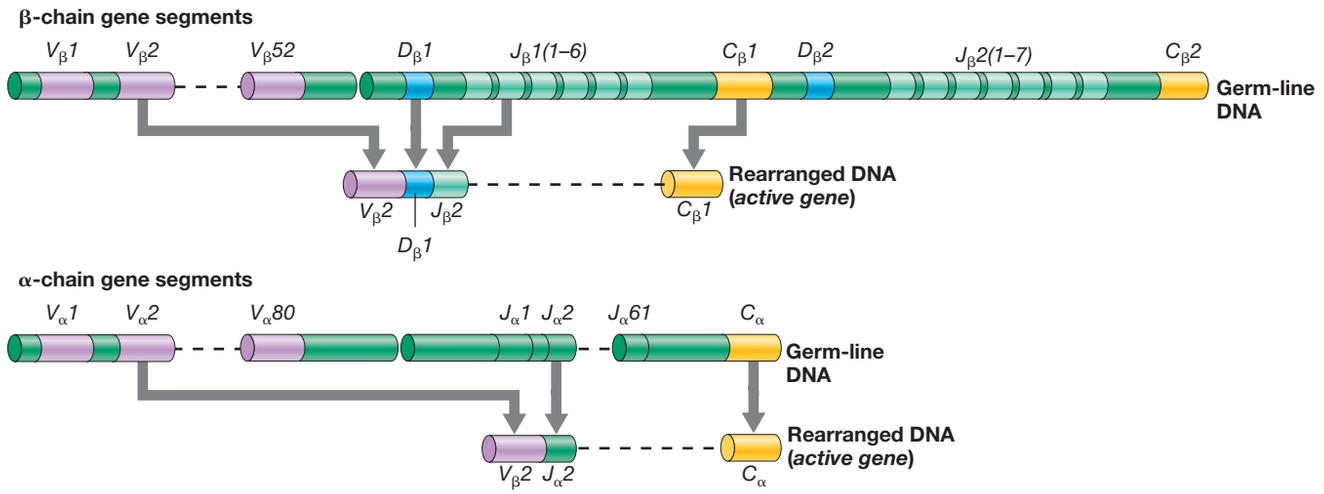


Figure 27.19 **Organization of the human TCR α- and β-chain genes.** The α-chain gene segments are located on chromosome 14, and the β-chain gene segments are on chromosome 6. Compare this figure with Figure 27.12.

expressed in the thymus, meaning that many T cell clones responsive to nonthymus self antigens avoid deletion. Yet, because of the requirement of a second signal for activation, these self-reactive T cells remain unresponsive and become anergic after they leave the thymus.

T cells that migrate from the thymus to secondary lymphoid organs and have not yet encountered specific antigen are naive and must be activated to become competent effector cells. For example, the first requirement for activation of a naive Tc cell is TCR binding of an MHC–peptide complex presented by an APC (**Figure 27.20*a***). The next step requires the interaction of two more proteins: B7, found on the APC, and CD28, expressed on the surface of the Tc cell. The binding of B7 to CD28 is the second signal necessary to activate the Tc cell and make it an effector cell; without the B7–CD28 interaction, the naive Tc cell is not activated (Figure 27.20). Once a T cell is activated, only the first signal (TCR binding to MHC–peptide) is required to induce an effector T cell. Thus, the activated (effector) Tc cell in Figure 27.20*b* can kill any target cell presenting the recognized peptide antigen on its MHC I, even those cells that do not display B7.

Mastering Microbiology
Art Activity: Figure 27.20 Immunoglobulin gene superfamily proteins

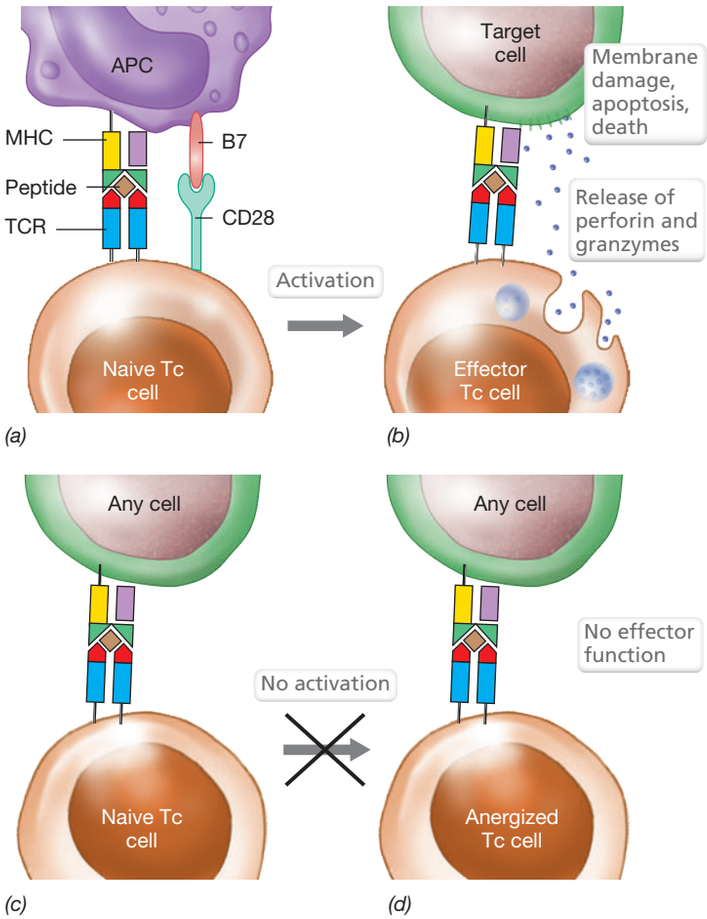


Figure 27.20 T cell activation. *(a)* A naive Tc cell interacts through its TCR with an MHC–peptide presented by an APC, the first required activation signal. In addition, CD28 on the Tc cell interacts with B7 on the APC, the second signal required to activate the naive T cell. *(b)* The activated Tc cell can kill any target cell bearing the same MHC–peptide. *(c)* A naive Tc cell interacts through its TCR with an MHC–peptide presented by any cell. Conditions for the first signal are met, but the second signal is not generated because only APCs display the B7 protein. *(d)* In the absence of the second signal, the Tc cell becomes permanently unresponsive, or *anergized*.

The requirement for a second activation signal has major implications for establishing and maintaining clonal anergy. A naive Tc cell that interacts with a self antigen on a cell that is not an APC will receive only an MHC–peptide signal because non-APCs do not express B7. In the absence of the B7–CD28 interaction, a T cell that engages MHC–peptide is permanently anergized and can never be activated (Figure 27.20*c* and *d*), thus providing protection against harmful autoimmune reactions.

Structural Similarities of Antigen-Binding Proteins

As we have seen, antigen-binding proteins of the adaptive immune response have common structural features. Ig, TCR, and MHC protein complexes all consist of two nonidentical polypeptides: MHC and TCR proteins are composed of α and β polypeptide chains, and Igs have separate heavy and light chains (Section 27.4). Therefore, these protein complexes are believed to have arisen from duplication and selection of genes encoding primordial antigen receptors. Because of their shared structural, evolutionary, and functional features, genes encoding Ig, TCR, and MHC proteins are part of an extended gene family called the **immunoglobulin gene superfamily**, and a comparison of Ig superfamily proteins is shown in **Figure 27.21**.

The constant (C) domain of each protein in the superfamily has a highly conserved amino acid sequence consisting of about 100 amino acids with an intrachain disulfide bond spanning 50–70 amino acids. C domains provide structural integrity for the antigen-binding molecules, anchor the antigen-binding V domains to the cytoplasmic membrane, and give each protein its characteristic shape. C domains can also provide recognition sites for accessory molecules. For example, C domains of most IgG and all IgM proteins are bound by the C1q component of complement, a critical first step in initiating the classical complement activation sequence (◀ Section 26.9). Likewise, MHC I C domains bind to the accessory CD8 protein on Tc cells, and homologous MHC II C domains bind CD4 on Th cells. As we have discussed, such interactions are critical steps for T cell activation and initiation of adaptive immunity (Section 27.5).

The variable (V) domains of TCR and Ig molecules are about the same length as the C domains, but the structures of V domains can vary considerably from one another and from C domains. As we have seen, Ig and TCR V domains interact specifically with a nearly limitless variety of antigens, whereas the V domains of MHC proteins, which have likely evolved independently of Ig and TCR V domains, interact with foreign peptides that share a common motif (Section 27.6).

Although all T cells produce and express antigen-binding TCRs, T cells are not all functionally equivalent. As we will see next, there are several different subsets of T cells, each with their own specific functions in the adaptive immune response.

Check Your Understanding

- Identify diversity-generating mechanisms unique to TCRs as compared to diversity-generating mechanisms in Igs.
- What does it mean when a T cell becomes "anergized," and how does this process relate to the prevention of autoimmunity?
- Describe and compare the structural features of Ig gene superfamily constant and variable domains.

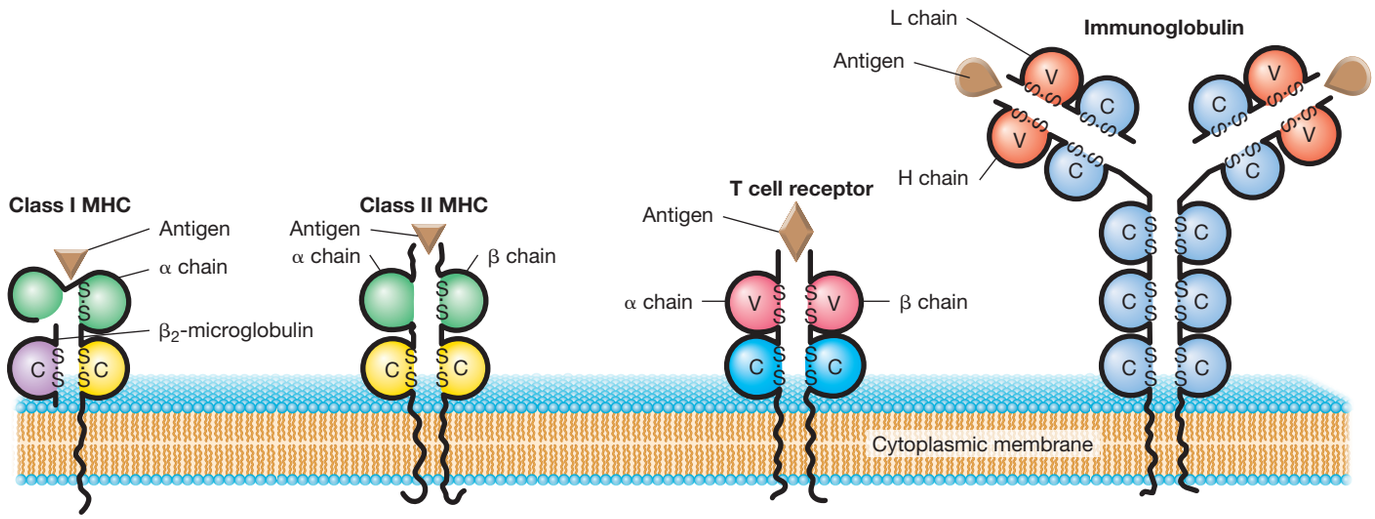


Figure 27.21 Immunoglobulin gene superfamily proteins. Constant (C) domains have homologous amino acid sequences and higher-order structures. The Ig-like C domains in each protein chain indicate evolutionary relationships that identify the proteins as members of the Ig gene superfamily. The variable (V) domains of Igs and TCRs are also Ig-like, but the peptide-binding domains of MHC class I and class II proteins (green) are not because their structures vary considerably from the basic features of the Ig domain.

27.8 T Cell Subsets and Their Functions

Antigen-reactive T cells consist of multiple T cell subsets having different functional properties. As we have discussed, **T-cytotoxic (Tc) cells**, also called *cytotoxic T lymphocytes* (*CTLs*), are CD8 T cells (Section 27.5) that recognize the peptide–MHC I complex on an infected cell. By contrast, **T-helper (Th) cells**, which can differentiate into several more specialized Th cell subsets, are CD4 T cells that interact with peptide–MHC II complexes on the surface of APCs and function to activate macrophages and stimulate antibody-mediated immunity.

T-Cytotoxic Cells

When an effector Tc cell (a CTL) recognizes a foreign peptide on an infected cell, it kills the peptide-bearing target cell. For example, a viral peptide embedded in MHC I, displayed on a virus-infected cell, marks the cell for interaction and killing by a CTL whose TCRs recognize the viral antigen. Contact between a CTL and the target cell is required to initiate killing of the infected cell (**Figure 27.22*a***). The point of initial contact is between the TCR and the peptide–MHC I complex. The CD8 protein on the CTL then binds the MHC I protein, strengthening the interaction and establishing an *immunological synapse* between the two cells. In addition to engaging and destroying virus-infected host cells, CTLs also have the ability to identify and kill cancer cells that present abnormal peptides (Figure 27.22*b*).

Upon contact with the target cell, granules in the CTL migrate to the contact site, where the contents of the granules—cytolytic proteins called *perforin* and *granzyme* (Section 27.5)—are released into the synapse (Figure 27.22). Perforin molecules enter the membrane of the target cell and combine to form a transmembrane pore through which granzyme molecules then enter the target cell. Granzymes are cytotoxins that induce *apoptosis* (programmed cell death), characterized by organized killing and degradation of the target cell from within. The released perforin and granzyme molecules affect only the target cell because degranulation within the synapse is confined to the contact surface between the CTL and the target cell bearing peptide–MHC I. Cells lacking the peptide recognized by the CTL do not make contact and are not killed.

Different Classes of T-Helper Cells

Interactions with APCs drive naive CD4 Th cells to differentiate into one of several Th subsets, each producing unique combinations of cytokines that direct the immune response (**Table 27.4**). Dendritic cells and, to a lesser extent, macrophages (◀ Section 26.4) play a central role as APCs in the activation of Th cells. A naive

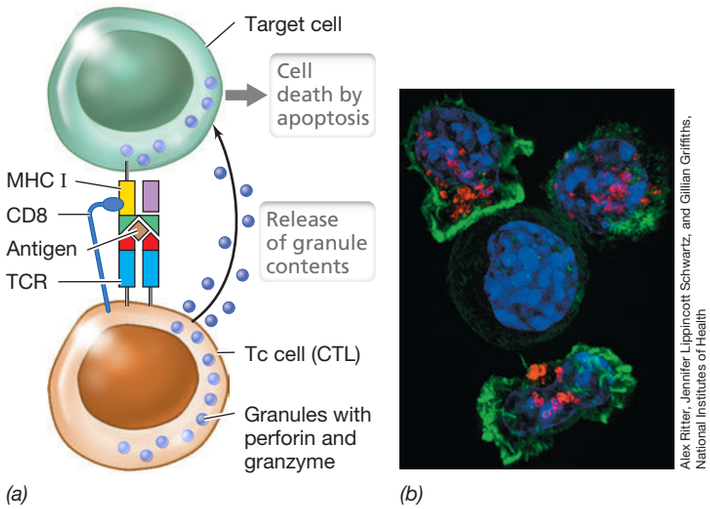


Figure 27.22 T-cytotoxic cells. *(a)* When the TCR on a cytotoxic T lymphocyte (CTL) binds MHC I–peptide complexes on any cell, degranulation of the CTL releases molecules of perforin and granzyme, cytotoxins that perforate the target cell and cause apoptosis, respectively. *(b)* Three CTLs containing granules of perforin and granzyme (red) surround a cancer cell (blue, center). The CTL on the top left is in the process of flattening its membrane surface (green) against the cancer cell to form the immunological synapse.

TABLE 27.4 T-helper cell subsets

Characteristic	Th1	Th2	Th17	Treg
Antigen-presenting cell	Macrophage	B cell	Activated dendritic cell	Nonactivated dendritic cell
Major cytokines produced	IL-2, IFN-γ, TNF-α	IL-4, IL-5	IL-17, IL-6	IL-10, TGF-β
Cellular effects	Activation of T cells (IL-2) and macrophages	Activation of B cells	Activation and recruitment of neutrophils	Suppression of adaptive immune cells
Systemic effects	Cell-mediated immunity	Antibody-mediated immunity	Amplification of innate immunity	Control of Th immunity

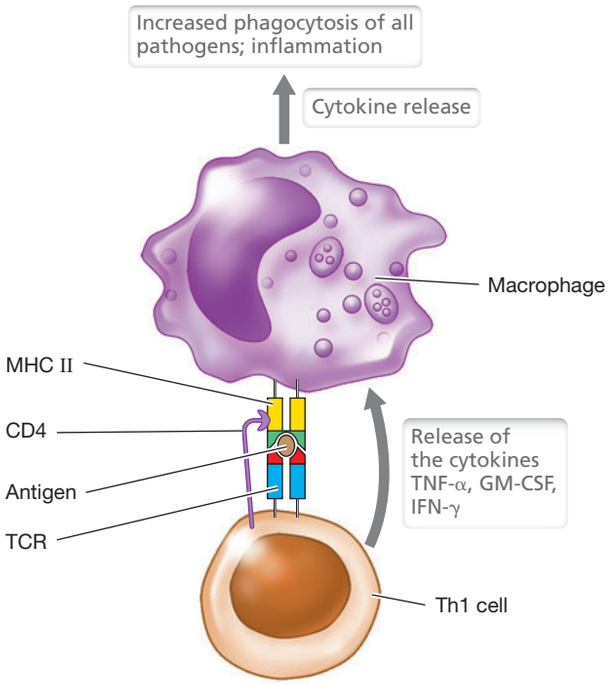
Th cell that recognizes peptide–MHC II through its TCR and also receives the costimulatory B7–CD28 second signal (Section 27.7) is further stimulated by the release of cytokines from the APC. This causes the Th cell to proliferate and differentiate into long-lived memory cells and effector Th cells that enter the circulation and migrate to sites of infection. The population of effector Th cells is comprised of different Th cell subsets, which may include *Th1*, *Th2*, and *Th17* cells.

Th1 cells produce IL-2, a cytokine that promotes growth and activation of other T cells, and also activate macrophages through the cytokines interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and granulocyte–monocyte colony-stimulating factor (GM-CSF) (**Figure 27.23*a***). Th1-activated macrophages take up and kill foreign cells more efficiently than nonactivated (resting) macrophages (Figure 27.23*b*), and they may also kill tumor cells because activated macrophages recognize as nonself the tumor-specific antigens expressed by cancer cells.

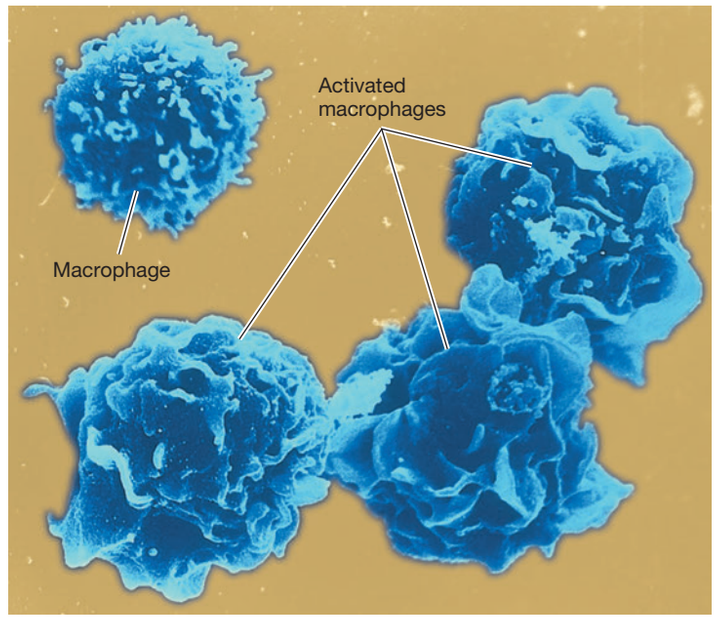
Mastering Microbiology
Art Activity: Figure 27.22 Th1 cells and macrophage activation

Activated macrophages also produce several cytokines and chemokines that function as proinflammatory mediators and chemoattractants, respectively. **Table 27.5** summarizes the source and activities of these and other important immune cytokines and chemokines. In the case of organ or tissue transplants, Th1-activated macrophages can actually harm the host if the host's Th1 cells recognize nonself MHC proteins on the transplant and trigger macrophage activation and transplant destruction (Section 27.6).

Th2 cells play a pivotal role in B cell activation and antibody production. As we have discussed (Section 27.3), B cells make antibodies, and differentiated B cells are coated with antibodies (BCRs)



(a)



(b)

Figure 27.23 Th1 cells and macrophage activation. *(a)* Th1 cells are activated by antigens presented on macrophages in the context of MHC II protein. Activated Th1 cells produce cytokines that activate macrophages, leading to increased phagocyte activity and inflammation. *(b)* Activated macrophages are generally larger than resting macrophages and have a ruffled surface, often with cytoplasmic extensions that "feel" for pathogens. In addition to carrying out more aggressive phagocytosis, activated macrophages express genes that encode bactericidal enzymes found in lysosomes, such as proteases and enzymes that produce reactive oxygen species, all of which are designed to quickly kill ingested microorganisms inside the phagolysosome (◀ Figure 26.11).

TABLE 27.5 Major immune cytokines and chemokines

Cytokine (chemokine)	Major producer cells	Major target cells	Major effect
IL-4[a]	Th2	B cells	Activation, proliferation, differentiation, IgG1 and IgE synthesis
IL-5	Th2	B cells	Activation, proliferation, differentiation, IgA synthesis
IL-2	Naive T cells, Th1, and Tc	T cells	Proliferation (often autocrine)
IFN-γ[b]	Th1	Macrophages	Activation
GM-CSF[c]	Th1	Macrophages	Growth and differentiation
TNF-α[d]	Th1	Macrophages	Activation, production of proinflammatory cytokines
	Macrophages	Vascular epithelium	Activation, inflammation
IL-1β	Macrophages	Vascular epithelium, lymphocytes	Activation, inflammation
IL-6	Macrophages, dendritic cells	Lymphocytes	Activation
IL-12	Macrophages, endothelial cells	NK cells, naive T cells	Activation, enhances differentiation to Th1
IL-17	Th17	Neutrophils	Activation
CXCL8 (chemokine)	Macrophages	Neutrophils, basophils, T cells	Chemotactic factor
CCL2 (MCP-1[e]) (chemokine)	Macrophages	Macrophages, T cells	Chemotactic factor, activator

[a]IL, interleukin; [b]IFN, interferon; [c]GM-CSF, granulocyte–monocyte colony-stimulating factor; [d]TNF, tumor necrosis factor; [e]MCP, macrophage chemoattractant protein.

that function as antigen receptors. When antigen binds the BCR (Figure 27.6), the antibody-bound antigen is taken into the B cell by endocytosis and degraded. Peptides from the degraded antigen are then loaded into the B cell's MHC II protein for presentation to a Th2 cell. The Th2 cell responds by producing IL-4 and IL-5, cytokines that activate the B cell (Table 27.5) and cause it to proliferate and differentiate into plasma cells that produce and secrete antibodies specific to the presented antigen (Section 27.3).

Th17 cells are important in the first stages of the immune response. Naive Th cells differentiate into Th17 cells primarily through the activity of dendritic cells. When dendritic cells encounter pathogens, they present antigen and secrete IL-6 and transforming growth factor-β (TGF-β), cytokines that catalyze differentiation of naive Th cells to Th17 cells (**Figure 27.24*a***). Th17 cells then produce IL-17, a cytokine that activates other tissue cells to produce cytokines and chemokines that attract proinflammatory neutrophils to the site of infection (Table 27.5). Thus, the function of Th17 cells is to produce IL-17, starting a cascade that draws neutrophils to infection sites. By recruiting neutrophils, Th17 cells amplify innate immunity triggered by interaction of a pathogen with the dendritic cell.

We conclude our consideration of T cells with a description of the **T regulatory (Treg) cell**, a Th cell subset that plays a critical role in *controlling* the immune response. Undifferentiated Th cells remain naive unless they are stimulated to mature and differentiate by certain cytokines, as is the case for IL-6 stimulation to produce Th17 cells. However, in the absence of a pathogen, Th cells can still interact with dendritic cells through MHC II–peptide–TCR (Figure 27.24*b*). In this case, the peptide is usually a self peptide, and an immune response to it could trigger autoimmunity. However, because the dendritic cells did not interact with a pathogen, they cannot produce IL-6 to promote Th17 differentiation. Instead, the absence of IL-6 pushes differentiation to Treg cells that make IL-10 and TGF-β, two cytokines that suppress immunity and inflammation. In the presence of self antigens and in the absence of IL-6, Treg cells shut down the immune response and inhibit inflammation. This is important for controlling immune responses to self antigen and preventing autoimmunity. In this regard, Treg cells play a key role in the maintenance of a healthy gut by interacting with short-chain fatty acids produced by the normal colonic microbiota to suppress host inflammatory responses against these "friendly" bacteria (◀ Section 24.9 and Figure 24.23).

Mastering Microbiology
Art Activity: Figure 27.23*a* Th17 and Treg cells

Check Your Understanding

- Describe the mechanism used by Tc cells to recognize infected host cells.
- Describe the effector system (the cell-killing mechanism) used by Tc cells.
- Compare and contrast the roles and activities of the different Th cells. What cytokines do they produce, and which effector cells do these cytokines act upon?

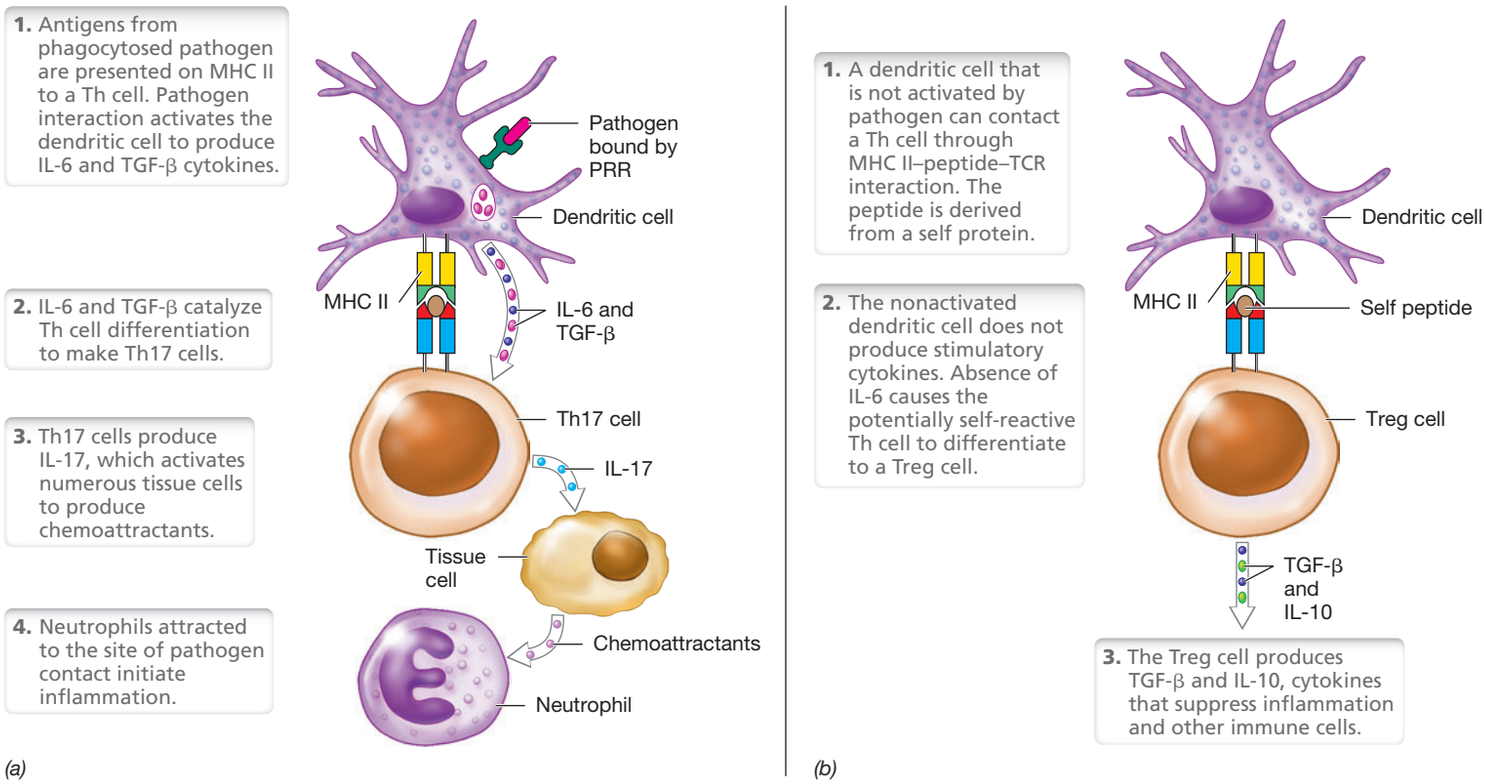


Figure 27.24 Th17 and Treg cells. *(a)* Th17 cells interact with pathogen-stimulated dendritic cells to draw neutrophils to the site of pathogen invasion, leading to inflammation and pathogen control. PRR, pattern recognition receptor. *(b)* Treg cells interact with nonactivated dendritic cells and respond by making immunosuppressive cytokines that control reactions to self antigens.

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CHAPTER REVIEW

I • Principles of Adaptive Immunity

27.1 The adaptive immune response is characterized by *specificity* for the antigen, the ability to respond more vigorously when reexposed to the same antigen (*memory*), and the acquired inability to interact with self antigens (*tolerance*). Tolerance in lymphocytes is acquired through a selection process. Immature T cells that do not interact with MHC–peptide (positive selection) or that react strongly with self antigens (negative selection) are eliminated by clonal deletion in the thymus. T cells that survive positive and negative selection leave the thymus and can participate in the immune response. Lymphocyte reactivity to self antigens is controlled through clonal deletion and anergy.

Q Why is it necessary for immature T lymphocytes to undergo a two-step selection process through which antigen-reactive cells are first selected followed by the elimination of cells that react strongly with self-antigens?

27.2 Immunogens are foreign macromolecules that induce an immune response. Immunogens initiate an immune response when introduced into a host. Antigens are molecules recognized (bound) by antibodies or TCRs. Adaptive immunity develops naturally and actively through immune responses to infections, or naturally and passively through antibody transfer from the placenta or breast milk. Artificial passive immunity occurs when antibodies or immune cells are transferred from an immune individual to a nonimmune individual. Immunization induces artificial active immunity and is widely used to prevent infectious diseases.

Q Immunity can be of many types: natural active immunity, natural passive immunity, artificial active immunity, and artificial passive immunity. Which of these forms of immunity confer immune memory?

II • Antibodies

27.3 Antibody production is initiated when an antigen contacts an antigen-specific B cell. Whereas B cell activation from T-independent antigens requires no T cell help, B cells that bind T-dependent antigens must process the antigen and present it to an antigen-specific Th cell. The Th cell becomes activated, producing cytokines that signal the B cell to clonally

expand and differentiate to produce soluble, antigen-specific antibodies. Each antibody (immunoglobulin) protein consists of two heavy and two light chains. The antigen-binding site is formed by the interaction of the variable regions of one heavy and one light chain. Each antibody class has different structural characteristics, expression patterns, and functional roles. Clones of activated B and T cells can live for years as memory cells and can rapidly expand and differentiate to produce high titers of antibodies after reexposure to antigen.

Q Describe the structural and functional differences among the five major classes of antibodies. What cellular and molecular interactions occur to initiate production of antibodies?

27.4 The antigen-binding site of an immunoglobulin (Ig) is composed of the V (variable) domains of one heavy chain and one light chain. Each V region contains three complementarity-determining regions, or CDRs, that are folded together to form the antigen-binding site. Ig diversity is generated by several mechanisms. Somatic recombination of gene segments allows shuffling of the various Ig gene segments. Random reassortment of the heavy- and light-chain genes, imprecise joining of VDJ and VJ gene segments, and hypermutation mechanisms contribute to nearly unlimited Ig diversity.

Q Which Ig chains are used to construct a complete antigen-binding site? Which domains? Which CDRs? Calculate the total number of germ-line-encoded V_H and V_L domains that can be constructed from the available Ig genes.

III • The Major Histocompatibility Complex (MHC)

27.5 T cells, with their TCRs, bind peptide antigens presented by MHC proteins on infected cells or APCs. Class I MHC proteins are expressed on all nucleated cells and present endogenous antigenic peptides to TCRs on Tc cells. Class II MHC proteins are expressed only on APCs. They function to present exogenously derived peptide antigens to TCRs on Th cells. These interactions activate T cells to kill antigen-bearing cells or to promote inflammation or antibody production.

Q Describe the basic structure of class I and class II major histocompatibility complex (MHC) proteins. In what functional ways do they differ?

27.6 Class I and class II MHC genes are highly polymorphic, and the many allelic variations challenge successful tissue transplantation. Different alleles of MHC class I and class II genes encode proteins that bind and present different peptide subsets, each characterized by a specific structural motif.

Q For the MHC class I polymorphisms, how many different MHC proteins are expressed in an individual? How many by the entire human population?

IV • T Cells and Their Receptors

27.7 T cell receptors bind to peptide antigens presented by MHC proteins. The CDR3 regions of both the α chain and the β chain bind to the antigen epitope; the CDR1 and CDR2 regions bind to the MHC protein. VDJ gene segments encode the β-chain V domain of TCRs, and VJ gene segments encode the α-chain V domain. TCR diversity, generated by a variety of mechanisms, is nearly unlimited. The immunoglobulin gene superfamily encodes proteins that are evolutionarily, structurally, and functionally related to immunoglobulins. The antigen-binding Igs, TCRs, and MHC proteins are members of this superfamily.

Q What diversity-generating mechanisms function to produce the nearly unlimited variety of antigen-specific TCRs? What structural and functional features are common to proteins classified within the immunoglobulin gene superfamily?

27.8 T-cytotoxic (Tc) cells recognize antigens on virus-infected host cells and tumor cells through antigen-specific TCRs, and recognition triggers killing via perforin and granzyme. T-helper (Th) cells differentiate into several subsets. Through the action of cytokines, Th1 inflammatory cells activate macrophage effector cells, whereas Th2 cells activate B cells. Th17 cells are activated by pathogen-activated dendritic cells and secrete IL-17 to recruit neutrophils to the site of infection. Treg cells produce cytokines that suppress adaptive immunity.

Q What mechanism do Tc cells use to identify and destroy infected cells in the body? How do Th cells differ from Tc cells, and how do the different subsets of Th cells differ from each other?

APPLICATION QUESTIONS

1. Antibodies of the IgA class are probably more prevalent than those of the IgG class. Explain why this might be the case. What benefits might this have for the host?
2. Although genetic recombination events are important for generating significant diversity in the antigen-binding site of Igs, post-recombination somatic events may be even more important in achieving overall Ig diversity. Do you agree or disagree with this statement? Explain.
3. Polymorphism implies that each different MHC protein binds a different peptide motif. However, for the MHC class I proteins, only 6 peptide motifs can be recognized in an individual, whereas over 6000 motifs can be recognized by the entire human population. What advantage does recognition of multiple motifs have for the individual? What potential advantage does recognition of the extremely large number of motifs have for the population? Can everyone process and present the same antigens?

CHAPTER GLOSSARY

Antigen a molecule capable of interacting with specific components of the immune system and that often functions as an immunogen to elicit an adaptive immune response

B cell receptor (BCR) a cell surface immunoglobulin that acts as an antigen receptor on a B cell

CD4 coreceptor a protein found on Th cells that interacts with MHC II on an antigen-presenting cell

CD8 coreceptor a protein found exclusively on Tc cells that interacts with MHC I on a target cell

Clonal anergy the inability to produce an immune response to specific antigens due to the neutralization of effector cells

Clonal deletion for T cell selection in the thymus or B cell selection in the bone marrow, the elimination by apoptosis of useless or self-reactive precursor lymphocytes

Clonal expansion following its activation, the proliferation and differentiation of a naive T or B lymphocyte into a clone of effector cells

Clone lymphocytes of identical antigen specificity that have arisen from a single, activated progenitor cell

Complementarity-determining region (CDR) a varying amino acid sequence within the variable domains of immunoglobulins or T cell receptors where contacts with antigen are made

Epitope the portion of an antigen that reacts with a specific antibody or T cell receptor

Human leukocyte antigen (HLA) an antigen-presenting protein encoded by a major histocompatibility complex gene in humans

Immune memory (memory) the ability to rapidly produce large quantities of specific immune cells or antibodies after subsequent exposure to a previously encountered antigen

Immunogen a molecule capable of eliciting an adaptive immune response

Immunoglobulin gene superfamily a family of genes that are evolutionarily, structurally, and functionally related to immunoglobulins

Memory cell a long-lived B or T lymphocyte responsive to a specific antigen

MHC class I protein an antigen-presenting molecule found on all nucleated vertebrate cells

MHC class II protein an antigen-presenting molecule found on macrophages, B cells, and dendritic cells

Motif in antigen presentation, a specific amino acid sequence found in all peptides that bind to a given MHC protein

Negative selection in T cell selection, the deletion of T cells that interact strongly with self antigens in the thymus (see also *clonal deletion*)

Polygeny the occurrence of two or more genetically, structurally, and functionally related gene loci due to an evolutionary gene duplication event

Polymorphism in a population, the occurrence of multiple alleles for a gene locus at a higher frequency than can be explained by recent random mutations

Positive selection in T cell selection, the growth and development of T cells that interact with self MHC–peptide in the thymus

Primary immune response the production of antibodies or immune T cells on first exposure to antigen; the antibodies are mostly of the IgM class

Secondary immune response the enhanced production of antibodies or immune T cells on second and subsequent exposures to antigen; the antibodies are mostly of the IgG class

Somatic hypermutation the mutation of immunoglobulin genes at rates higher than those observed in other genes

Specificity the ability of cells of the adaptive immune response to interact with particular antigens

T cell receptor (TCR) an antigen-specific receptor protein on the surface of T cells

T-cytotoxic (Tc) cell a lymphocyte that interacts with MHC I–peptide complexes through its T cell receptor and produces cytotoxins that kill the interacting target cell

T-helper (Th) cell a lymphocyte that interacts with MHC II–peptide complexes through its T cell receptor and produces cytokines that act on other cells. Th subsets include **Th1** cells, which activate macrophages; **Th2** cells, which activate B cells; **Th17** cells, which activate neutrophils; and **Treg** cells, which suppress adaptive immunity

Tolerance the acquired inability to produce an immune response to particular antigens

Vaccination (immunization) the inoculation of a host with inactive or weakened pathogens or pathogen products to stimulate protective active immunity

Immune Disorders and Antimicrobial Therapy 28

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II Vaccines and Immunotherapy 925

III Drug Treatments for Infectious Diseases 930

MICROBIOLOGYNOW

Preventing Autoimmunity with . . . Parasitic Worms?

Some immune disorders are a result of *autoimmunity*, a harmful condition in which an immune response is directed against "self" antigens. For example, type 1 diabetes (T1D) is an autoimmune disorder in which immune cells attack and destroy insulin-secreting β cells in the pancreas, resulting in an inability to metabolize sugars and control blood glucose levels. With no cure, type 1 diabetics must treat their condition by strict diet control and regular injection (or subcutaneous infusion) of insulin.

The prevalence of T1D has risen markedly in recent years; currently, nearly 80,000 children around the world are diagnosed annually. Interestingly, most of these cases occur in developed nations associated with elevated socioeconomic conditions and modern sanitation. The "hygiene hypothesis" has been offered as an explanation for the rising prevalence of autoimmune diseases. The hypothesis asserts that the overly sanitized, primarily indoor upbringing typical for those in developed nations precipitates the onset of allergies and autoimmunity due to reduced early exposure to parasites and other microbes that traditionally associate with humans and shape immune development.

In this connection, researchers have reported that infection with parasitic helminths such as *Schistosoma mansoni* (photo), the causative agent of schistosomiasis, may improve and even prevent T1D. Recent studies have shown that *Schistosoma* infection can suppress Th1 cell activation and instead trigger the propagation of regulatory T (Treg) cells, thereby inhibiting inflammation and controlling the immune response. A similar helminth-mediated immune dampening has been demonstrated for other autoimmune disorders, such as the treatment of Crohn's disease by introducing whipworm (*Trichuris suis*) infection.

These studies suggest that short-term parasitic worm infection (cured using antihelminthic drugs) may bring about long-term autoimmune protection. The next step is to determine what components of the worms actually cause the immunoregulatory effect so that it might become possible to administer only those purified products and avoid the intentional helminth infection.

Source: Tang, C.-l., et al. 2019. Helminths protect against type 1 diabetes: Effects and mechanisms. *Parasitol. Res. 118*: 1087. doi:10.1007/s00436-019-06247-4.

We discussed the key features of the innate and adaptive immune responses in Chapters 26 and 27, respectively, but of course the immune system does not always effectively halt microbial infection and disease progression. In fact, the immune response itself can be detrimental if self antigens are targeted. Such events cause immune disorders and immunodeficiency syndromes, which we explore in the first part of this chapter. We then discuss strategies to both prevent and treat diseases by either optimizing the immune response through vaccinations and immunotherapy or administering antimicrobial drugs to kill or inhibit established pathogens. We conclude the chapter with a discussion of the important implications of antimicrobial drug resistance.

Mastering Microbiology
Art Activity: Figure 27.24 Immediate hypersensitivity

I • Disorders and Deficiencies of the Immune System

In some cases, the immune system fails to provide an adequate or beneficial response, and this may result in hypersensitivity, autoimmunity, superantigen-induced overstimulation, or immunodeficiency. All of these immune disorders are harmful, and some can even be fatal.

In some cases, reactions of the adaptive immune response can damage the host. For example, **hypersensitivity** is an immune response that results in host damage and, in some cases, even host death. Hypersensitivities are grouped according to the antigens and the mechanisms that produce disease. Likewise, exposure to *superantigens*, proteins produced by certain bacteria and viruses that initiate massive inflammatory responses, also causes severe immune reactions that result in host damage. We consider both of these along with immunodeficiency, a condition in which a host's immune response is either absent or insufficient to effectively fight infections.

28.1 Allergy, Hypersensitivity, and Autoimmunity

There are two major types of hypersensitivity: antibody-mediated and cell-mediated. Antibody-mediated, or **immediate hypersensitivity**, is more commonly called *allergy*. Cell-mediated hypersensitivities also cause allergy-like syndromes but, because of the typically delayed onset of symptoms, are termed **delayed-type hypersensitivity (DTH)**. As discussed in MicrobiologyNow on type 1 diabetes in the opening of this chapter, **autoimmunity** is a harmful immune reaction directed against self antigens. Hypersensitivities are categorized as type I, II, III, or IV based on immune effectors, antigens, and symptoms (Table 28.1), and we consider these first.

Immediate Hypersensitivity

Immediate (type I) hypersensitivity occurs because mast cells coated with IgE release substances that either increase or decrease blood pressure or heart rate (vasoactive products) (Figure 28.1). Immediate hypersensitivity reactions occur within minutes after exposure to an **allergen**, the antigen that caused the type I hypersensitivity. Depending on the individual and the allergen, immediate hypersensitivity reactions can be mild allergic reactions or can cause a life-threatening reaction called *anaphylaxis*.

Nearly 25% of the population suffers from immediate hypersensitivity allergies to pollens, molds, animal dander, certain foods (strawberries, nuts, and shellfish), insect venoms, dust mites, and other agents. Most allergens enter the body at the surface of mucous membranes, especially those lining the lungs and the gut. Initial exposure to allergens stimulates Th2 cells to produce cytokines that induce B cells to make IgE antibodies. The allergen-specific IgE antibodies bind to IgE receptors on mast cells (Figure 28.1). Mast cells are nonmotile granulocytes (◀ Section 26.4) associated with connective tissue adjacent to capillaries throughout the body. With any subsequent exposure to the immunizing allergen, the mast-cell-bound IgE molecules bind the antigen. Cross-linking of IgEs by an antigen triggers *degranulation*—the release of soluble allergic mediators from the mast cells. These mediators cause allergic symptoms within minutes of antigen exposure. After initial sensitization by an allergen, the allergic individual responds to each subsequent reexposure to the allergen.

The principal chemical mediators released from mast cells are *histamine* and *serotonin*, modified amino acids that cause rapid dilation of blood vessels and contraction of smooth muscle, initiating symptoms ranging from mild local discomfort to systemic *anaphylactic shock*. Local symptoms typically include mucus production, rash, sneezing, itchiness, watery eyes, and hives (Figure 28.2). Symptoms of anaphylactic shock may include vasodilation (causing a sharp

UNIT 6

TABLE 28.1 Hypersensitivity[a]

Classification	Description	Immune mechanism	Time of latency	Examples
Type I	Immediate	IgE sensitization of mast cells	Minutes	Reaction to bee venom (sting) Hay fever
Type II	Cytotoxic	IgG interaction with cell surface antigen	Hours	Drug reactions (penicillin)
Type III	Immune complex	IgG interaction with soluble or circulating antigen	Hours	Systemic lupus erythematosus (SLE)
Type IV	Delayed type	Th1 inflammatory cell activation of macrophages	Days (24–48 h)	Poison ivy Tuberculin test

[a]Autoimmune diseases may be caused by type II, type III, or type IV reactions.

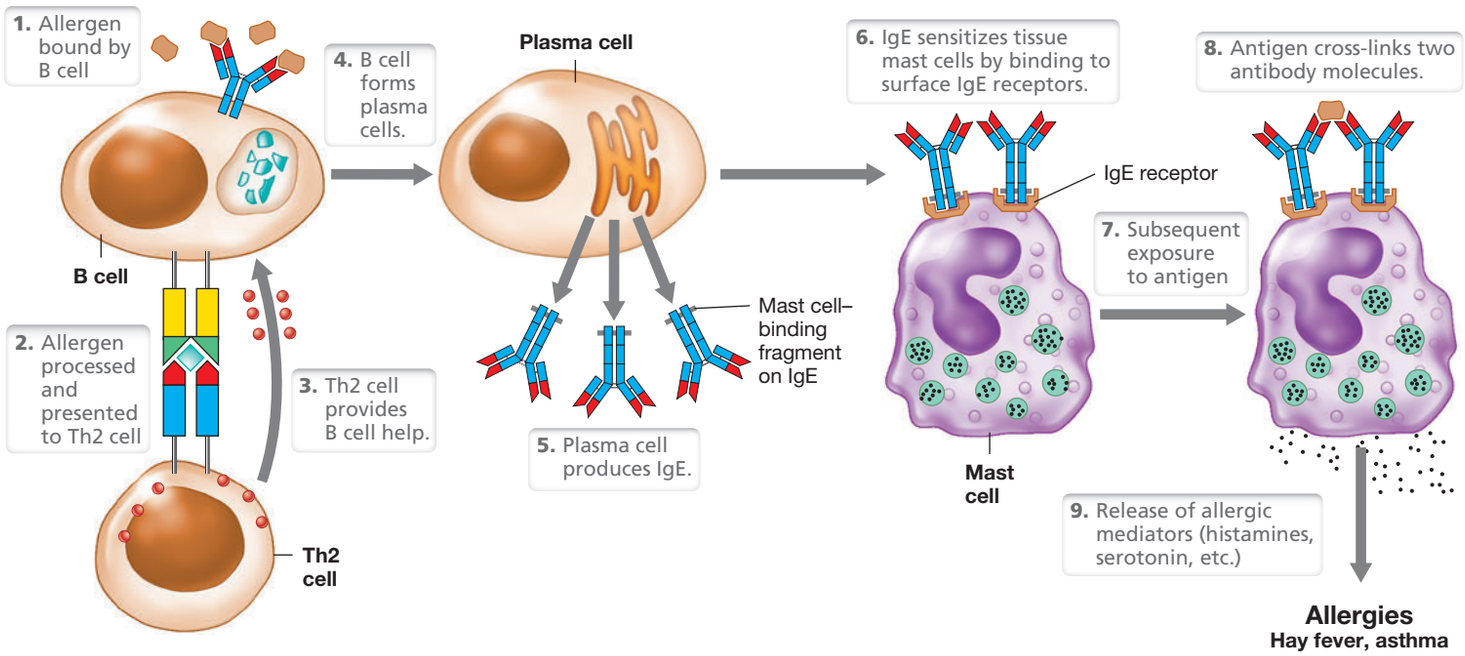
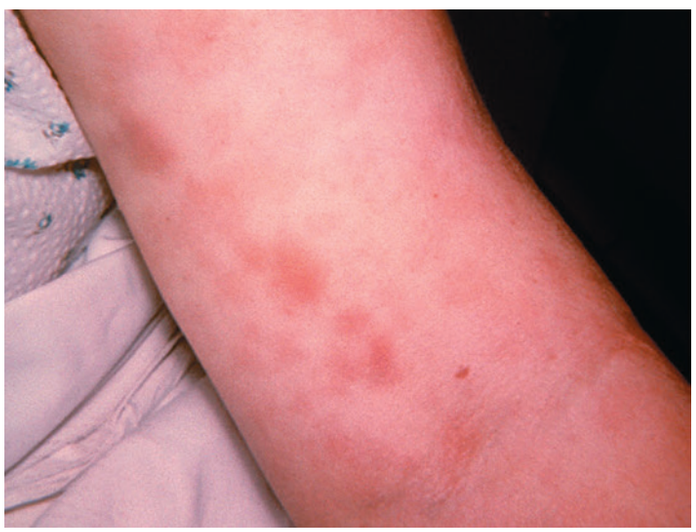


Figure 28.1 Immediate hypersensitivity. Certain antigens, such as pollens, stimulate IgE production. IgE binds to mast cells by means of a high-affinity surface receptor and arms the mast cell. Antigen cross-links surface IgE, causing release of soluble mediators, including histamine. These mediators produce symptoms ranging from mild allergic symptoms to life-threatening anaphylaxis.

drop in blood pressure) and asthma due to smooth muscle constriction in the lungs.

Severe anaphylaxis is treated immediately with the hormone epinephrine to counter smooth muscle contraction, promote breathing, and increase blood pressure. Less serious allergic symptoms may be treated with *antihistamines*, which are drugs that neutralize histamine, or with anti-inflammatory steroids. Finally, immunization with increasing doses of the allergen (beginning with a tiny amount) can be used to shift antibody production from IgE to IgG and IgA.



CDC/PHIL, Emory University, T.F. Sellers, Jr.

Figure 28.2 Hives due to immediate hypersensitivity. The raised, red areas are typical symptoms after contact with allergens that cause immediate hypersensitivity.

The IgG and IgA then interact with the allergen during future exposures and thereby block antigen binding to IgE on sensitized mast cells. This process, called *desensitization*, inhibits IgE production and arrests allergic symptoms.

Delayed-Type Hypersensitivity

Delayed-type (type IV) hypersensitivity (DTH) is cell-mediated hypersensitivity characterized by tissue damage due to inflammation initiated by Th1 cells (Table 28.1). DTH symptoms appear several hours after secondary exposure to the eliciting antigen, with a maximal response usually occurring in 24 to 48 hours. Typical DTH antigens include chemicals that are not normally immunogens but become so when they covalently bind to skin proteins, creating new antigens and eliciting a DTH response. Hypersensitivity to these newly created antigens is known as *contact dermatitis* and results in, for example, skin reactions to poison ivy (**Figure 28.3**), jewelry, cosmetics, latex, and other chemicals that react with host tissues. Several hours after a second or subsequent exposure to the antigen, the skin feels itchy at the site of contact. Erythema (reddening) and edema (swelling) appear, often with localized tissue destruction in the form of blistering (Figure 28.3) and reach a maximum in several days. The delayed onset and the progress of the inflammatory response are the hallmarks of the DTH reaction. As discussed below, certain self antigens may also elicit DTH responses, resulting in autoimmune disease.

Another example of delayed-type hypersensitivity is the development of protective immunity to the bacterium that causes tuberculosis, *Mycobacterium tuberculosis* (▶ Section 31.4). The German physician-turned-microbiologist Robert Koch discovered this cellular immune response during his classic studies on tuberculosis over a century ago (◀ Section 1.12). When antigens derived from

Figure 28.3 Delayed-type hypersensitivity. Poison ivy blisters on an arm. The raised rash appears 24–48 hours after exposure to plants of the genus *Rhus* as a result of macrophage activation by Th1 cells sensitized to *Rhus* antigens.

the bacterium were injected subcutaneously into a person previously infected with *M. tuberculosis*, Koch observed that a skin reaction called the *tuberculin reaction* developed within 24–48 hours (**Figure 28.4**). We now know that during this DTH, local Th1 cells stimulated by the introduced *M. tuberculosis* antigens release cytokines that attract and activate large numbers of macrophages, which in turn produce a characteristic local inflammation, including induration (hardening), edema, erythema, pain, and heating of the skin. The activated macrophages then ingest and destroy the invading antigen. Today, the DTH-based tuberculin skin test is used to test for a current or previous infection with *M. tuberculosis* or previous immunization with the tuberculosis vaccine (▶ Section 29.5).

A number of other infectious diseases due to intracellular pathogens elicit DTH reactions. These include bacterial diseases such as leprosy, brucellosis, and psittacosis; viral diseases such as mumps; and fungal diseases such as coccidioidomycosis, histoplasmosis, and blastomycosis (many of these are covered in Chapters 31–34). Visible, antigen-specific skin responses similar to the tuberculin reaction occur after injection of antigens derived from the pathogens, indicating pathogen exposure and Th1-mediated immunity.

Autoimmune Conditions

As lymphocytes develop, T and B cells that react with self antigens are normally eliminated (◀ Section 27.1). Autoimmune diseases result when these cells are instead activated to produce immune reactions against self proteins (**Table 28.2**). For example, Th1-mediated DTH can cause autoimmune responses directed against self antigens, as in the case of the Th1-mediated response to brain-derived antigens in allergic encephalitis. In type 1 diabetes mellitus (see MicrobiologyNow in the chapter opener), Th1 cells mount an immune response directed to antigens on insulin-producing β cells in the pancreas. The destruction of these cells leads to insulin insufficiency and an inability to control blood sugar levels without an insulin supplement.

In addition to these examples, some autoimmune diseases are caused by **autoantibodies**, antibodies that bind self antigens, many of which are organ-specific. For example, in *Hashimoto's disease* (hypothyroidism), autoantibodies are made against thyroglobulin, a protein product of the thyroid gland that assists in the synthesis of thyroid hormones. In this disease, antibodies to thyroglobulin bind complement proteins (◀ Section 26.9), leading to local inflammation and the destruction of host cells, the hallmarks of a type II hypersensitivity disease (Table 28.2).

Systemic lupus erythematosus (*SLE*) is an autoimmune disease caused by a type III hypersensitivity. This disease and others like it are caused by autoantibodies directed against soluble, circulating self antigens. In SLE, the antigens include nucleoproteins and even DNA itself. Autoantibodies that bind to soluble proteins produce insoluble immune complexes, and disease symptoms result when these circulating antigen–antibody complexes deposit in different body tissues, such as the kidney, lungs, and spleen. In the tissues, the antibodies bind complement, resulting in inflammation and local, often severe, tissue damage. Type III hypersensitivities are also called *immune complex disorders* (Table 28.1).

Treating Autoimmunity

Organ-specific autoimmune diseases are sometimes more easily treated than diseases that affect multiple organs. For example, the product of organ function, such as thyroxine in autoimmune hypothyroidism or insulin in type 1 diabetes, can often be supplied in pure form from another source, such as genetically modified bacteria (◀ Section 12.6 and Table 12.1). SLE, rheumatoid arthritis, and other autoimmune diseases that affect multiple organs and sites can often be controlled only by general immunosuppressive therapy, such as the use of steroid drugs. Unfortunately, the immunosuppression associated with these treatments significantly increases the chance of developing infections from opportunistic pathogens.

As an alternative to traditional immunosuppressive treatments, monoclonal antibodies (a single antibody type, ▶ Section 29.5) have emerged as a promising therapeutic strategy to combat autoimmune diseases. For example, adalimumab (marketed as the drug Humira®) is a monoclonal antibody that neutralizes tumor

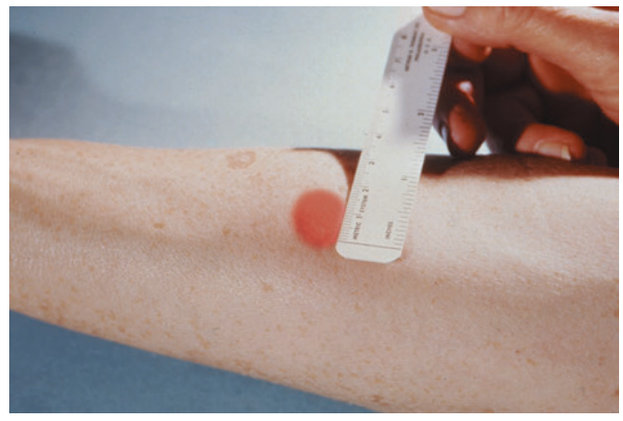


Figure 28.4 The tuberculin test and macrophage activation. This tuberculin test shows a positive reaction. Macrophages activated by antigen-specific Th1 cells cause the localized, delayed-type reaction to a tuberculosis antigen, tuberculin, at the site of injection. The raised area of inflammation on the forearm is about 1.5 cm in diameter.

TABLE 28.2 Some autoimmune diseases of humans

Disease	Organ, cell, or molecule affected	Mechanism (hypersensitivity type[a])
Type 1 diabetes (insulin-dependent diabetes mellitus)	Pancreas	Cell-mediated immunity and autoantibodies against surface and cytoplasmic antigens of beta cells of pancreatic islets (II and IV)
Myasthenia gravis	Skeletal muscle	Autoantibodies against acetylcholine receptors on skeletal muscle (II)
Goodpasture's syndrome	Kidney	Autoantibodies against basement membrane of kidney glomeruli (II)
Rheumatoid arthritis	Cartilage	Autoantibodies against self IgG antibodies, which form complexes deposited in joint tissue, causing inflammation and cartilage destruction (III)
Hashimoto's disease (hypothyroidism)	Thyroid	Autoantibodies to thyroid surface antigens (II)
Male infertility (some cases)	Sperm cells	Autoantibodies agglutinate host sperm cells (II)
Pernicious anemia	Intrinsic factor	Autoantibodies prevent absorption of vitamin B_{12} (III)
Systemic lupus erythematosus (SLE)	DNA, cardiolipin, nucleoprotein, blood clotting proteins	Autoantibody response to various cellular constituents results in immune complex formation (III)
Addison's disease	Adrenal glands	Autoantibodies to adrenal cell antigens (II)
Allergic encephalitis	Brain	Cell-mediated response against brain tissue (IV)
Multiple sclerosis	Brain	Cell-mediated and autoantibody response against central nervous system (II and IV)

[a]See Table 28.1.

necrosis factor alpha (TNF-α), an inflammatory cytokine linked to several autoimmune diseases including rheumatoid arthritis, Crohn's disease, and psoriasis. Similarly, the monoclonal antibody belimumab (marketed as the drug Benlysta®) targets B-cell activating factor, a cytokine that stimulates B cell maturation but is overexpressed in SLE patients, causing the persistence of B cells that produce autoantibodies. Other immunotherapeutic strategies to control autoimmunity focus on limiting the proliferation of proinflammatory Th17 cells by stimulating the production of regulatory T (Treg) cells; the latter function as "brakes" of the immune system to limit harmful inflammatory responses (◀ Section 27.8). We explore immunotherapeutic treatments further in Section 28.4.

Heredity often influences the incidence, type, and severity of autoimmune diseases. Many autoimmune diseases correlate strongly with the presence of certain major histocompatibility proteins (◀ Section 27.6). Studies of autoimmune diseases in mice support a genetic link, but the precise conditions necessary for developing autoimmunity may also depend on other factors, such as prior infections, gender, age, and health status. Women, for example, are about ten times more likely to develop SLE than are men. It is thus likely that the balance between a normal immune response and autoimmunity is tipped by a combination of factors in which genetic predisposition plays a central role.

Check Your Understanding

- Discriminate between immediate hypersensitivity and delayed-type hypersensitivity with respect to antigens and immune effectors.
- Provide examples and mechanisms for an antibody-mediated autoimmune disease directed against a specific organ and one that produces circulating immune complexes.

28.2 Superantigens and Immunodeficiency

Extremes in the adaptive immune response, whether overactive or deficient, can have devastating effects on the host. The exotoxins (◀ Sections 25.6 and 25.7) called *superantigens* damage host cells indirectly by subverting the immune system such that T cells and their cytokine products destroy host tissues through an exaggerated immune response. By contrast, some diseases—either genetic or infectious—cause *immunodeficiency*, resulting in increased susceptibility of the host to infectious diseases. We now consider these opposing extremes in the adaptive immune response.

Superantigens

Superantigens are proteins that, upon exposure to the immune system, activate many more T cells than normal and are therefore capable of eliciting an unusually strong immune response. A variety of viruses and bacteria produce superantigens. For example, streptococci and staphylococci, especially certain strains of *Streptococcus pyogenes* and *Staphylococcus aureus*, produce several different extremely potent superantigens in the condition known as *toxic shock syndrome* (**Figure 28.5**)(▶ Sections 31.2 and 31.9).

Superantigen interaction with TCRs differs from conventional antigen–TCR binding. Ordinarily, foreign antigens presented by MHC proteins, such as those on the surface of an antigen-presenting cell, bind to a TCR at a defined antigen-binding site (◀ Figure 27.18). By contrast, superantigens bind to sites on TCR and MHC proteins that are outside the antigen-specific binding site (**Figure 28.6**). A superantigen binds to all TCRs that share a common structure, and many different TCRs share the same structure outside the antigen-binding site. Whereas less than 0.01% of all available T cells interact with a conventional foreign antigen in a typical immune response, some superantigens can bind up to 25% of all T cells in the body. These interactions mimic conventional antigen

Figure 28.5 Toxic shock syndrome. This individual has a swollen and bumpy tongue called "strawberry tongue," a symptom of toxic shock syndrome caused by a *Staphylococcus aureus* superantigen.

presentation and activate large numbers of T cells to grow and divide. As in normal immune responses, the activated T cells produce cytokines that stimulate phagocytes and other immune cells. However, the unusually large cytokine production by the enhanced population of superantigen-activated T cells triggers a widespread cell-mediated response characterized by systemic inflammatory reactions. The resulting fever, diarrhea, vomiting, mucus production, and even systemic shock may be fatal in extreme cases. The clinical symptoms of superantigen shock are indistinguishable from those of septic shock, a condition in which a bacterial infection has spread throughout the body (◀ Section 26.8).

One of the most common superantigen diseases is *Staphylococcus aureus* food poisoning, characterized by fever, vomiting, and diarrhea, and caused by one of several superantigen staphylococcal enterotoxins (▶ Section 33.8). *S. aureus* also produces the superantigen responsible for *toxic shock syndrome* (Figure 28.5), and *Streptococcus pyogenes* produces *erythrogenic toxin*, the superantigen responsible for scarlet fever (▶ Section 31.2).

Immunodeficiency

Active adaptive immunity is critical for infectious disease resistance. We know this because of problems caused by genetic defects and diseases that affect the adaptive immune system. For example, animals that cannot produce antibodies because of genetic defects in their B cells acquire serious infections from extracellular pathogens, especially bacteria. In addition, animals with genetic defects that prevent development of T cells suffer from recurrent infections with viruses and other intracellular pathogens.

Severe combined immune deficiency syndrome (*SCID*) is a genetic disorder that prevents proper formation of B and T cells. Individuals with SCID essentially have no effective adaptive immunity. A lack of proper T cell function results in a deficiency of cell-mediated immunity and indirectly causes a loss of antibody-mediated immunity because B cell activation against most antigens requires functional Th cells. Unless SCID patients receive supportive therapy, such as a bone marrow transplant and antibiotic treatments, SCID eventually causes death from multiple recurrent infections. The transplantation of compatible bone marrow tissue provides the afflicted individual with hematopoietic stem cells (◀ Sections 26.3 and 26.4) free of the genetic defects that cause SCID.

Gene therapy has also shown promise as a curative treatment option for some forms of SCID. In this procedure, defective genes in hematopoietic stem cells are replaced through a transduction process using viral vectors (◀ Section 9.7). As with bone marrow transplantation, successful gene therapy restores function of the adaptive immune system. However, using viral vectors—even genetically engineered vectors—can be risky because of secondary effects that the viruses may have, such as activating host genes that initiate tumor growth. Because of this, powerful new tools for genome editing (◀ Section 12.13) that do not require viral vectors are being developed, and thus safe genetic therapies for SCID may be on the horizon.

In some cases, immunodeficiency is not the result of a genetic disorder but rather is caused by microbial infection. The best-studied example of this is the loss of the adaptive immune response due to *acquired immunodeficiency syndrome* (*AIDS*). Human immunodeficiency virus (HIV) infects host cells that express the CD4 cell surface protein. The virus initially infects macrophages but later attacks and replicates primarily in Th cells (**Figure 28.7**). Once infected, Th cells cease dividing and eventually die. Therefore, in untreated cases, HIV infection causes a gradual depletion of Th cells, resulting in a lack of effective immunity and the eventual onset of AIDS (▶ Section 31.15 and Figure 31.45).

In most AIDS patients, the actual cause of death is not directly attributed to HIV but rather to secondary microbial infections caused by *opportunistic pathogens* (◀ Section 25.4). Many of these infections are caused by fungal, bacterial, and viral pathogens that only rarely cause serious disease symptoms in individuals that have

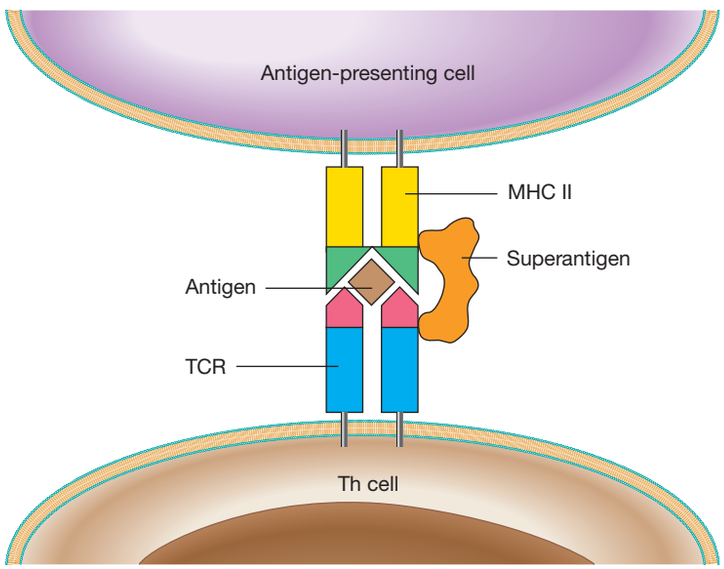
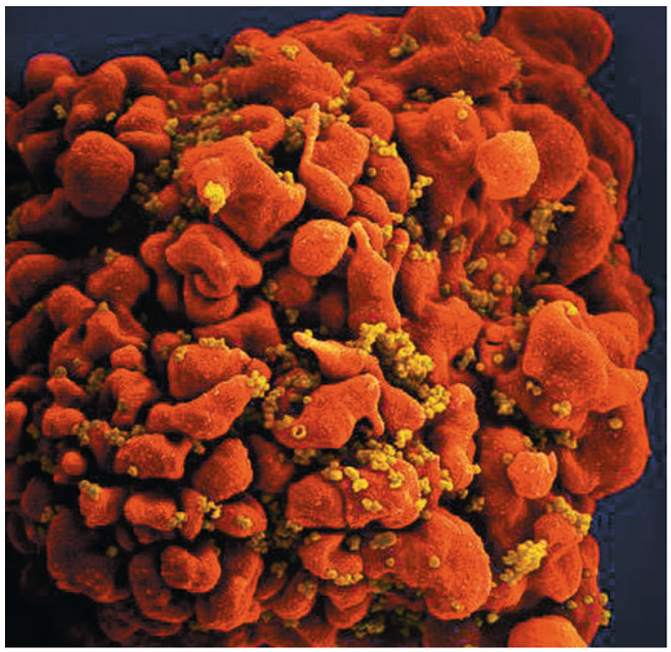


Figure 28.6 Superantigens. Superantigens bind to conserved regions of both the MHC and TCR proteins at positions outside the normal binding site. Superantigens interact with large numbers of T cells, causing large-scale T cell activation, cytokine release, and systemic inflammation.



NIAID/CDC-PHIL

Figure 28.7 HIV infection of a Th cell. Colorized scanning electron micrograph of HIV virions (yellow) attacking a Th cell (red).

a fully functional immune system. The deficiency of adaptive immunity in AIDS patients allows these opportunistic pathogens to colonize and invade body tissues, eventually leading to death. We discuss the pathogenicity, symptoms, treatment, and other aspects of HIV/AIDS in more detail in Chapter 31.

Check Your Understanding

- Describe the binding site for superantigens on T cells and antigen-presenting cells. How does this correlate to the activation of a much larger population of T cells than normal upon exposure to superantigens?
- Compare and contrast the immunodeficiency observed in SCID patients to that of AIDS patients. What cell types are affected by each condition?
- In the absence of treatment, what is the prognosis for individuals afflicted with SCID and AIDS?

II • Vaccines and Immunotherapy

Vaccines are indispensable preventive tools to train the immune system to recognize and attack specific pathogens upon exposure. Immunotherapy is a treatment option that uses therapeutic agents to either activate or suppress immune responses to specific diseases, including cancer.

As we have seen, malfunctions of the immune system can either contribute to disease signs and symptoms (hypersensitivity and autoimmunity) or result in a failure to contain them (immunodeficiency). Here we move on to discuss ways in which attributes of the immune system can be harnessed to develop tools and strategies to both *prevent* and *treat* diseases.

28.3 Vaccination Against Infectious Diseases

A major means of disease prevention is **vaccination (immunization)**, in which deliberate exposure to an antigen triggers an adaptive immune response intended to protect an individual against future infection by a pathogen. The immunogen used to induce this artificial active immunity is called a **vaccine**. A summary of major diseases for which vaccines are available for human use is given in Table 28.3.

TABLE 28.3 Vaccines for infectious diseases in humans

Bacterial diseases	Type of vaccine used
Anthrax	Toxoid
Cholera	Killed cells or cell extract (*Vibrio cholerae*)
Diphtheria	Toxoid
Haemophilus influenzae type b meningitis	Conjugated vaccine (polysaccharide of *Haemophilus influenzae* type b conjugated to protein)
Meningitis	Purified polysaccharide from *Neisseria meningitidis*
Paratyphoid fever	Killed bacteria (*Salmonella enterica* [*paratyphi*])
Pertussis	Killed bacteria (*Bordetella pertussis*) or acellular proteins
Plague	Killed cells or cell extract (*Yersinia pestis*)
Pneumonia (bacterial)	Purified polysaccharide from *Streptococcus pneumoniae* or polysaccharide–toxoid conjugate
Tetanus	Toxoid
Tuberculosis	Attenuated strain of *Mycobacterium tuberculosis*
Typhoid fever	Killed bacteria (*Salmonella enterica* [*typhi*])
Typhus	Killed bacteria (*Rickettsia prowazekii*)
Viral diseases	**Type of vaccine used**
Hepatitis A	Recombinant DNA vaccine
Hepatitis B	Recombinant DNA vaccine or inactivated virus
Human papillomavirus (HPV)	Recombinant DNA vaccine
Influenza (seasonal or H1N1)	Inactivated or attenuated virus
Japanese encephalitis	Inactivated virus
Measles and mumps	Attenuated virus
Polio	Attenuated virus (Sabin) or inactivated virus (Salk)
Rabies	Inactivated virus (human) or attenuated virus (animal)
Rotavirus	Attenuated virus
Rubella	Attenuated virus
Smallpox and monkeypox	Cross-reacting virus (vaccinia)
Varicella (chicken pox/shingles)	Attenuated virus
Yellow fever	Attenuated virus

The Nature of Vaccines

Infants are immune to many common infectious diseases during the first several months of life because they acquire natural passive immunity from maternal antibodies transferred across the placenta and in breast milk (◀ Section 24.8). However, it is recommended that infants be immunized against key infectious diseases as soon as possible so that their own active immunity can replace the temporary maternal passive immunity (**Figure 28.8**). As discussed in Section 27.3, a single exposure to antigen does not lead to a high antibody titer. After an initial immunization, a series of "booster" immunizations are given to produce a secondary response and a high antibody titer.

It is well established that the introduction of effective vaccines into a population has reduced the incidence of formerly epidemic childhood diseases such as measles and mumps (▶ Section 31.6) and has eliminated smallpox altogether (▶ Section 30.5). However, lifelong immunity is rarely achieved by vaccination because the immune cells and antibodies generated by immunization gradually disappear from the body. In the absence of environmental antigenic stimulation, the length of effective immunity varies considerably with different vaccines. For example, the tetanus toxoid vaccine provides effective immunity for ten years, but immunity induced by inactivated influenza virus vaccine generally disappears within a year or two (the reasons for this short-term immunity in the case of influenza are considered in Section 31.8).

Immunizations against bacteria	Immunizations against viruses
Haemophilus influenzae type B (Hib)	Hepatitis A virus
Meningococcal (*Neisseria meningitidis*)	Hepatitis B virus
Pneumococcal (*Streptococcus pneumoniae*)	Human papillomavirus (HPV)
Tetanus, diphtheria, pertussis (DTaP, Tdap)	Influenza virus
	Inactivated poliovirus (IPV)
	Measles, mumps, rubella (MMR)
	Rotavirus
	Varicella virus (chicken pox)

Figure 28.8 Immunization recommendations for infants and children in the United States. The U.S. Centers for Disease Control and Prevention website (http://www.cdc.gov) has recommendations for timing and dose of immunizations for all age groups and for special populations, such as international travelers, women of childbearing age, and those with immunodeficiency or chronic disease.

To minimize the risk of infection or other adverse reactions, pathogens or pathogen products used in vaccines must be made harmless. To achieve this, many vaccines consist of pathogens killed either by heat treatment or by chemical agents. For example, formaldehyde is used to inactivate polio virions for preparation of the Salk polio vaccine. In addition, many exotoxins can be chemically modified to create a *toxoid*, a molecule that retains its antigenicity but is no longer toxic. Toxoid vaccines, such as the vaccine for *Clostridium tetani* (the causative agent of tetanus, ▶ Section 32.9), safely induce long-term protective immunity against the exotoxin.

Immunization with intact pathogens is usually more effective than immunization with dead or inactivated material. It is often possible to isolate *attenuated strains* of pathogens—those that have lost their virulence but still retain the immunizing antigens (◀ Figure 25.10). However, because attenuated strains of pathogens are still viable, some individuals may inadvertently acquire active disease from the vaccination. Nevertheless, attenuated vaccines are widely used because they tend to provide long-lasting immunity and a strong secondary booster response. By contrast, killed virus vaccines generally provide short-lived immune responses with less long-term memory.

Most bacterial vaccines are composed of key cellular antigens in an inactivated form, such as the toxoids that protect against tetanus and diphtheria. Inactivated bacterial vaccines induce antibody-mediated protection without exposing recipients to the risk of infection. However, variability in primary and secondary responses with each vaccine and individual make periodic reimmunization necessary to establish and maintain immunity.

Synthetic and Genetically Engineered Vaccines

An alternative approach to vaccine development is to deploy the tools of genetic engineering (Chapter 12) to produce *synthetic peptides*. To make such a vaccine, a genetic engineer can synthesize a peptide that corresponds to an antigen from an infectious agent. For example, the toxin from the foot-and-mouth disease virus, an important animal pathogen, must be modified from its native form to render it harmless for use as a vaccine. The toxin contains a peptide of 20 amino acids that is an important antigenic determinant in the protein, but the peptide is too small to be an effective vaccine by itself. However, genetic engineers attached the small peptide to a larger, innocuous protein that acts as a carrier molecule, creating a *conjugate vaccine* against foot-and-mouth disease virus. This strategy has great promise for creating vaccines for a number of other pathogens as well, especially because the complete genomic sequences of many pathogens are now known, and this provides the insight necessary to pinpoint the most likely antigenic molecules.

Two widely available conjugate vaccines couple extracted bacterial polysaccharides, which are poorly immunogenic by themselves, to a protein toxoid, thereby inducing a more robust immune response

with better immune memory than injection of the polysaccharide antigen alone. The first of these is a pneumococcal vaccine that pairs diphtheria toxoid to polysaccharide capsule antigens derived from pathogenic strains of *Streptococcus pneumoniae*, the causative agent of bacterial pneumonia (▶ Section 31.2) (**Figure 28.9**). The second is the *Haemophilus influenzae* type b (Hib) vaccine, which couples Hib polysaccharide to tetanus toxoid. The conjugation of polysaccharide antigens to protein toxoids efficiently activates Th2 cells, resulting in a strong immune response that confers immune memory.

Genomic information is particularly useful for making viral vaccines. Genes that encode antigens from virtually any virus can be cloned into the vaccinia virus genome and expressed (◀ Section 12.8). Inoculation with the antigen-producing vaccinia virus can induce immunity to the product of the cloned gene. Such a preparation is called a *recombinant-vector vaccine*, an example of which is the recombinant vaccinia–rabies vaccine used in animals. A second immunization strategy uses proteins made from cloned DNA as immunogens. After a pathogen gene is cloned and expressed in a suitable microbial host, the pathogen protein is harvested and used to produce a *recombinant-antigen vaccine*. For example, the current vaccine for hepatitis B virus (▶ Section 31.11) is a major hepatitis B surface protein antigen (HbsAg) expressed in genetically modified yeast cells. Similarly, a vaccine effective against human papillomavirus (HPV, ▶ Section 31.14) is also a recombinant-antigen vaccine made in yeast cells.

Nucleic Acid Vaccines and Plant-Based Vaccines

A novel vaccine delivery method is seen with the *DNA vaccines*, vaccines in which the antigen(s) of interest are not injected into the animal but are actually *synthesized* by the animal. DNA vaccines are bacterial plasmids that contain cloned DNA that encodes the antigen of interest. Typically, the vaccine is injected intramuscularly into a host animal. Once host cells take up the plasmid, the DNA is transcribed and translated to produce immunogenic proteins, triggering a conventional immune response of activated T cells and antibodies directed to the protein encoded by the cloned DNA. A major advantage of DNA vaccines is that, because only a single pathogen gene is cloned into the plasmid and administered to the host, there is no chance of an infection, as there might be with an attenuated vaccine.

A second nucleic acid–based vaccine methodology that has shown promise as an alternative to conventional methods is *mRNA vaccines*. In this approach, RNA that encodes an antigen of interest and has been engineered to resemble a fully processed, mature mRNA suitable for translation in eukaryotic cells (◀ Section 6.6) is administered to the animal, and production of the protein by host cells produces an adaptive immune response to the antigen. As with DNA vaccines, there is no risk of infection from mRNA vaccines, but in addition, the short half-life of mRNA allows for greater control of protein expression and immunogenicity. Early challenges associated with instability and inefficient in vivo delivery of mRNA vaccines have largely been overcome, and therefore this vaccine platform may be of great importance for future disease control and prevention.

Lastly, a novel variation of the recombinant-antigen vaccine described above uses fast-growing plants as the transgenic host to produce *plant-based vaccines*. In this method, the pathogen-derived target gene is transferred to the plant using the plant pathogen *Agrobacterium tumefaciens* as a bacterial vector (◀ Sections 12.7 and 23.6).

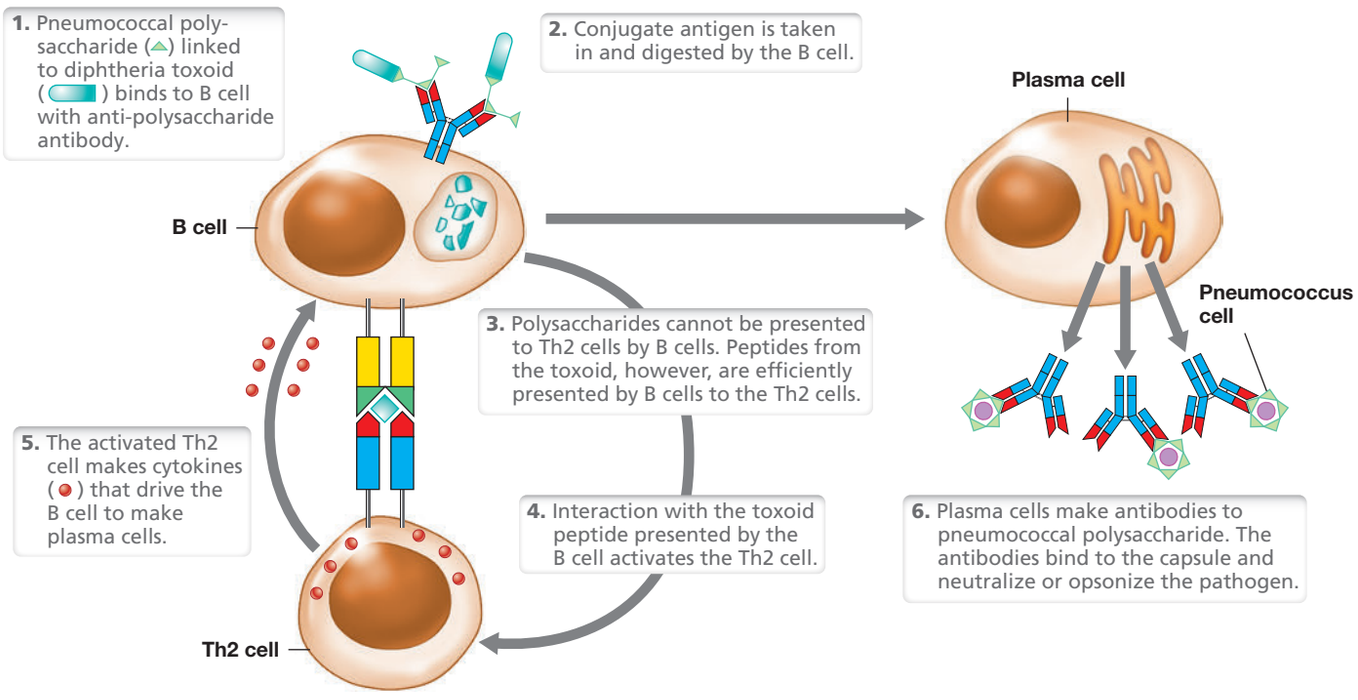


Figure 28.9 Conjugate vaccines. Conjugate vaccines, such as *Streptococcus pneumoniae* (pneumococcus) polysaccharide covalently linked to diphtheria toxoid (shown) or *Haemophilus influenzae* type B (Hib) polysaccharide coupled to tetanus toxoid provide effective immunity to polysaccharide antigens, which are poor immunogens in the absence of protein carriers.

Following plant infection by *A. tumefaciens*, recombinant proteins are extracted from the plants and purified to produce *virus-like particles* (*VLPs*), lipid bilayer constructs embedded with pathogen-specific protein antigens. VLPs produce a strong immune response, but because they lack the genetic material of the native pathogen, they cannot replicate or cause infection. Promising VLP vaccines are in development for a wide variety of viral pathogens, including rotavirus, norovirus, West Nile virus, hepatitis B virus, and pandemic influenza.

Check Your Understanding

- Compare and contrast live attenuated vaccines, inactivated vaccines, and toxoids. Which of these has the greatest potential to cause active disease in the recipient? Which typically provides the longest-lasting immunity?
- Identify the advantages of alternative immunization strategies as compared to traditional immunization procedures.

28.4 Immunotherapy

As we have seen, the immune system can be manipulated to provide protection against future pathogen exposures through vaccination strategies. In addition to this, **immunotherapy** is a burgeoning field in clinical medicine that seeks to harness cells and other components of the immune system to fight or prevent diseases, including various forms of cancer. Diagnostic tests for cancer typically begin with either a tissue biopsy for solid tumors or a blood sample for hematological cancers or circulating cancer cells. Key immunotherapeutic approaches currently in development to combat cancer include *anticancer vaccination*, the use of *checkpoint inhibitors* to stimulate the antitumor immune response, and *adoptive T cell transfer*, an encouraging approach that optimizes T cell activity against a variety of cancers (**Figure 28.10**).

Anticancer Vaccines

Attempts to develop anticancer vaccines have historically been ineffective in practice, but due to recent advances in molecular immunology, interest is once again growing in this area. Although a universal vaccine to protect against all cancer is unlikely to be realized considering the many different types of cancer that exist, immunotherapeutic vaccines are currently in development for certain cancers, including melanoma, pancreatic cancer, and glioblastoma, an aggressive brain cancer with an otherwise poor prognosis.

Anticancer vaccines can be either *prophylactic* (preventive) or *therapeutic*. Rather than preventing cancer directly, prophylactic vaccines target oncogenic pathogens, such as human papillomavirus, the causative agent of most cervical and anal cancers, and hepatitis B virus, which can cause liver cancer. Therapeutic anticancer vaccines are designed to stimulate the body's natural immune response to cancer cells. Advances in genomics, including digital innovations that allow for detailed data analyses, have led to *personalized cancer vaccines*. By mapping specific oncogenic mutations in extracted tumor cells, a patient's dendritic cells can be sensitized to new antigens (called "neoantigens") that appear on the transformed tumor cells, resulting in a potent anticancer response mediated by activated T cells (Figure 28.10).

Checkpoint Inhibitors

The tumor microenvironment has been shown to be immunosuppressive, which is an obstacle to cancer treatment. This is largely due to the production and activity of immune checkpoint proteins, including programmed cell death protein-1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), which function to suppress the adaptive immune response. PD-1 and CTLA-4 inhibit T cell activation by regulating the TCR costimulatory protein CD28 and competing with CD28 for protein B7 on APCs, respectively (◀ Figure 27.20). Normally, these functions prevent uncontrolled inflammation and autoimmune responses, but cancer cells exploit these checkpoint proteins to suppress immunity and evade anticancer effector cells.

The goal of checkpoint inhibitor therapy (also called *checkpoint blockade*), therefore, is to prevent neutralization of cytotoxic T cells and stimulate their tumoricidal effector activity. The most successful checkpoint inhibitors are monoclonal antibody (mAb)-based drugs (▶ Section 29.5), such as pembrolizumab (marketed as Keytruda®), that target PD-1 on T cells (or its ligand PD-L1 on cancer cells) in solid tumors and block inhibition of the antitumor immune response (Figure 28.10). Checkpoint inhibitors are often combined with steroids or other immunity-modulating drugs to boost their efficiency, but the safety and efficacy of these combined treatment strategies are still being assessed in clinical trials. Recall from Section 28.1 that mAbs are also used to treat autoimmune disorders, and therefore it is important to note that, depending on their activity, these drugs may be used to either suppress (to combat autoimmunity) or stimulate (to treat cancer) the immune response.

Adoptive T Cell Transfer

Adoptive T cell transfer (ACT) is a novel type of cancer immunotherapy that focuses on harnessing the tumor-targeting abilities of T cells. Two main approaches have been developed for ACT therapy: tumor-infiltrating lymphocytes (TILs) and chimeric antigen receptor (CAR) T cells (Figure 28.10).

TILs are effector T cells having natural anticancer activity that are extracted from tumors, propagated in the laboratory and then infused back into the patient to amplify the immune response against the cancer cells. TIL therapy has proven especially effective in treating melanoma—a dangerous and often metastasizing (spreading) form of cancer—and clinical trials using TILs against this and other forms of cancer, including ovarian, renal cell, and nasopharyngeal cancers, are under way.

CAR T cell therapy is a form of ACT that employs genetically engineered T cells to treat various cancers, especially B cell malignancies such as leukemia and lymphoma. CARs are engineered receptors that are an amalgam of components from TCRs, costimulatory molecules such as CD28, and the antigen-binding domains of antibodies (◀ Figure 27.11) specific to tumor neoantigens (Figure 28.10). The synthesis of CAR T cells is complex and, like TIL therapy, requires extraction of the patient's T cells for laboratory

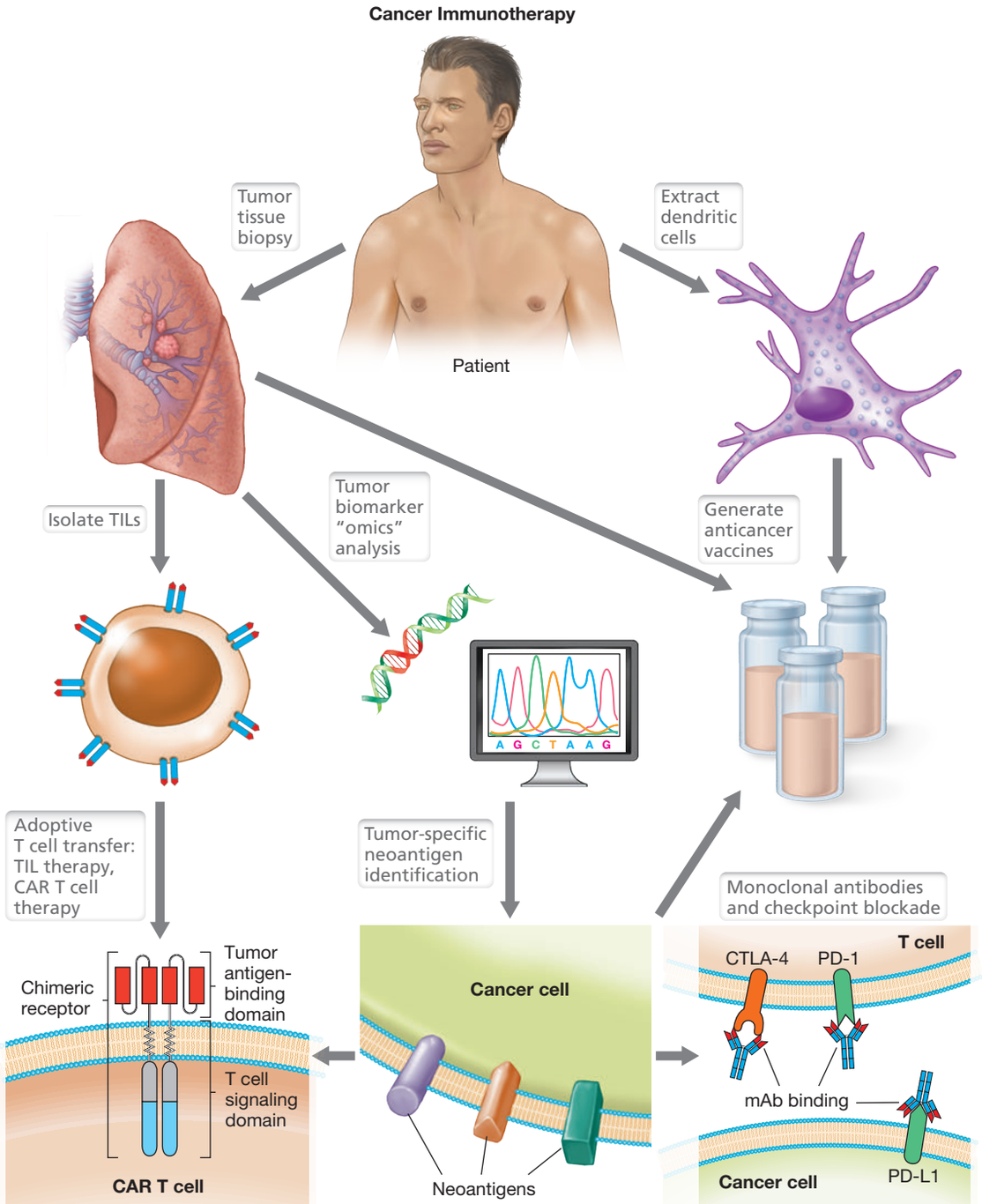


Figure 28.10 Overview of cancer immunotherapy. Anticancer vaccines can potentially be designed from properties of extracted tumor cells or the patient's dendritic cells. Checkpoint blockade uses monoclonal antibodies to neutralize immunosuppressive components, such as PD-1/PD-L1 and CTLA-4, in the tumor microenvironment. CARs are genetically engineered receptors that reprogram cytotoxic T cells to recognize and be directly activated by tumor-specific antigens. CAR, chimeric antigen receptor; mAb, monoclonal antibody; TIL, tumor-infiltrating lymphocyte.

manipulation. The manipulation includes introducing a CAR-containing viral vector and clonal expansion of the transgenic T cells before infusion back into the patient. Essentially, CAR T cells are customized to recognize tumor-specific antigens and kill cancer cells *even in the absence of MHC antigen presentation* (◀ Section 27.5). The latter is possible because the antigen-binding domain of CARs is of immunoglobulin origin rather than TCR origin.

Although treatments such as checkpoint inhibitors and CAR T cells are undeniably powerful immunotherapeutic tools, these technologies are also accompanied by several unresolved challenges, including the requirement to overcome immunosuppressive tumor microenvironments and side effects such as neurologic toxicity and excessive proinflammatory cytokine release. In addition, reactions to the same therapies can vary widely in different individuals, a response that could be the result of person-to-person differences in our gut microbiota (Chapter 24), a consideration we turn to now (**Figure 28.11**).

Immunotherapeutic Efficacy and the Gut Microbiome

The composition of each person's gut microbiota plays a significant role in the efficacy of a given immunotherapeutic treatment. For example, studies in mice have shown that administering *Bifidobacterium* as an oral probiotic produces an antitumor effect, and the use of PD-1/PD-L1 checkpoint inhibitors in addition to the probiotic can eliminate tumor growth entirely. Subsequent studies revealed that the abundance of the bacterium *Bifidobacterium* is particularly high in feces of melanoma patients that respond favorably to PD-1/PD-L1 blockade, and transplanting samples of the feces into germ-free mice (fecal transplant, ◀ Sections 24.9 and 24.11) improved the anticancer activity of checkpoint inhibitors and CD8 T cells in the mice (Figure 28.11). Using data from mouse studies as proof-of-concept, clinical trials are now under way to determine whether fecal transplants from patients who respond favorably to PD-1 checkpoint inhibitor therapy into those who do not respond to the treatment can ameliorate the effect of checkpoint blockade in nonresponders.

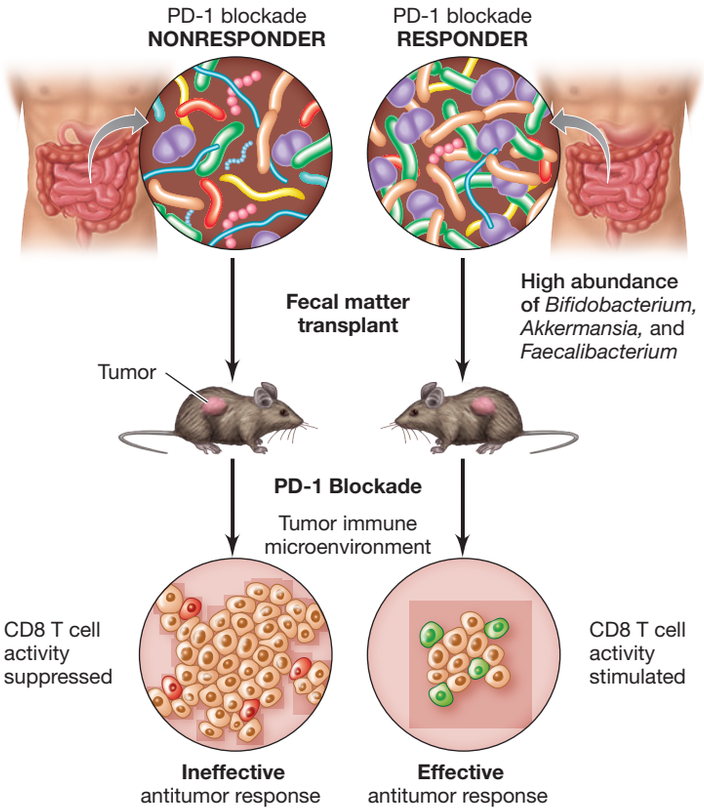


Figure 28.11 Immunotherapy and the gut microbiome. Checkpoint blockade responders tend to have high abundance of *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* spp. in their gut microbiota, whereas nonresponders do not. A fecal transplant from responders into germ-free mice expressing tumors results in increased efficacy of checkpoint inhibition and tumor reduction. Fecal transplants from nonresponders confers no antitumor effect on mice.

Mastering Microbiology
Art Activity: Figure 28.27 Mechanisms of action of major antibacterial agents

The mechanism responsible for the beneficial effect of the transplants has been linked to the release of immune-stimulating cytokines by gut-associated lymphoid tissue upon interaction with the beneficial bacteria. The goal now is to streamline the procedure and increase its reproducibility by determining as precisely as possible which microbes are conferring the beneficial effect and formulating a probiotic suspension of just those organisms. In addition to *Bifidobacterium*, research has focused on species of *Bacteroides*, *Faecalibacterium*, and *Akkermansia* (Figure 28.11) and species of the family *Ruminococcaceae*. Knowledge of the major contributors will minimize potential problems with quality control, as well as the risk of inadvertent transfer of pathogens or immunosuppressive strains in fecal transplants, both of which could undermine the benefits of the treatment.

Check Your Understanding

- How do checkpoint inhibitors stimulate the immune response in the tumor microenvironment? How do chimeric antigen receptors (CARs) differ from T cell receptors (TCRs)?
- Explain how the composition of the gut microbiome may influence the efficacy of cancer immunotherapy. What evidence do we have for this phenomenon?

UNIT 6

III • Drug Treatments for Infectious Diseases

Antimicrobial drugs kill or inhibit microorganisms by a variety of mechanisms and are of immense importance in clinical medicine. However, antimicrobial drug resistance is an escalating threat and a major impediment to the treatment of certain infections.

28.5 Antibacterial Drugs

In cases where immunization is either not an option or fails to prevent disease, antimicrobial drug therapy is the primary weapon against infections. Antimicrobial drugs either kill or inhibit the growth of microorganisms in the host (in vivo) and are classified based on their molecular structure, mechanism of action (Figure 28.12), and spectrum of antimicrobial activity. **Antibiotics** are antimicrobial agents that are produced naturally by certain microorganisms, typically species of *Bacteria* or fungi. In addition to natural antibiotics, many other drugs are produced synthetically. Regardless of their origin, it is desirable that antimicrobial drugs exhibit **selective toxicity**—they inhibit or kill pathogens without adversely affecting the host. Although thousands of antibiotics are known, fewer than 1% are clinically useful, often because of problems with host toxicity or lack of uptake by host cells.

The susceptibility of individual microbes to different antimicrobial agents varies significantly. For example, gram-positive *Bacteria* are often sensitive to natural penicillin, whereas gram-negative *Bacteria* are generally resistant; thus, natural penicillin exhibits a relatively *narrow spectrum* of activity. By contrast, antibiotics that exhibit *broad-spectrum* activity, such as tetracycline, are generally effective against both groups. Although broad-spectrum antibiotics often find wider medical use than narrow-spectrum antibiotics, antibiotics with a limited spectrum of activity may be quite useful against certain pathogens, especially those that fail to respond to other antibiotics. A good example is vancomycin, a narrow-spectrum antibiotic that is an effective bactericidal agent for penicillin-resistant, gram-positive *Bacteria*, including certain enterococci, staphylococci, and clostridia.

Important targets of antibiotics in *Bacteria* include the cell wall, ribosomes, enzymes that facilitate nucleic acid synthesis or catalyze metabolic processes, and the cytoplasmic membrane (Figure 28.12). We provided an overview of antibiotic targets in Section 8.11 and now consider the mechanisms of drug activity in further detail.

The Bacterial Cell Wall as a Drug Target

Worldwide, an estimated 100,000 metric tons of antibacterial drugs are manufactured and used annually (Figure 28.13*a*), and the vast majority of these target the bacterial cell wall. **β-lactam antibiotics**, which include penicillins and cephalosporins, inhibit cell wall synthesis and account for nearly two-thirds of all antibiotics produced and used worldwide (Figure 28.13*b*). These antibiotics share a characteristic structural component, the *β-lactam ring* (Figure 28.14), and historically have been some of the most effective weapons against many different types of bacterial infections.

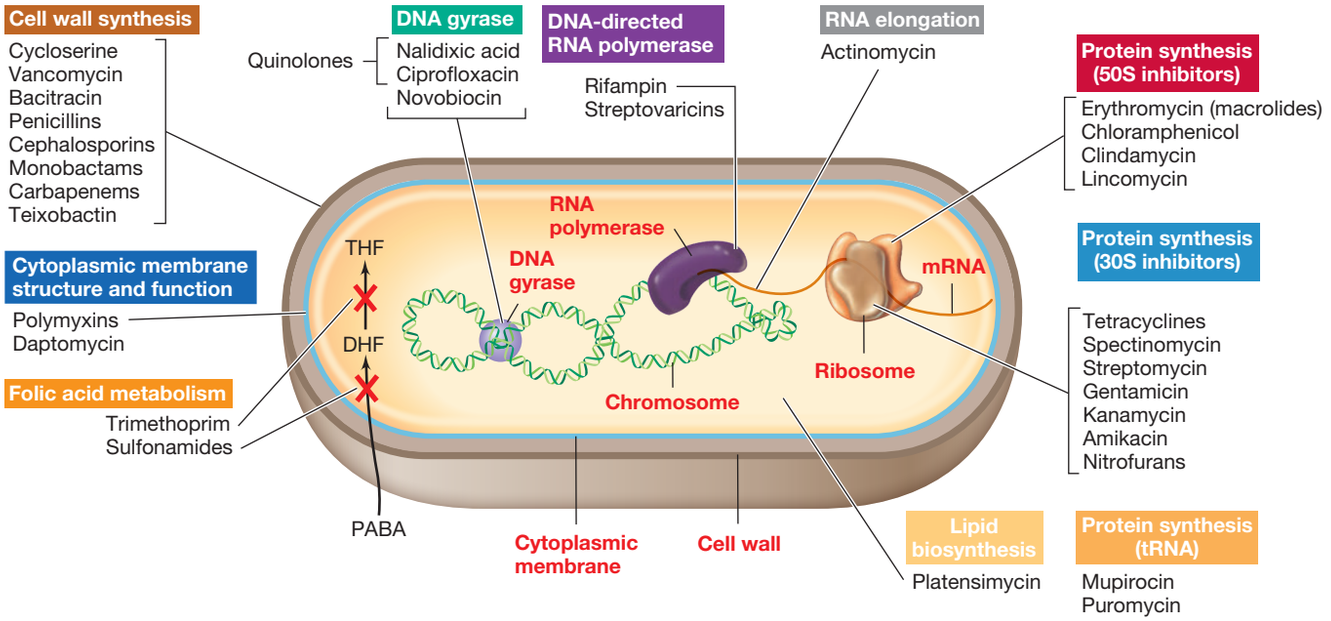
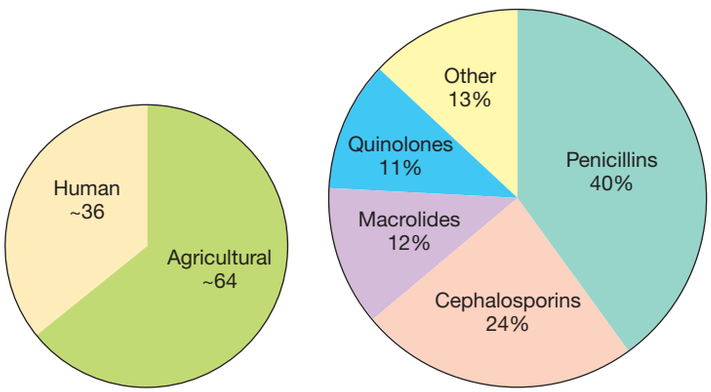


Figure 28.12 Mechanisms of action of major antibacterial agents. Agents are classified according to their target structures in the bacterial cell. THF, tetrahydrofolate; DHF, dihydrofolate; PABA, *p*-aminobenzoic acid.

The first β-lactam antibiotic ever characterized was penicillin G, isolated from the fungus *Penicillium chrysogenum* in 1929 by Alexander Fleming. This new drug was dramatically effective for controlling staphylococcal and pneumococcal infections and was more effective for treating streptococcal infections than the synthetic sulfa drugs, discussed later in this section. Penicillin and other β-lactam antibiotics interfere with an important feature of bacterial cell wall synthesis called *transpeptidation*, the reaction catalyzed by transpeptidases that results in the cross-linking of two glycan-linked peptide chains (◀ Sections 2.3 and 8.5). Because peptidoglycan synthesis mechanisms are unique to *Bacteria*, the β-lactam antibiotics are highly selective and nontoxic to host cells.



(a) **Antibiotic usage in metric tons** *(b)* **Frequency of antibiotic usage**

Figure 28.13 Annual worldwide production and use of antibiotics. *(a)* An estimated 100,000 metric tons of antimicrobial agents are manufactured worldwide per year, and nearly two-thirds of this amount is used in an agricultural context. *(b)* By far, the β-lactam antibiotics (penicillins and cephalosporins) constitute the most important and widely used class of antibiotic. "Other" includes trimethoprim combinations, tetracyclines, chloramphenicol, aminoglycosides, and all other antimicrobial drug classes. Data are from the Center for Disease Dynamics, Economics, and Policy, Washington, DC.

Penicillin G is active primarily against gram-positive *Bacteria* because gram-negative *Bacteria* are impermeable to the antibiotic. However, chemical modification of the *N*-acyl group of penicillin G produces *semisynthetic* penicillins, such as ampicillin and carbenicillin, which have broader activity and are effective against certain gram-negative *Bacteria* (Figure 28.14). The structural differences in these semisynthetic penicillins allow them to be transported across the gram-negative outer membrane (◀ Section 2.4), where they inhibit cell wall synthesis. Penicillin G is also sensitive to *β-lactamases*, enzymes that destroy β-lactam antibiotics and are produced by many penicillin-resistant *Bacteria* (see Section 28.7). Oxacillin and methicillin are widely used β-lactamase-resistant semisynthetic penicillins (Figure 28.14).

Cephalosporins, produced by species of the fungus *Cephalosporium*, differ structurally from the penicillins. They retain the β-lactam ring but have a six-member dihydrothiazine ring joined to it instead of the five-member thiazolidine ring of penicillins (**Table 28.4** and Figure 28.14). The cephalosporins have the same mode of action as the penicillins; they bind irreversibly to transpeptidases and prevent the cross-linking of peptidoglycan. Clinically important cephalosporins are semisynthetic antibiotics with a broader spectrum of activity than the penicillins. In addition, cephalosporins are typically more resistant to β-lactamases. For example, ceftriaxone (Table 28.4) is highly resistant to β-lactamases and has replaced penicillin for treatment of gonorrhea because many *Neisseria gonorrhoeae* strains have become resistant to penicillin (see Section 28.7).

Some antimicrobial drugs mimic *growth factors*, organic nutrients obtained from an organism's environment that are required by the organism for growth and survival (◀ Section 3.1). A **growth factor analog** is a synthetic compound that chemically resembles a growth

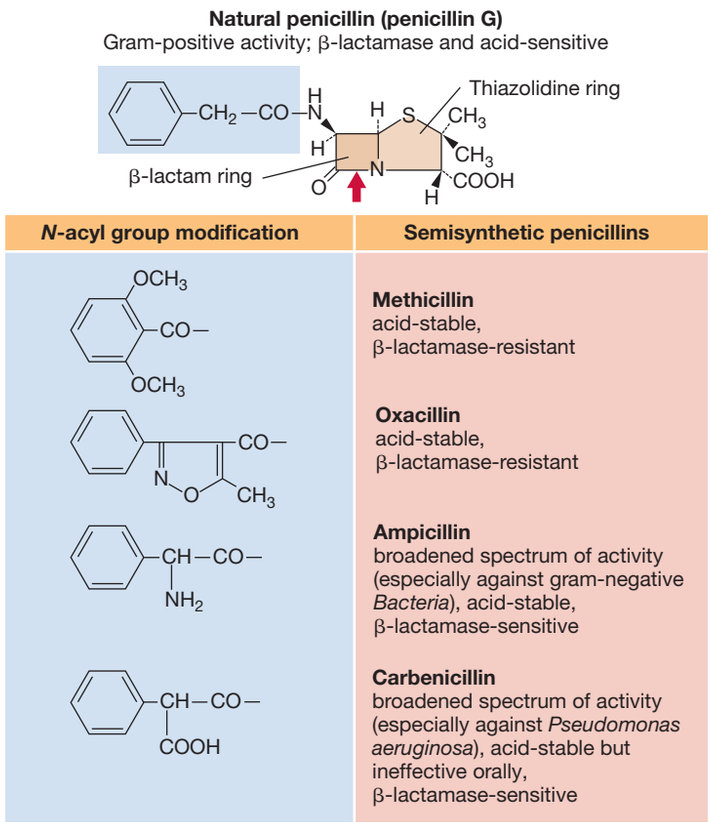


Figure 28.14 Structure of selected penicillins. The red arrow (top panel) indicates the site within the β-lactam ring cleaved by most β-lactamases, enzymes that destroy penicillin and other β-lactam antibiotics. Although the *N-acyl* group (blue shading) varies among different penicillin drugs, all penicillins have a common nucleus consisting of a β-lactam ring and a thiazolidine ring. Whereas penicillin G must be injected, most acid-stable penicillins can be administered orally (carbenicillin is an exception).

factor, but whose subtle structural differences prevent the analog from functioning as a growth factor in the cell, thereby disrupting cell metabolism. *Isoniazid* (Table 28.4) is an important growth factor analog with a very narrow spectrum of activity. Effective against mycobacteria only, isoniazid is an analog of nicotinamide, a vitamin required for mycolic acid synthesis, which is, in turn, required to construct the mycobacterial cell wall. Isoniazid is one of the most effective drugs for treatment of tuberculosis (▶ Section 31.4), but isoniazid resistance in strains of *Mycobacterium tuberculosis* is increasing.

Protein Synthesis as a Drug Target

Some antibiotics inhibit bacterial pathogens by disrupting protein synthesis (translation), often through interactions with the ribosome that may include binding to ribosomal RNA (rRNA) (Figure 28.12). Most of these drugs target bacterial (70S) ribosomes only and, therefore, have no effect on the structurally distinct, cytoplasmic ribosomes of eukaryotic cells. However, because mitochondria also contain 70S ribosomes (◀ Section 13.4), many antibiotics that inhibit protein synthesis in *Bacteria* also potentially inhibit mitochondrial protein synthesis in eukaryotic cells. Nevertheless, these drugs are still medically useful because 70S ribosomes in organelles are affected only at higher concentrations than are used for antimicrobial therapy.

The **aminoglycosides** are antibiotics that inhibit translation by targeting the 30S (small) subunit of the ribosome. Aminoglycosides contain amino sugars linked by glycosidic bonds, and clinically useful examples include streptomycin (produced by the bacterium *Streptomyces griseus*), kanamycin (Table 28.4), neomycin, and gentamicin. These broad-spectrum antibiotics are useful for treating infections caused by gram-negative *Bacteria*, but because of host toxicity effects that include kidney and hearing damage, aminoglycoside use has decreased since the development of the semisynthetic penicillins and tetracyclines. With the exception of gentamicin, which is routinely used to combat *Pseudomonas* infections, and neomycin, which is used in topical antibiotic ointments, aminoglycosides are generally administered only when other antibiotics fail.

Like the aminoglycoside antibiotics, **tetracyclines** interfere with the function of the 30S subunit of the ribosome. Tetracyclines are broad-spectrum antibiotics produced by several species of *Streptomyces*, and they inhibit most clinically relevant gram-positive and gram-negative *Bacteria*. The basic structure of tetracycline consists of a naphthacene ring system (Table 28.4), and side-chain substitutions (either natural or synthetic) to the various rings form new tetracycline analogs. Physicians are cautioned against the administration of tetracyclines to pregnant women and to young children because these antibiotics bind calcium in bones and teeth, weakening them and causing permanent staining of the latter (**Figure 28.15**). Tetracyclines are widely used in veterinary medicine, though, and in some countries, they are used routinely in healthy animals as nutritional supplements to suppress the growth of pathogens in poultry and swine, a practice that strongly selects for antibiotic resistance (◀ Section 8.11).

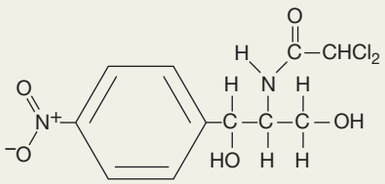
Macrolide antibiotics inhibit translation by targeting the 50S (large) subunit of the bacterial ribosome. Their basic structure contains a lactone ring bonded to sugars (as in erythromycin in Table 28.4), and variations in these constituents result in a diversity of macrolide antibiotics. Macrolides account for about 12% of global antibiotic production (Figure 28.13) and include, for example, erythromycin (produced by *Streptomyces erythreus*), clarithromycin, and azithromycin. The partial inhibition of protein synthesis by erythromycin, in particular, leads to preferential translation of some proteins and restricts translation of others, resulting in an imbalance in the proteome and potentially disrupting metabolic functions at all levels. Often used clinically in patients allergic to penicillin or other β-lactam antibiotics, erythromycin is particularly useful for treating legionellosis (▶ Section 33.4).

Nucleic Acid Synthesis as a Drug Target

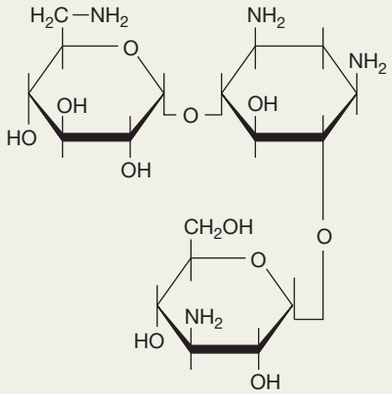
The **quinolones** are synthetic antibacterial compounds that disrupt bacterial metabolism by interfering with DNA gyrase, thus preventing the supercoiling and packaging of DNA in the bacterial cell (◀ Section 6.1). Because DNA gyrase is found in all *Bacteria*, quinolones are effective for treating both gram-positive and gram-negative bacterial infections. *Fluoroquinolones*, such as ciprofloxacin (Table 28.4), are fluorinated derivatives of quinolones that are routinely used to treat urinary tract infections and have been widely

TABLE 28.4 Selected antibacterial compounds

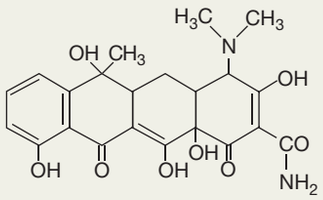
Mode of action	Antibiotic class	Example(s)	Representative structures
Inhibit cell wall synthesis	β-lactams	Penicillins, cephalosporins	Ceftriaxone
	Isoniazids	Isoniazid	Isoniazid
	Polypeptide antibiotics	Vancomycin, bacitracin	Vancomycin (see Figure 28.19)
Inhibit protein synthesis	Aminoglycosides	Streptomycin, kanamycin, gentamicin	Kanamycin
	Tetracyclines	Tetracycline, doxycycline	Tetracycline
	Macrolides	Erythromycin, azithromycin	Erythromycin
	Chloramphenicol	Chloramphenicol	Chloramphenicol



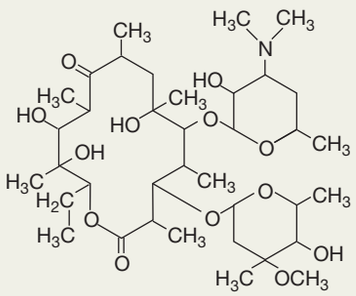
Chloramphenicol



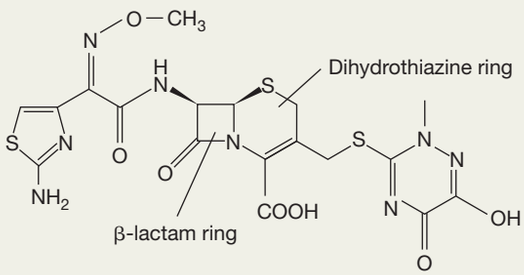
Kanamycin



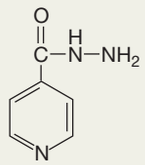
Tetracycline



Erythromycin



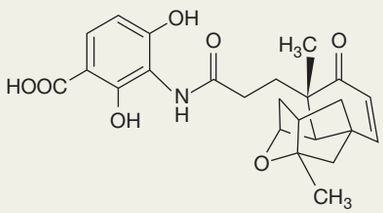
Ceftriaxone



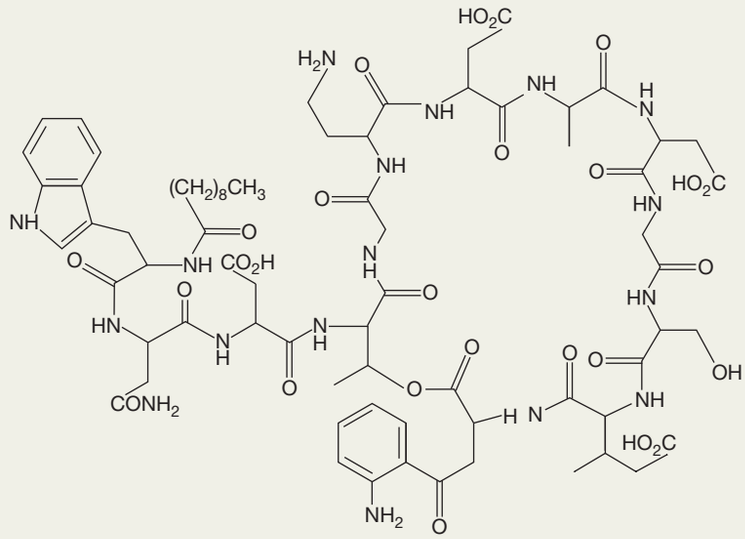
Isoniazid

TABLE 28.4 Selected antibacterial compounds *(continued)*

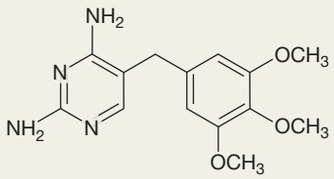
Mode of action	Antibiotic class	Example(s)	Representative structures
Inhibit nucleic acid synthesis	Quinolones and fluoroquinolones	Nalidixic acid, ciprofloxacin, moxifloxacin	Ciprofloxacin
	Rifamycins	Rifampin	Rifampin
Inhibit metabolite synthesis	Trimethoprim	Trimethoprim	Trimethoprim
	Sulfa drugs	Sulfanilamide, sulfamethoxazole	Sulfanilamide
Damage to the cytoplasmic membrane	Lipid biosynthesis disruptor	Platensimycin	Platensimycin
	Membrane structure disruptor	Daptomycin, polymyxins	Daptomycin



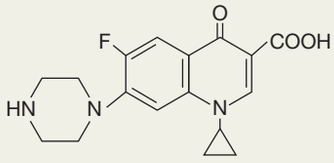
Platensimycin



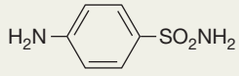
Daptomycin



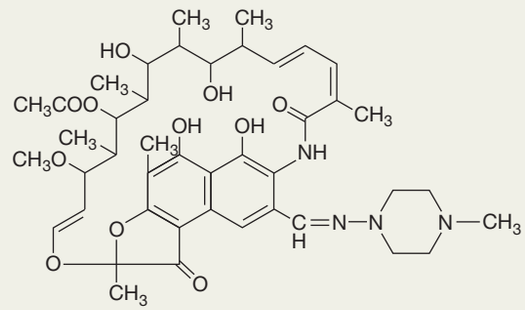
Trimethoprim



Ciprofloxacin



Sulfanilamide



Rifampin

Figure 28.15 Staining of teeth from the use of tetracycline. Tetracycline binds calcium in developing bones and teeth, weakening them and causing permanent staining of tooth enamel. Therefore, tetracycline should not be administered to pregnant women or children unless absolutely necessary.

used in the beef and poultry industries for prevention and treatment of respiratory diseases in animals. Ciprofloxacin is also the drug of choice for treating anthrax because some strains of *Bacillus anthracis*, the causative agent of anthrax (▶ Section 32.8), are resistant to penicillin. The fluoroquinolone moxifloxacin is effective for treatment of tuberculosis; in combination with other anti-tuberculosis drugs, moxifloxacin may significantly reduce treatment time, a major problem with isoniazid-based treatments. However, in some individuals the use of fluoroquinolones has been accompanied by serious side effects, including tendon and organ damage and neurotoxicity, although why these occur is not well understood.

Some antibiotics disrupt transcription by inhibiting RNA synthesis (Figure 28.12). For example, rifamycins, such as *rifampin* (Table 28.4), inhibit RNA synthesis by binding to the β-subunit of RNA polymerase in *Bacteria*. Rifampin, often used in concert with isoniazid for treatment of tuberculosis, has the odd side effect of causing body secretions, including tears, urine, and sweat, to turn reddish-orange (**Figure 28.16**). *Actinomycin* inhibits RNA synthesis by combining with DNA and blocking RNA elongation. This agent binds most strongly to DNA at guanine–cytosine base pairs, fitting into the major groove in the double strand where RNA is synthesized.

Other Antibacterial Drug Targets

Like isoniazids, **sulfa drugs** (also called *sulfonamides*) are synthetic growth factor analogs. However, instead of affecting the cell wall, sulfa drugs block a key biosynthetic pathway in *Bacteria*. Sulfanilamide (Table 28.4), the simplest sulfa drug, is an analog of *p*-aminobenzoic acid (PABA), which is a component of the vitamin folic acid, a nucleic acid precursor. By mimicking PABA, sulfanilamide blocks the synthesis of folic acid, thereby inhibiting nucleic acid synthesis. Sulfanilamide is selectively toxic because bacteria synthesize their own folic acid, unlike humans and most animals, which obtain folic acid from their diet. Antimicrobial therapy with sulfamethoxazole (a sulfa drug) plus trimethoprim, a related folic acid synthesis competitor, is highly effective because the drug combination blocks two sequential steps in the folic acid synthesis pathway (Figure 28.12); resistance to this drug combination therefore requires two mutations in genes of the same pathway, a relatively rare event. However, resistance to sulfonamides is increasing as many formerly susceptible pathogens develop the ability to import folic acid from their environment.

Some antibiotics have atypical structures or targets. For example, the antibiotic *daptomycin* (Table 28.4) is produced by a species of *Streptomyces* and is a cyclic lipopeptide with a unique mode of action. Used mainly to treat infections by gram-positive *Bacteria*, including pathogenic streptococci and staphylococci, daptomycin binds specifically to bacterial cytoplasmic membranes, forms a pore, and induces rapid depolarization of the membrane. The depolarized cell quickly loses its ability to synthesize macromolecules, such as nucleic acids and proteins, resulting in cell death. However, mutations leading to alterations in cytoplasmic membrane structure may lead to resistance.

Platensimycin (Table 28.4), produced by *Streptomyces platensis*, is an unusual antibiotic that inhibits fatty acid and lipid biosynthesis. Platensimycin is effective against a broad range of gram-positive *Bacteria*, including nearly untreatable infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci. Platensimycin has a unique mode of action, shows no host toxicity, and there is no known potential for development of resistance by pathogens. We discuss the discovery of platensimycin in Section 28.7.

Check Your Understanding

- Explain the concept of selective toxicity in terms of antimicrobial therapy.
- How does the activity of each antibiotic class lead to control of the affected pathogens?
- What are the sources of aminoglycosides, tetracyclines, macrolides, daptomycin, and platensimycin?

Figure 28.16 Reddish-orange urine from rifampin usage. The antibiotic rifampin is often administered to tuberculosis patients. However, a side effect of rifampin usage is that it turns urine and other body fluids reddish-orange.

28.6 Antimicrobial Drugs That Target Nonbacterial Pathogens

Many pathogens are nonbacterial—such as viruses or eukaryotes—and are often difficult to treat. Antiviral drugs often adversely affect the host as well as the pathogen because viruses depend on host cell biosynthetic machinery for their replication (Chapter 5). As a result, selective toxicity for viruses is achieved only with agents that preferentially affect unique viral replication pathways or the assembly of viral components. In spite of these limitations, a number of drugs are more toxic for viruses than for the host, and a few agents specifically target individual viruses. Fungi, protozoa, and helminths (worms) pose special problems for the development of effective drugs because these pathogens are eukaryotic, and therefore their cellular biology is similar to that of humans.

Antiviral Drugs

The most successful and commonly used agents for antiviral chemotherapy are the *nucleoside analogs*. These drugs are **nucleoside reverse transcriptase inhibitors (NRTIs)**, which work by inhibiting elongation of the viral nucleic acid chain by a nucleic acid polymerase. The first NRTI to be widely used was *zidovudine*, also called *azidothymidine* (*AZT*) (Table 28.5; ▶ Figure 31.49*a*), which effectively blocks reverse transcription and production of complementary DNA (cDNA) in HIV and other retroviruses (▶ Section 31.15). It is structurally similar to thymidine but is a dideoxy derivative, lacking the 3′-hydroxyl group. Another widely used nucleoside analog, *acyclovir* (Table 28.5), resembles guanosine and has been successfully used to control the symptoms of herpesvirus infections (▶ Section 31.14).

Some antiviral agents target the key enzyme of retroviruses, reverse transcriptase. *Nevirapine* (▶ Figure 31.49*b*), a **nonnucleoside reverse transcriptase inhibitor (NNRTI)**, binds directly to reverse transcriptase and inhibits reverse transcription (Table 28.5). Phosphonoformic acid, an analog of inorganic pyrophosphate, inhibits normal internucleotide linkages, preventing synthesis of viral nucleic acids. Because their action may affect normal host cell nucleic acid synthesis, both NRTIs and NNRTIs often induce some host toxicity.

Other anti-retroviral drugs include **protease inhibitors** and *enfuvirtide*, a **fusion inhibitor** (Table 28.5). Protease inhibitors disrupt viral replication by binding the active site of HIV protease (see Figure 28.20*a*), preventing this enzyme from processing large viral polyproteins into several individual viral components (◀ Section 11.8) including reverse transcriptase and integrase. Enfuvirtide (marketed as Fuzeon®) is a 36-amino-acid synthetic peptide that binds to the gp41 membrane protein of HIV; this stops the conformational changes necessary for the fusion of HIV with T lymphocyte membranes, thus preventing infection of host immune cells by HIV (▶ Figure 31.44).

A single category of drugs limits influenza infection: The *neuraminidase inhibitors* include oseltamivir (marketed as Tamiflu®), zanamivir (marketed as Relenza®), and peramivir (marketed as Rapivab®). These drugs block the active site of neuraminidase (◀ Figure 11.24) in influenza A and B viruses, inhibiting virus release from infected cells (Table 28.5). Oseltamivir is used for both treatment and prophylaxis of influenza, but its use has, in some cases, been linked to adverse side effects that include behavioral abnormalities, especially in children. Zanamivir and peramivir are suitable for children, but because zanamivir is an inhaled drug, it is not recommended for those with asthma or other respiratory conditions.

TABLE 28.5 Antiviral compounds

Examples	Mechanism of action	Virus affected
Enfuvirtide	Blocks fusion of HIV with T lymphocyte membrane	HIV (human immunodeficiency virus)
α-, β-, γ-Interferon	Induces proteins that inhibit viral replication	Broad spectrum (host-specific)
Oseltamivir and zanamivir	Block active site of influenza neuraminidase	Influenza A and B
Nevirapine	Reverse transcriptase inhibitor	HIV
Acyclovir (▶ Figure 31.43)	Viral polymerase inhibitor	Herpesviruses, *Varicella zoster*
Zidovudine (AZT) (▶ Figure 31.49*a*)	Reverse transcriptase inhibitor	HIV
Ribavirin	Blocks capping of viral RNA	Respiratory syncytial virus, influenza A and B, Lassa fever
Cidofovir	Viral polymerase inhibitor	Cytomegalovirus, herpesviruses
Tenofovir (TDF)	Reverse transcriptase inhibitor	HIV
Indinavir, saquinavir (Figure 28.20*b*)	Viral protease inhibitors	HIV

Recall from Chapter 26 that virus-infected cells release small cytokine proteins called *interferons* that trigger a defensive response in neighboring host cells (◀ Section 26.10). If produced and administered properly, interferons may have potential use as prescribed antimicrobial agents. The clinical utility of interferons depends on whether they can be delivered to specific areas in the host to stimulate the production of antiviral proteins in uninfected host cells. Alternatively, appropriate interferon stimulators, such as viral nucleotides, nonvirulent viruses, or even synthetic nucleotides, if given to host cells prior to infection, may induce natural production of interferon.

Drugs That Target Eukaryotic Pathogens

Fungi cause a number of serious diseases (▶ Sections 34.1 and 34.2), and their treatment is complicated by the fact that many antifungal agents target metabolic pathways that are shared by fungi and their host cells, thus making the drugs toxic. As a result, many antifungal drugs can be used only for topical (surface) applications (Table 28.6).

A major group of antifungal compounds are *ergosterol inhibitors*, which work either by interacting directly with ergosterol or inhibiting its synthesis (Table 28.6). Ergosterol is present in fungal cytoplasmic membranes in place of the cholesterol found in animal cell cytoplasmic membranes. Important ergosterol inhibitors include

TABLE 28.6 Antifungal agents

Category	Target	Examples	Use
Allylamines	Ergosterol synthesis	Terbinafine	Oral, topical
Aromatic antibiotic	Mitosis inhibitor	Griseofulvin	Oral
Azoles	Ergosterol synthesis	Clotrimazole	Topical
		Fluconazole	Oral
		Miconazole	Topical
Chitin synthesis inhibitor	Chitin synthesis	Nikkomycin Z	Experimental
Echinocandins	Cell wall synthesis	Caspofungin	Intravenous
Nucleic acid analogs	DNA synthesis	5-Fluorocytosine	Oral
Polyenes	Ergosterol synthesis	Amphotericin B	Oral, intravenous
		Nystatin	Oral, topical
Polyoxins	Chitin synthesis	Polyoxin A and B	Agricultural

the *polyene* antibiotics, which are produced by species of *Streptomyces* bacteria. Polyenes bind specifically to ergosterol, causing membrane permeability and cell death (**Figure 28.17**). By contrast, *azoles* and *allylamines* are broad-spectrum antifungal drugs that work by selectively inhibiting ergosterol biosynthesis. Treatment with these drugs causes membrane damage and alteration of critical membrane transport activities.

Echinocandins are cell wall inhibitors that block the activity of 1,3-β-D-glucan synthase, the enzyme that forms β-glucan polymers in the fungal cell wall (Figure 28.17 and Table 28.6). Because mammalian cells do not have 1,3-β-D-glucan synthase (or cell walls), the activity of these agents selectively kills fungal cells. Echinocandins are often used to treat *Candida* yeast infections, as well as some fungal pathogens that are resistant to azoles and other agents.

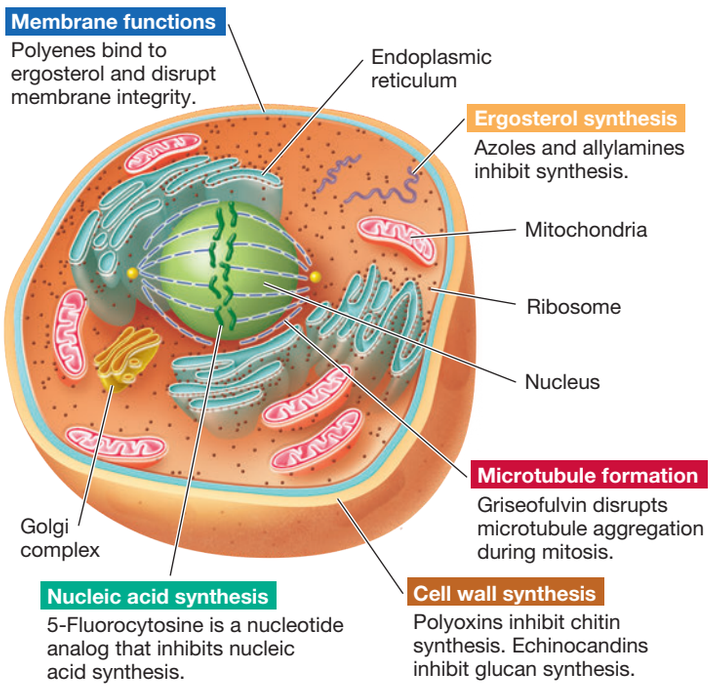


Figure 28.17 Targets of some antifungal agents. Traditional antibacterial agents are generally ineffective because fungi are eukaryotic cells. The cell wall and ergosterol (present in the cytoplasmic membrane) of fungal cells make good selective targets because they are unique structures not present in vertebrate host cells.

Fungal cell walls also contain chitin, a polymer of *N*-acetylglucosamine found only in fungi and insects. Several *polyoxins* inhibit cell wall synthesis by interfering with chitin biosynthesis (Figure 28.17). Although not used clinically, polyoxins are widely used as agricultural fungicides. Other antifungal drugs inhibit folate biosynthesis, interfere with DNA topology during replication, or, in the case of drugs such as griseofulvin, disrupt microtubule aggregation during mitosis (Figure 28.17). Moreover, the nucleic acid analog 5-fluorocytosine (flucytosine) is an effective nucleic acid synthesis inhibitor in fungi.

Historically, protozoan-caused diseases, especially malaria, have been treated with *quinine* or, more recently, quinine derivatives, such as chloroquine and mefloquine. However, extensive resistance to these drugs among the *Plasmodium* spp. that cause malaria (▶ Section 34.5) has led to the development of artemisinin-based alternatives. Artemisinin is produced in low amounts by the Chinese wormwood plant (*Artemisia*), which has traditionally been used to control the cyclic fever associated with malaria. A more recent discovery has shown artemisinin to also be an effective treatment for certain helminth diseases, such as schistosomiasis (▶ Section 34.7). Using the techniques of synthetic biology, a genetically engineered yeast strain is now being used to produce artemisinin, making the drug more widely available (◀ Section 12.11 and Figure 12.37).

The drug of choice to combat many parasitic protozoans is *metronidazole* (or the related drug *tinidazole*), which targets anaerobic pathogens and is therefore also useful against infections caused by clostridia. Infections with *Giardia intestinalis* (giardiasis), *Trichomonas vaginalis* (trichomoniasis), and especially *Entamoeba histolytica* (amebic dysentery) have all been treated successfully with these drugs (▶ Sections 34.3 and 34.4). The related protist *Cryptosporidium parvum* (cryptosporidiosis) is often treated with *nitazoxanide*, a drug that has also been effective against helminths. However, perhaps the most widely used antihelminthic drugs are *praziquantel*, for treating

Mastering Microbiology
Art Activity: Figure 28.32 Targets of some antifungal agents

schistosomiasis and tapeworm infections, and *mebendazole* (and similar drugs), for treating a variety of helminth infections, including tapeworms, pinworms, hookworms, trichinosis, and ascariasis (▶ Section 34.7).

We now tackle the topic of drug resistance, a consequence of the widespread use of antimicrobial agents in clinical and veterinary medicine and animal husbandry.

Check Your Understanding

- What steps in the viral maturation process are inhibited by nucleoside analogs? By protease inhibitors? By interferons?
- Why are there fewer clinically effective antifungal and antiparasitic agents than antibacterial agents?

28.7 Antimicrobial Drug Resistance and New Treatment Strategies

Antimicrobial drug resistance is the acquired ability of a microorganism to resist the effects of an antimicrobial agent to which it was formerly susceptible. As we have discussed, many microorganisms produce antibiotics, and genes encoding antibiotic resistance are present in virtually all of them. Horizontal gene transfer (Chapters 9 and 13) between and among microorganisms disseminates antimicrobial drug resistance.

Antimicrobial Drug Resistance

Common mechanisms of bacterial resistance to antibiotics were discussed in the context of microbial growth in Section 8.11 and depicted in Figure 8.28, and some examples are listed in **Table 28.7**.

Antibiotic resistance can be genetically encoded on the bacterial chromosome, but more often, antibiotic-resistant bacteria isolated from patients contain drug-resistance genes located on horizontally transmitted *R* (*resistance*) *plasmids* (◀ Section 6.2). Enzymes encoded by genes on R plasmids confer resistance by any of three classes of mechanisms: modifying and inactivating the drug, preventing uptake of the drug, or actively pumping the antibiotic out of the cell, a process called *efflux* (Table 28.7).

The widespread use of antibiotics (Figure 28.13*a*) provides favorable conditions for the spread of R plasmids because they carry genes that confer an immediate selective advantage. The ubiquity of resistance genes limits the long-term use of any single antibiotic as an effective antimicrobial agent. A classic example is the development of multidrug resistance in the bacterium *Neisseria gonorrhoeae*. Penicillin, widely used to treat gonorrhea into the 1980s, became ineffective and was replaced by ciprofloxacin, which, in turn, lost much of its efficacy after just 10 years of use (**Figure 28.18**). This prompted a switch in treatment to the β-lactamase-resistant ceftriaxone, and more recently, a combination of ceftriaxone and azithromycin. Such combinations of two unrelated antimicrobial agents often reduce resistance because it is less likely that a mutant strain resistant to one antibiotic will also be resistant to the second antibiotic. However, certain R plasmids confer multiple drug resistance and can thwart multiple antibiotic therapy as a clinical strategy.

Overuse of antibiotics also accelerates resistance. In addition to their traditional use as a treatment for infections, antibiotics are widely used as supplements to farm animal feeds both as growth-promoting substances and as prophylactic additives to prevent the occurrence of disease. For example, broad-spectrum antibiotics

TABLE 28.7 Bacterial resistance to antibiotics

Resistance mechanism	Antibiotic example	Genetic basis of resistance	Mechanism present in
Reduced permeability	Penicillins	Chromosomal	Gram-negative bacteria
Inactivation of antibiotic Examples: βlactamases; modifying enzymes, such as methylases, acetylases, phosphorylases, and others	Penicillins	Plasmid and chromosomal	*Staphylococcus aureus* Enteric bacteria
	Chloramphenicol	Plasmid and chromosomal	*Neisseria gonorrhoeae* *Staphylococcus aureus*
	Aminoglycosides	Plasmid	Enteric bacteria
Alteration of target Examples: RNA polymerase, rifamycin; ribosome, erythromycin and streptomycin; DNA gyrase, quinolones	Erythromycin	Chromosomal	*Staphylococcus aureus*
	Rifamycin		Enteric bacteria
	Streptomycin		Enteric bacteria
	Norfloxacin		Enteric bacteria *Staphylococcus aureus*
Development of resistant biochemical pathway	Sulfonamides	Chromosomal	Enteric bacteria *Staphylococcus aureus*
Efflux (pumping out of cell)	Tetracyclines	Plasmid	Enteric bacteria
	Chloramphenicol	Chromosomal	*Staphylococcus aureus* *Bacillus subtilis*
	Erythromycin	Chromosomal	*Staphylococcus aureus*

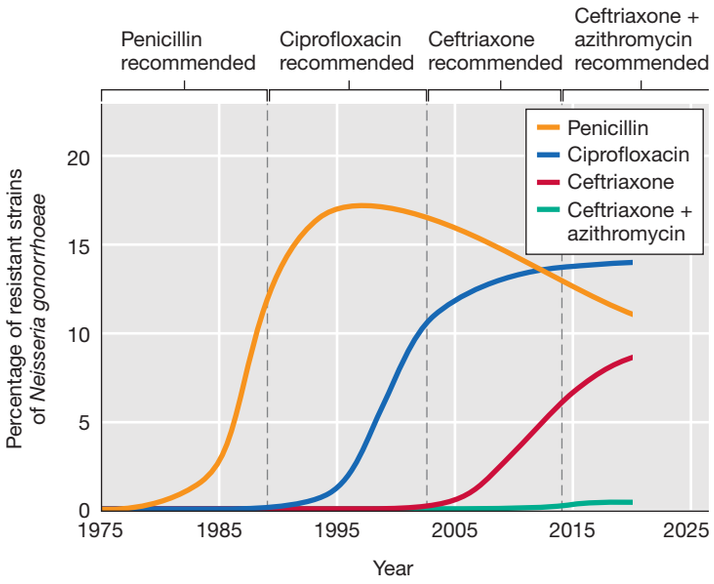


Figure 28.18 The emergence of multidrug resistance in *Neisseria gonorrhoeae*. Resistance to penicillin developed in the 1980s to the point that it could no longer be recommended for treatment of gonorrhea, at which point ciprofloxacin, a quinolone, became the drug of choice. By the early 2000s, resistance to ciprofloxacin prompted a change to ceftriaxone. The current gonorrhea treatment recommendation is a combination of ceftriaxone plus azithromycin.

widely used clinically, such as the fluoroquinolone ciprofloxacin, were used extensively as a feed additive, especially by the poultry industry. In addition, although the trend is improving, antibiotics are used in clinical practice far more often than necessary, and this problem is compounded by patient noncompliance—many patients stop taking antibiotics as soon as they feel better. Antibiotic resistance can be minimized if drugs are used only for treatment of susceptible pathogens and are given in sufficiently high doses and for sufficient lengths of time to eradicate the pathogen before resistant mutants can propagate.

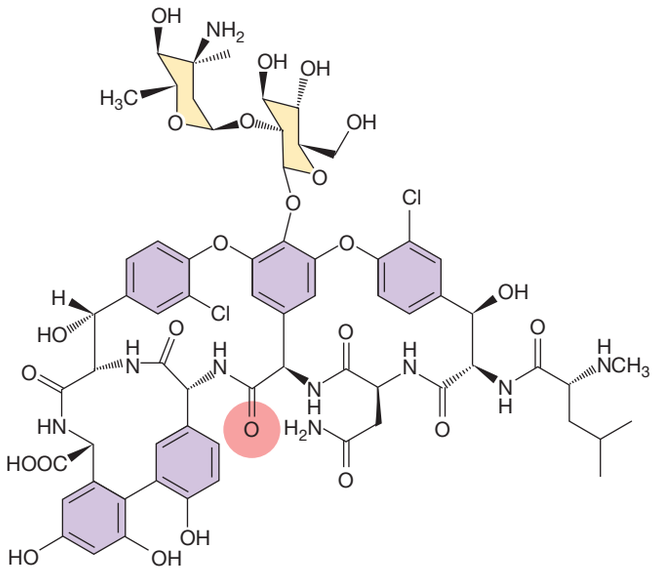
To prevent the further emergence of multidrug-resistant pathogens, the U.S. Centers for Disease Control and Prevention (CDC) publishes guidelines stressing the importance of vaccination, rapidly and accurately diagnosing and treating infections, using antimicrobial agents prudently, and preventing pathogen transmission.

New Drugs and New Treatment Strategies

Conservative, appropriate administration of antibiotics can prolong or even resurrect the clinical usefulness of available drugs, but the long-term solution to antimicrobial drug resistance requires continuous development of new drugs through design or discovery. Developing new analogs of existing antimicrobial compounds is usually more cost effective than discovering new drugs, and analogs may actually have greater antimicrobial activity than the parent compound. For example, using natural penicillin as the starting compound, systematic chemical substitution of the *N*-acyl group can generate hundreds of penicillin derivatives, many with broad-spectrum activity (Figure 28.14). Using this basic strategy, semisynthetic analogs of β-lactam antibiotics, tetracycline, and vancomycin (**Figure 28.19**) have been synthesized.

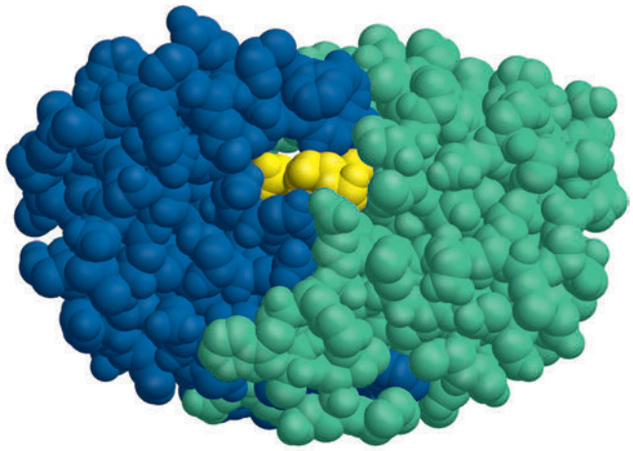
Novel antimicrobial compounds are more difficult to identify than analogs of existing drugs because new antimicrobial compounds must work at unique sites in metabolism or be structurally dissimilar to existing compounds to avoid resistance mechanisms. Computer-based methods accelerate novel compound design by maximizing molecular binding in a virtual environment. A dramatic success in computer-directed drug design was the development of *saquinavir*, a protease inhibitor that impedes the multiplication of HIV in infected individuals by binding the active site of the HIV protease enzyme (**Figure 28.20**). As an analog of the HIV precursor protein, saquinavir (marketed as Invirase®) displaces the authentic protease substrate and inhibits virus maturation. Several other computer-designed protease inhibitors, including *indinavir* (marketed as Crixivan®) (Figure 28.20*b*), are currently in use for the treatment of HIV/AIDS (▶ Section 31.15).

Another strategy to overcome antimicrobial resistance is to select for antibiotics that interact with unexploited targets. Platensimycin is the first antimicrobial drug targeted to disrupt bacterial lipid biosynthesis (Table 28.4 and Figure 28.12). Platensimycin is especially active against gram-positive pathogens, including drug-resistant staphylococci and enterococci. To select an agent for a defined target, in this case an enzyme in the bacterial lipid synthesis pathway, scientists used antisense RNA (◀ Section 7.12) to limit the amount of accessible mRNA required to produce a key lipid biosynthesis enzyme in *Staphylococcus aureus*. This reduced fatty acid synthesis in the cells

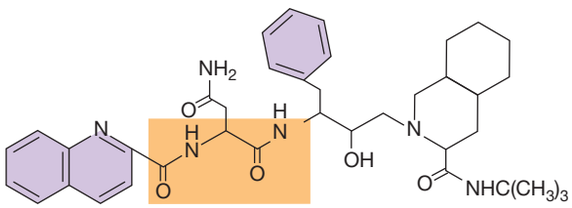


Vancomycin

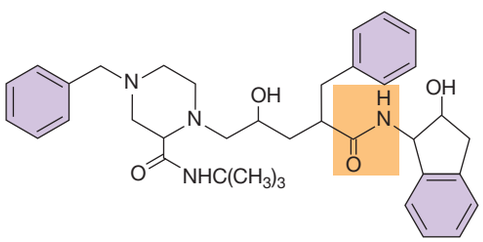
Figure 28.19 Vancomycin. Many pathogenic strains now show intermediate drug resistance to the parent structure of vancomycin, but chemically substituting the carbonyl oxygen at the position shown in red with a methylene ($=CH_2$) group restores much of the lost activity. Like penicillin, vancomycin prevents cross-linking of peptidoglycan and is most effective against gram-positive pathogens.



(a) **HIV protease**



Saquinavir



Indinavir

(b)

Figure 28.20 Computer-generated anti-HIV drugs. *(a)* The HIV protease homodimer. Individual polypeptide chains are shown in green and blue. A peptide (yellow) is bound in the active site. HIV protease cleaves an HIV precursor protein, a necessary step in virus maturation. Blocking of the protease site by the bound peptide inhibits precursor processing and HIV maturation. This structure is derived from coordinates in the Protein Data Bank. *(b)* These anti-HIV drugs are peptide analogs called protease inhibitors that were designed by computer to block the active site of HIV protease. The areas highlighted in orange show the regions analogous to peptide bonds in proteins.

and increased the sensitivity of the crippled *S. aureus* strain to antibiotics that inhibit fatty acid synthesis. After screening thousands of natural products from potential antibiotic producers, scientists isolated platensimycin from the soil bacterium *Streptomyces platensis*, a species of the filamentous *Actinobacteria* (◀ Section 16.12). This strategy identifies target-specific antibiotics present in low concentrations and is applicable to virtually any target for which the gene sequence (and, hence, the corresponding antisense RNA sequence) is known.

The efficacy of some antibiotics can be preserved if they are administered with compounds that thwart antibiotic resistance mechanisms. For example, several β-lactam *antibiotics* can be combined with β-lactamase *inhibitors* to preserve antibiotic activity in β-lactam-resistant microbes. For instance, amoxicillin (similar to ampicillin, Figure 28.14) can be mixed with the β-lactamase inhibitor clavulanic acid to produce the combination drug Augmentin®. The inhibitor binds β-lactamase (produced by the resistant pathogen) irreversibly. This prevents amoxicillin from being degraded and maintains the efficacy of the antibiotic.

Drug combination therapy has also revolutionized treatment of HIV infections. Currently, a combination therapy consisting of nucleoside analogs and a protease inhibitor is recommended. This drug treatment protocol is termed HAART, for *h*ighly *a*ctive *a*nti*r*etroviral *t*herapy (▶ Section 31.15). As with antibacterial combination regimens, HAART is designed to target two independent viral functions: nucleoside analogs target virus replication, and protease inhibitors target virus maturation. Because the probability of a virus developing resistance to multiple drugs is much less than the probability of it becoming resistant to a single drug, HAART therapy has been a successful strategy in controlling HIV infections.

Check Your Understanding

- Identify the basic mechanisms of antibiotic resistance in bacteria and describe what practices encourage their dissemination.
- What does vancomycin have in common with penicillin? How can native vancomycin be improved?
- Explain the advantages and disadvantages of developing new drugs based on existing drug analogs. What other methods exist for developing new drugs?

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CHAPTER REVIEW

I • Disorders and Deficiencies of the Immune System

28.1 Hypersensitivity is the induction by foreign antigens of antibody-mediated or cell-mediated immune responses that damage host tissue. In autoimmunity, the immune response is directed against self antigens. Damage to host tissue is caused by the inflammation produced by immune mechanisms.

Q How do immediate and delayed-type hypersensitivities differ from one another in terms of immune effectors, target tissues, antigens, and clinical outcome?

28.2 Superantigens are components of certain bacterial and viral pathogens that bind outside the antigen-specific binding site of TCRs and, therefore, activate large numbers of T cells. Superantigen-activated T cells may produce diseases characterized by systemic inflammatory reactions. Immunodeficiency is the inability to generate a proper immune response, resulting in recurrent, uncontrollable infections by opportunistic pathogens. Some of the most severe immunodeficiency syndromes are SCID, caused by a genetic disorder, and AIDS, caused by HIV infection.

Q How do superantigens differ from conventional antigens in terms of initial T cell activation and clinical outcome? How does immunodeficiency resulting from SCID differ from that caused by HIV infection and AIDS?

II • Vaccines and Immunotherapy

28.3 Immunization induces artificial active immunity and is widely used to prevent infectious diseases. Vaccines are either attenuated or inactivated pathogens or pathogen products or are genetically engineered antigens. The latter eliminate exposure to pathogens and, in some cases, even to protein antigen. Application of this strategy is providing safer vaccines targeted to individual pathogen antigens.

Q List the immunizations recommended for children in the United States. For which of these have you been immunized? List the diseases for which you may have acquired immunity naturally.

28.4 A growing number of diseases, including a variety of cancers and autoimmune disorders, are being treated by immunotherapy, a clinical strategy that optimizes the adaptive immune response to a disease using bioengineered immune components. The identification of tumor-specific neoantigens in cancer cells can allow for production of customized monoclonal antibodies (mAbs), chimeric antigen receptor (CAR) T cells, and anticancer vaccines. The efficacy of immunotherapeutic treatments varies between individuals, and this may be linked to the composition of their gut microbiota.

Q What is a "neoantigen"? How are they identified, and how can they be used to develop mAbs, CAR T cells, and anticancer vaccines?

III • Drug Treatments for Infectious Diseases

28.5 Antibiotics are chemically diverse antimicrobial compounds produced by microorganisms. Each antibiotic works by inhibiting a specific cellular process in the target microorganisms. The β-lactam antibiotics, including penicillins and cephalosporins, target bacterial cell wall synthesis and are the most important class of clinical antibiotics. The aminoglycosides, macrolides, and tetracycline antibiotics selectively interfere with protein synthesis in *Bacteria*. The quinolones are an important class of synthetic antibacterial drugs that inhibit DNA synthesis. Daptomycin and platensimycin are structurally novel antibiotics that target cytoplasmic membrane functions and lipid biosynthesis, respectively.

Q What are the common sources for natural antibiotics? How do these antimicrobial drugs differ from growth factor analogs, such as the sulfa drugs? Why are β-lactam antibiotics generally more effective against gram-positive bacteria than against gram-negative bacteria?

28.6 Antiviral agents selectively target virus-specific enzymes and processes. Useful agents include analogs and compounds that inhibit nucleic acid polymerases and viral genome replication. Protease inhibitors interfere with viral maturation steps. Because fungi are *Eukarya*, antifungal agents exhibiting selective toxicity are difficult to find. Nevertheless, some effective antifungal agents are available, and they primarily target fungi-specific structures and biosynthetic processes.

Q Why is host toxicity a common problem with antiviral and antifungal drugs? Identify the targets that allow for selective toxicity of antifungal agents.

28.7 The use of antimicrobial drugs inevitably leads to resistance in the targeted microorganisms. The development of resistance can be accelerated by the indiscriminate use of antimicrobial drugs, and many pathogens have developed resistance. New antimicrobial compounds must continually be discovered and developed to deal with drug-resistant pathogens and to enhance our ability to treat infectious diseases. Computer-based modeling and other novel strategies are helping to address this challenge.

Q What practices contribute to the spread of antibiotic resistance? Explain how antisense RNA strategies can extend traditional methods of natural product selection for antibiotic discovery.

APPLICATION QUESTIONS

1. What problems would arise if a person had a hereditary deficiency that resulted in an inability to present antigens to Tc cells? What would the problems be if the person had a deficiency in presenting antigen to Th1 cells? To Th2 cells? To all T cells? What molecules might be deficient in each situation? Could a person having any one of these deficiencies survive in a normal environment? Explain for each.
2. Viruses and fungi present special problems for drug therapy. Explain the issues inherent in drug treatment of both groups and explain whether you agree with the preceding statement. Give specific examples and suggest at least one group of agents that might target both types of infectious agents.
3. Describe three important reasons why semisynthetic penicillins were first developed. Which clinical challenges do each of these reasons address? What key part of the penicillin molecule must be retained for any semisynthetic penicillin to be active?

CHAPTER GLOSSARY

Allergen an antigen that elicits an immediate hypersensitivity (allergic) reaction upon exposure

Aminoglycoside an antibiotic, such as streptomycin, containing amino sugars linked by glycosidic bonds

Antibiotic a chemical substance produced by a microorganism that kills or inhibits the growth of another microorganism

Antimicrobial drug resistance the acquired ability of a microorganism to resist the effects of an antimicrobial agent to which it was formerly susceptible

Autoantibody an antibody that reacts to self antigens

Autoimmunity a harmful immune reaction directed against self antigens

β-lactam antibiotic penicillin, cephalosporin, or a related antibiotic that contains the four-membered heterocyclic β-lactam ring

Delayed-type hypersensitivity (DTH) an inflammatory allergic response mediated by Th1 lymphocytes

Fusion inhibitor a synthetic polypeptide that binds to viral glycoproteins, inhibiting fusion of viral and host cell membranes

Growth factor analog a chemical agent that has a similar structure to and blocks the uptake or utilization of a growth factor

Hypersensitivity an immune response leading to damage to host tissues

Immediate hypersensitivity an allergic response mediated by vasoactive products released from IgE-sensitized mast cells

Immunotherapy the treatment of a disease, especially cancer, using targeted therapeutic agents that either enhance or suppress the immune response

Macrolide erythromycin or a related antibiotic that contains a lactone ring bonded to sugars

Nonnucleoside reverse transcriptase inhibitor (NNRTI) a nonnucleoside compound that inhibits the action of retroviral reverse transcriptase by binding directly to the catalytic site

Nucleoside reverse transcriptase inhibitor (NRTI) a nucleoside analog compound that inhibits the action of retroviral reverse transcriptase by competing with nucleosides

Protease inhibitor a compound that inhibits the action of viral protease by binding directly to the catalytic site, preventing viral protein processing

Quinolone a synthetic antibacterial compound that inhibits DNA gyrase and prevents supercoiling of bacterial DNA

Selective toxicity the ability of a compound to inhibit or kill a pathogen without adversely affecting the host

Sulfa drugs synthetic growth factor analogs that inhibit folic acid biosynthesis in *Bacteria*

Superantigen a pathogen product capable of eliciting an inappropriately strong inflammatory immune response by stimulating greater than normal numbers of T cells

Tetracycline an antibiotic characterized by the four-member naphthacene ring structure

Vaccination (immunization) the inoculation of a host with inactive or weakened pathogens or pathogen products to stimulate protective active immunity

Vaccine an inactivated or attenuated pathogen, or a harmless pathogen product, used to induce artificial active immunity

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MICROBIOLOGY**NOW**

Shedding New Light on Diagnosing Tuberculosis

Few pathogens have caused more human deaths than *Mycobacterium tuberculosis* (photo), the causative agent of tuberculosis (TB). Due to the slow growth of *M. tuberculosis* and the sluggish progression of symptoms associated with TB, it can be difficult to rapidly and reliably detect active disease using traditional diagnostic tools. Further complicating the matter is that the prevalence of TB is high in less developed countries where adequate medical facilities or diagnostic supplies are often lacking. These factors can cause delayed diagnosis of TB, which compromises both patient care and public health efforts to control the disease.

For these reasons, a quick and reliable point-of-care diagnostic test for TB is sorely needed, and one may soon be on the way. Trehalose, a sugar that has been increasingly used as a food additive in recent years, is a precursor building block for the structurally unusual cell wall of *M. tuberculosis*. With this in mind, researchers have recently targeted trehalose as a chemical marker to reveal the presence of *M. tuberculosis* in clinical specimens, such as in the sputum samples of patients suspected of having TB.

To use trehalose incorporation as a diagnostic assay, the sugar molecule had to be chemically modified to make it easily detectable. The indicator dye 4-*N*,*N*-dimethylamino-1, 8-naphthalimide (DMN) was chosen for this purpose because it fluoresces brightly in a hydrophobic environment. By conjugating DMN to trehalose (creating DMN-Tre) and subsequently allowing living *M. tuberculosis* cells to incorporate the metabolized DMN-Tre into their waxy (and thus hydrophobic) cell wall, the researchers were, within minutes, able to easily and specifically visualize cells of the glowing pathogen in sputum samples. In addition, because only healthy, growing cells incorporate DMN-Tre and fluoresce, this method may also provide a quick way to determine antibiotic susceptibility for individualized treatment plans.

Source: Kamariza, M., et al. 2018. Rapid detection of *Mycobacterium tuberculosis* in sputum with a solvatochromic trehalose probe. *Sci. Transl. Med. 10*: eaam6310. doi:10.1126/scitranslmed.aam6310.

I • Microbiology and the Healthcare Environment

Considering the prevalence of multidrug-resistant pathogens and healthcare-associated infections, the importance of safety protocols and personal protective equipment in the clinical microbiology laboratory cannot be overstated.

Clinical microbiology is a subdiscipline of microbiology whose focus is diagnosing infectious diseases by identifying pathogenic microbes and advising medical providers on treatment. Clinical laboratories must identify pathogens safely, efficiently, and reliably. The clinical microbiologist examines patient samples using direct observation, culture, immunological assays, and molecular tools to identify particular pathogens and rule out infection by others. Rapid and accurate identification of pathogens is critical for developing successful treatment strategies and maintaining disease control.

29.1 The Clinical Microbiology Laboratory

Clinical laboratories handle dangerous materials, and thus laboratory workers must adhere to strict safety protocols to prevent the spread of infectious agents. Standard laboratory practices for handling clinical samples have been established to minimize the risk of accidental laboratory infections. One only has to be reminded of the 2014 Ebola hemorrhagic fever outbreak in West Africa (▶ Section 31.12) to appreciate how treating infected persons without paying rigorous attention to every safety detail can endanger the lives of medical personnel.

TABLE 29.1 Microbiology laboratory safety standards

Rule	Implementation
Restrict access	Only laboratory workers and trained support personnel have access.
Practice good personal hygiene	Eating, drinking, applying cosmetics, and manipulating contact lenses are forbidden in the laboratory. Hand washing prevents spread of pathogens.
Use personal protective equipment (PPE)	Lab coats, gloves, eye protection, and respirators are recommended or required depending on the pathogens being handled. Face masks are part of PPE if respirators are not required.
Vaccinate	Personnel must be vaccinated against agents to which they may be exposed.
Handle specimens safely	Assume all clinical specimens are infectious and handle appropriately.
Decontaminate	After use or exposure, decontaminate specimens, surfaces, and materials by disinfecting, autoclaving, or incinerating. Wash health-care clothing (scrubs) frequently.

Mastering Microbiology
Art Activity: Table 29.1 Microbiology laboratory safety standards

CDC/PHIL

Figure 29.1 Standard apparel for clinical laboratory safety. This technician is wearing proper personal protective equipment (PPE) for a clinical laboratory, including gloves, eye protection, lab coat, and face mask.

Laboratory Safety

The clinical laboratory has potential biohazards for all personnel and is especially dangerous for untrained personnel or those who do not employ the necessary precautions. All laboratories that handle human or primate tissue must have an occupational exposure control plan for handling bloodborne pathogens. This plan is specifically designed to protect workers from infection by hepatitis B virus (HBV, the cause of infectious hepatitis, ▶ Section 31.11) and human immunodeficiency virus (HIV, the cause of acquired immunodeficiency syndrome [AIDS], ▶ Section 31.15). The occupational exposure plan limits infection by all pathogens and typically includes the use of appropriate *personal protective equipment* (PPE), which includes a lab coat, gloves, eye protection, and face mask (**Figure 29.1**).

Proper training and enforcement of established safety procedures can prevent most accidental infections, which usually do not result from identifiable exposures like culture spills but instead from routine handling of patient specimens. Infectious aerosols generated during microbiological procedures are the most common causes of laboratory infections. Clinical laboratories follow the safety rules outlined in **Table 29.1** to minimize laboratory infections. These general standards apply to all laboratories that handle potentially infectious agents and are the basis for all aspects of healthcare infection control. However, as discussed next, laboratories that handle particularly dangerous or transmissible agents adhere to additional rules and procedures to ensure a safe work environment.

Figure 29.2 Conducting research in a BSL-4 (biosafety level 4) laboratory. BSL-4 is the highest level of biological control, affording maximum worker protection and pathogen containment. The researcher has a whole-body sealed suit with an outside air supply and ventilation system. Air locks control all access to the laboratory. All material leaving the laboratory is autoclaved or chemically decontaminated.

Biological Containment and Biosafety Levels

The level of containment used to prevent accidental infections or accidental environmental contamination (escape) in clinical, research, and teaching laboratories must be proportional to the biohazard potential of the organisms handled in the laboratory. Laboratories are classified according to their containment capabilities from least to greatest by their *biosafety level* (*BSL*), designated as *BSL-1*, *BSL-2*, *BSL-3*, or *BSL-4* (**Figure 29.2**). Personnel in laboratories working at all biosafety levels must follow standard laboratory practices that ensure basic cleanliness and limit contamination (Table 29.1). The precautions, equipment, and operational costs increase with each biosafety level, but the rationale in each case is the same: Keep any potentially infectious agents confined to the laboratory.

Most colleges and universities have BSL-1 and BSL-2 laboratories for teaching and research. Standard clinical laboratories operate at BSL-2. The specialized physical requirements for BSL-3 facilities limit them to major clinical centers and research settings. Because BSL-4 facilities must ensure total isolation and physical containment of pathogens, only about fifty BSL-4 laboratories are operational worldwide. Most BSL-4 laboratories are associated with government facilities, such as the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) and the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID; Fort Detrick, Maryland, USA).

Check Your Understanding

- The use of personal protective equipment (PPE) is required for clinical laboratory technicians. What protective apparel does PPE include?
- Identify and discuss the standard safety procedures adopted by microbiology laboratories. Under what biosafety level do most clinical laboratories operate? With what type of facility are most BSL-4 laboratories associated?

29.2 Healthcare-Associated Infections

The universal safety measures described in the previous section are implemented to contain infectious agents and prevent their transmission in laboratory settings. But despite the routine use of gloves and clean clinical clothing ("hospital scrubs") in healthcare facilities, the accidental transfer of pathogens to patients in these environments is a common occurrence.

Mechanisms of Transfer of Healthcare-Associated Infections

A **healthcare-associated infection (HAI)**, also called a *nosocomial infection* (from the Latin *nosocomium*, meaning hospital), is an infection acquired by a patient during a stay at a healthcare facility (clinic, hospital, rehabilitation facility, etc.). HAIs cause significant morbidity (incidence of *disease* in a population) and mortality (incidence of *death* in a population). An estimated 10% of patients admitted to healthcare facilities in the United States acquire HAIs, and up to 2 million HAIs occur annually, leading directly or indirectly to about 75,000 deaths. Some of the common risk factors for acquiring infectious diseases in healthcare settings are summarized in **Table 29.2**.

TABLE 29.2 Risk factors for hospital-acquired infections (HAIs)

Risk factor for HAI	Rationale
Patients	Patients are already ill or immunocompromised
Newborn infants and the elderly	Not fully immune competent
Infectious disease patients	Pathogen reservoirs
Patient proximity	Increases cross-infection
Healthcare personnel	Can transfer pathogens between and among patients; healthcare personnel may be asymptomatic disease carriers
Medical procedures (blood draws, etc.)	Breaching the skin barrier can introduce pathogens
Surgery	Exposes internal organs, may introduce pathogens, and causes stress, which lowers resistance to infection
Anti-inflammatory drug treatment	Lower resistance to infection
Antibiotic treatment	May select for resistant and opportunistic pathogens

Some HAIs are acquired from patients with communicable diseases, but others are caused by pathogens that are selected for and maintained within the hospital environment, spread by cross-infection from patient to patient or from healthcare personnel. Healthcare-associated pathogens are often present as normal microbiota in either patients or healthcare staff. Therefore, healthcare facilities are high-risk environments for the spread of infections because these facilities concentrate individuals who have infectious disease or are at risk for acquiring infectious disease because of underlying health conditions. Such conditions often lead to a compromised immune system and increased susceptibility to pathogens. The frequency of HAIs at different sites of the body is shown in **Figure 29.3**.

Common Causative Agents of HAIs

Most HAIs are caused by a relatively short list of pathogens (**Table 29.3**), but many other infectious agents can cause HAIs. *Staphylococcus aureus* is one of the most important and widespread HAI pathogens (▶ Section 31.9). It is the most common cause of pneumonia, the third most common cause of blood infections, and is particularly problematic in nurseries. Many hospital strains of *S. aureus* are unusually virulent and are resistant to common antibiotics, making treatment especially difficult (see Explore the Microbial World, "MRSA—A Formidable Clinical Challenge"). The staphylococci are the most common cause of bloodborne HAIs and are also prevalent in pus-forming wound infections.

Staphylococcus and *Enterococcus*, as well as *Escherichia coli, Klebsiella pneumoniae*, and various other *Enterobacteriaceae*, all have the potential to cause HAIs, but they are also members of the normal microbiota of most individuals, making it essentially impossible to eliminate these potential pathogens from healthcare settings. In addition, these organisms can acquire multidrug resistance by horizontal gene flow (Chapter 9). Pathogens that are not part of the normal microbiota, such as species of *Acinetobacter* and *Mycobacterium*, can be eliminated from the healthcare environment. These pathogens are carried into the healthcare facilities by infected individuals or, in the case of some mycobacteria, as airborne contaminants.

Prevention of HAIs requires cooperation between the healthcare facility infection-control team and the rest of the facility staff, including direct healthcare workers and supporting staff, such as housekeeping. Infection control starts with management of incoming patients at the point of entry to the healthcare facility; incoming patients should be assessed for possible infections and isolated as necessary to prevent spread of infections to staff and other patients. In addition, periodic testing of healthcare personnel for key pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), can identify healthy carriers. From this point, the healthcare facility staff employs standard procedures that limit infection, applying the same general precautions outlined for laboratory technicians in Table 29.1.

Mastering Microbiology
Art Activity: Figure 29.3 Frequency of healthcare-associated infections (HAIs) in different sites of the body

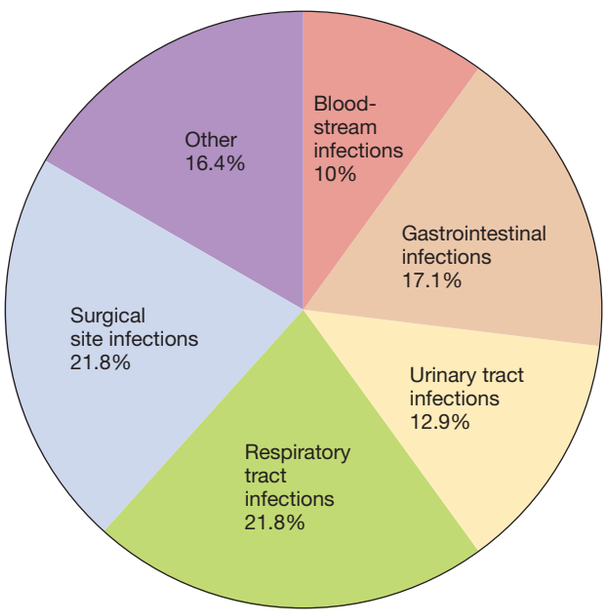


Figure 29.3 Frequency of healthcare-associated infections (HAIs) in different sites of the body. Up to 2 million HAIs occur annually in the United States. Data are from the Centers for Disease Control and Prevention.

Check Your Understanding

- Why are patients in healthcare facilities especially susceptible to pathogens?
- How can the spread of HAIs be controlled?

II • Isolating and Characterizing Infectious Microorganisms

Although the emphasis on molecular diagnostic tools is growing in clinical laboratories, traditional specimen collection and pathogen cultivation techniques are universally used and provide accurate disease diagnosis in a timely manner.

The growth and observation of pathogens from patient specimens are important strategies for identification of the causative agent of an infectious disease. Identification leads to antimicrobial drug susceptibility testing and development of a specific treatment plan. We begin by looking at methods for collecting, culturing, and identifying pathogens, followed by methods used to determine drug susceptibility.

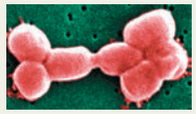
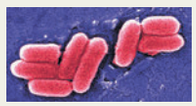
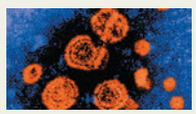
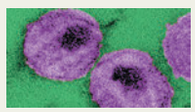
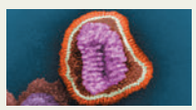
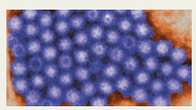
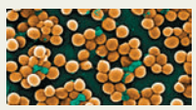
29.3 Workflow in the Clinical Laboratory

Collecting specimens from infectious patients and subsequently culturing pathogens using a variety of growth media is a necessary and routine practice in clinical medicine. Combined with microscopic observation, these methods allow for direct detection and identification of causative agents of disease.

Collecting Specimens and Detecting and Culturing Pathogens

Proper clinical diagnosis of infectious diseases requires that pathogens be identified from tissue or fluid samples using a variety of

TABLE 29.3 Common healthcare-associated pathogens

Pathogen	Common infection sites and diseases	Micrographs[b]	
[a]*Acinetobacter*	Wound/surgical site, bloodstream, pneumonia, urinary tract		*Acinetobacter*
Burkholderia cepacia	Pneumonia		*B. cepacia*
Clostridioides (Clostridium) difficile	Gastrointestinal tract		*C. difficile*
[a]*Enterobacteriaceae*, carbapenem-resistant, especially *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*	Urinary tract, pneumonia, wound/surgical site, bloodstream		*E. coli*
			Klebsiella
[a]Vancomycin-resistant *Enterococcus*	Wound/surgical site, bloodstream, urinary tract		*Enterococcus*
Hepatitis	Chronic liver infection		Hepatitis B virus
Human immunodeficiency virus (HIV)	Immunodeficiency		HIV
Influenza virus	Pneumonia		Influenza virus
[a]*Mycobacterium tuberculosis*	Chronic lung infection		*M. tuberculosis*
Mycobacterium abscessus	Skin and soft tissue infections		
Norovirus	Gastroenteritis		Norovirus
[a]*Staphylococcus aureus* Methicillin-resistant (MRSA) and vancomycin-intermediate and resistant strains	Bloodstream, pneumonia, endocarditis		*S. aureus*

[a]Antibiotic-resistant organisms that exhibit multiple drug resistance. Because of the ease by which multiple-drug-resistant plasmids can be transmitted, many of the pathogens listed as well as members of the normal microbiota could be or become drug resistant in the highly selective nature of the healthcare environment where antibiotics are used routinely and extensively. In addition to the pathogens listed, other extremely pathogenic agents (such as Ebola virus) could be localized to an isolation unit of a healthcare facility, thus making that part of the facility especially dangerous for disease transmission.
[b]All inset photographs are colorized scanning or transmission electron micrographs obtained from CDC/PHIL. Additional micrograph credits (numbers run top to bottom): 1–5, 10, and 12, Janice Haney Carr; 6, Peta Wardell; 7, Erskine Palmer; 8, A. Harrison and P. Feorino; 9, Frederick Murphy; 11, Charles D. Humphrey.

MRSA—A Formidable Clinical Challenge

Staphylococcus aureus is an opportunistic human pathogen that colonizes an estimated one-third of the population. In such individuals, this gram-positive bacterium usually exists as an innocuous member of the normal microbiota of the skin and mucous membranes, especially in the nasal epithelium. Although most complications from staphylococci occur in the form of local skin infections, *S. aureus* may in some cases invade other tissues of the body, including the bloodstream, where a host of virulence factors it produces may transform this bacterium from a harmless commensal to a serious pathogen capable of causing life-threatening disease.

Virulence factors of *S. aureus* include coagulase and clumping factor, two proteins that precipitate blood plasma components and promote the formation of a protective matrix around the bacterial cells that inhibits the activity of phagocytes at sites of infection. In addition, protein A is a surface protein on *S. aureus* that obstructs opsonization by antibodies and complement (◀ Section 26.9), effectively camouflaging the pathogen and further impeding phagocytosis. *S. aureus* also produces several potent exotoxins, including α-hemolysin (lyses blood cells), enterotoxin B (causes food poisoning), and toxic shock syndrome toxin-1 (causes life-threatening toxic shock).

Many *S. aureus* infections are effectively treated using β-lactam antibiotics, such as various penicillins, but the number of strains resistant to these drugs is increasing as global use of antibiotics remains extremely high (see Figure 28.13*a*). Infections caused by multidrug-resistant strains of *S. aureus* are often treated with *methicillin*, a powerful semisynthetic β-lactam drug that is among the last treatment options available in these cases. However, an alarming percentage of *S. aureus* strains have also developed resistance to methicillin in recent years. These methicillin-resistant *S. aureus* (MRSA) strains have been especially prevalent as healthcare-associated infections (Section 29.2).

Clinical diagnosis of MRSA infections depends upon either culture-based techniques, which often include the use of chromogenic agar media (**Figure 1**) or nucleic acid–based tests (Section 29.8). Confirmation of infection must be followed by antibiotic susceptibility testing (Section 29.4) to determine the best treatment strategy. Typically, treatments are case-specific and require a non-β-lactam antibiotic such as vancomycin, tetracycline, or sulfa drugs (◀ Section 28.5), often administered intravenously.

The global incidence of MRSA infection remains high; almost 80,000 cases are documented every year in the United States alone, and several regions of the world have reported increases in MRSA prevalence in recent years, especially in India. Interestingly, the prevalence of healthcare-associated MRSA infections has steadily declined in recent years while that of community-associated MRSA infections has increased. The United States Centers for Disease Control and Prevention estimates that MRSA carriers comprise 2% of the population. Considering its prevalence and extensive multidrug resistance, MRSA may continue to prove a difficult pathogen to control.

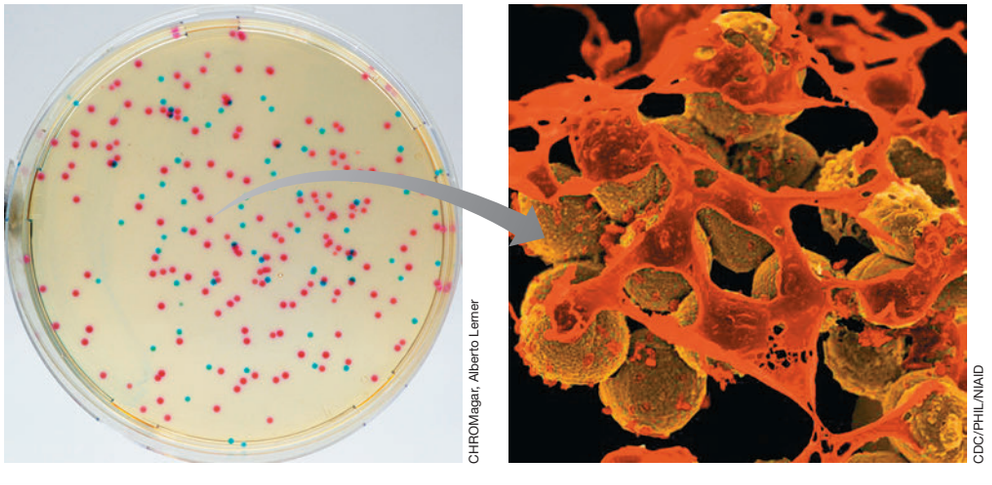


Figure 1 **Methicillin-resistant *Staphylococcus aureus* (MRSA).** Colonies of MRSA on this chromogenic agar medium (left) appear pink, whereas colonies of other bacteria appear blue. The colorized scanning electron micrograph on the right shows cells of MRSA (yellow) embedded in a matrix of cellular debris (orange).

Mastering Microbiology
Art Activity: Figure 29.4 Laboratory identification of microbial pathogens

microbiological, immunological, and molecular biological techniques (**Figure 29.4**). Patient specimens must be collected aseptically from the site of the infection, and the sample size must be large enough to ensure an inoculum sufficient for growth. In addition, the requirements for organism survival, such as oxic versus anoxic conditions, must be maintained at all times, and the sample should be processed quickly to avoid degradation. Sterile swabs are often used to obtain samples from infected areas, including wounds, nares, or throat (**Figure 29.5**), and the swab is then used to inoculate a suitable growth medium.

Many pathogens can be detected by direct means using one or more of several diagnostic tests. For example, the microscopic observation of gram-negative diplococci, especially inside neutrophil inclusions, in a urethral exudate sample is diagnostic for infection with *Neisseria gonorrhoeae* (**Figure 29.6*a***), the causative agent of the sexually transmitted disease gonorrhea (▶ Section 31.13). However, the reliability of any diagnostic test depends on both the *specificity* and the *sensitivity* of the test. **Specificity** is the ability of the test to recognize a single pathogen. High specificity reduces the

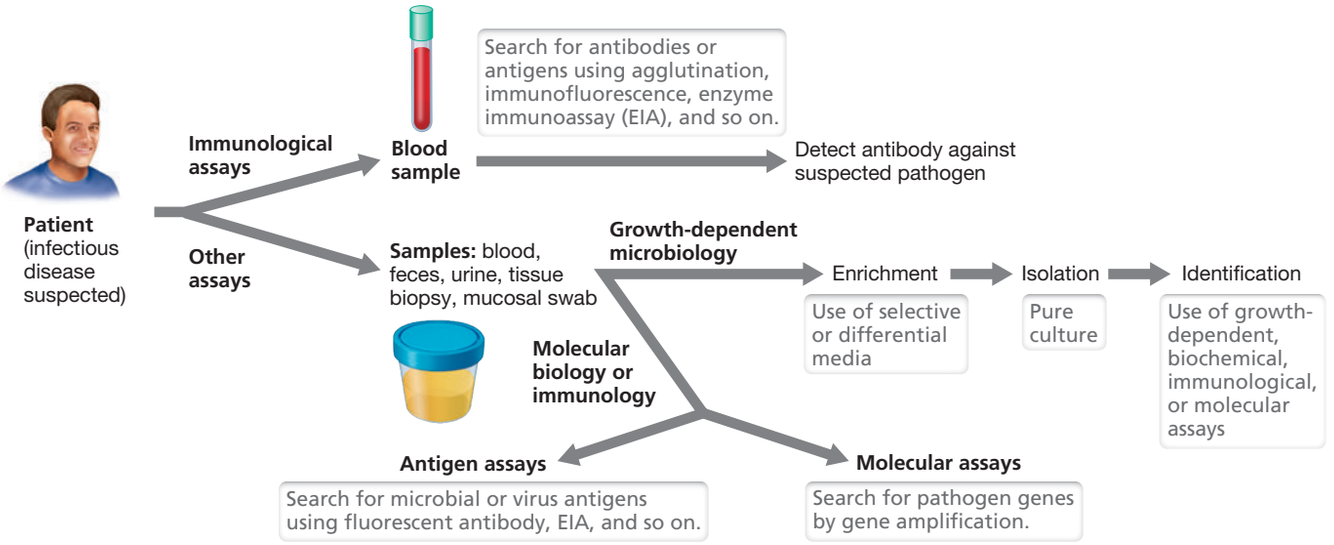


Figure 29.4 Laboratory identification of microbial pathogens. The flowchart shows alternative paths for identifying pathogens or pathogen exposure in the clinical laboratory.

likelihood of a *false-positive* result. **Sensitivity** defines the smallest quantity of a pathogen or a pathogen product that can be detected. High sensitivity minimizes the likelihood of a *false-negative* reaction. For the detection of *N. gonorrhoeae*, the specificity of Gram-stained smears of urogenital exudates is high for both men and women (≥95%), so false-positive tests for gonorrhea are rare. By contrast, the sensitivity of Gram-stained smears of urogenital exudates for the detection of *N. gonorrhoeae* is about 80% greater for men than for women. Thus, in suspected cases of gonorrhea in females, *false-negative* Gram stains are relatively common. Females must therefore be examined by more sensitive methods, including culture techniques (Figure 29.6*b*), to establish or confirm a diagnosis of gonorrhea.

Specific pathogens can be selectively grown, isolated, and identified from patient specimens using specialized growth media and incubation conditions to establish an *enrichment culture* (◀ Sections 19.1 and 19.2). For primary enrichment, clinical samples are inoculated on general-purpose media, such as *blood agar* (**Figure 29.7*a***) and *chocolate agar* (so called because it contains heat-lysed blood, making it brown in color; Figure 29.7*b*), which support the growth of a variety of microorganisms. To aid in the isolation and identification of specific pathogens, medical technologists use various *selective* and *differential* growth media. **Selective media** are specialized culture media that contain inhibitory agents for the purpose of allowing some organisms to grow but not others. **Differential media** allow identification of organisms based on the appearance of the culture after growth. For example, eosin–methylene blue (EMB) agar is a selective medium because the methylene blue it contains inhibits the growth of gram-positive bacteria. EMB agar is also a differential medium because it distinguishes gram-negative

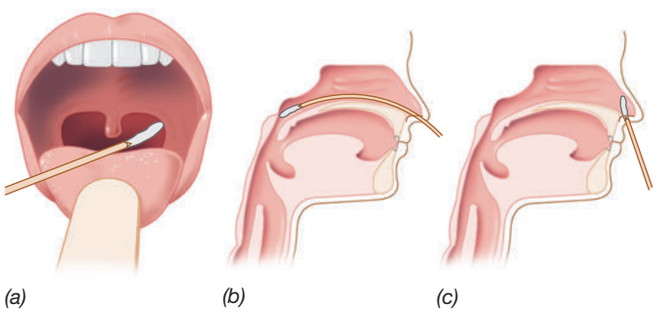


Figure 29.5 Specimens from the upper respiratory tract. *(a)* Throat swab. *(b)* Nasopharyngeal swab passed through the nose. *(c)* Swabbing the inside of the nose.

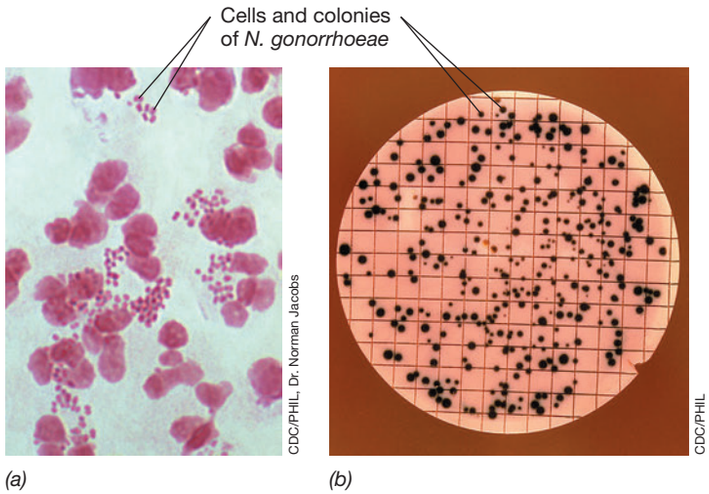
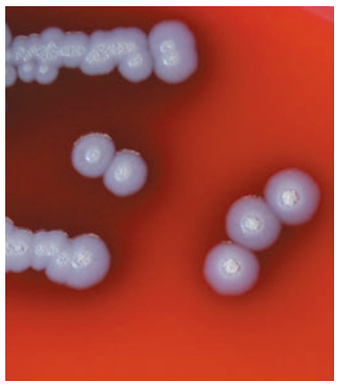


Figure 29.6 Identification of *Neisseria gonorrhoeae*, the cause of gonorrhea. *(a)* Cells of gram-negative *N. gonorrhoeae* within human polymorphonuclear leukocytes (neutrophils) from a urethral exudate. *(b)* Colonies of *N. gonorrhoeae* growing on a filter placed on Thayer–Martin agar. Oxidase reagent, which turns colonies dark purple if they contain cytochrome *c*, has been added to the filter. The dark color of the colonies shows that *N. gonorrhoeae* is oxidase-positive.



(a)

CDC/PHIL, Dr. Todd Parker

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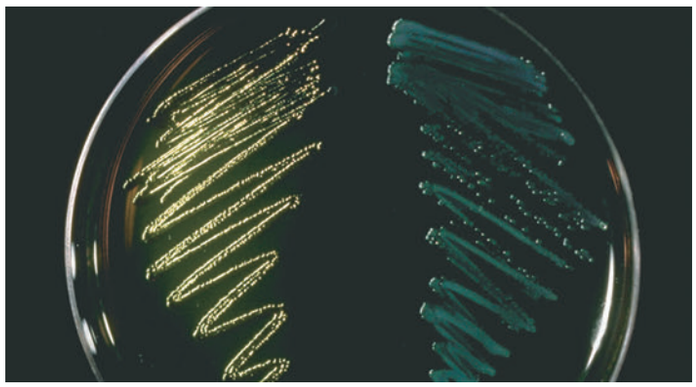


John Martinko and Cheryl Broadie

(c)

Figure 29.7 Enriched media. *(a) Burkholderia* growing on sheep blood agar (SBA); the red color is from blood suspended in the trypticase soy agar medium. *(b) Francisella tularensis* growing on chocolate agar; the brown color is due to heat-lysed blood in the trypticase soy agar medium. *(c) Escherichia coli*, a lactose fermenter (left), and *Pseudomonas aeruginosa*, a non–lactose fermenter (right) growing on eosin–methylene blue (EMB) agar. The reflective, greenish-yellow sheen of the colonies on the left identifies *E. coli* as a lactose fermenter.

bacteria that can ferment lactose from those that cannot. Lactose fermenters, such as *Escherichia coli*, acidify the medium and produce dark colonies that may have a reflective metallic green sheen; non–lactose fermenters, such as *Pseudomonas aeruginosa*, produce opaque or translucent colonies (Figure 29.7*c*).

Blood and Cerebrospinal Fluid Specimens

Pathogens in liquid tissue samples, such as blood and cerebrospinal fluid (CSF), are routinely detected using automated culture systems. For a suspected case of meningitis, a CSF specimen is obtained by a procedure called a *lumbar puncture* (spinal tap) in which 3–5 ml of fluid is collected drop-by-drop from a needle inserted between lumbar vertebrae. CSF is sterile and clear in a healthy individual, and therefore fluid turbidity and high leukocyte counts are indicators of infection. Similar to other liquid specimens (blood, urine, sputum, wound exudates, etc.), CSF is routinely examined by Gram staining and used to inoculate selective culture media.

The standard procedure for obtaining a blood sample is to aseptically draw 10–20 ml of blood from a vein and inject it into two culture bottles containing general-purpose growth media and an anticoagulant. One bottle is incubated aerobically, while the other is incubated anaerobically (**Figure 29.8**); both are kept at 35°C for several days. Automated culture systems (Figure 29.8*b*) detect growth by measuring turbidity or fluorescence and by periodically monitoring the consumption of O_2 or the production of CO_2. Most clinically significant bacteria are recovered within 2 days, but growth of some pathogens, including mycobacteria and certain fungi, may take 3 to 5 days or longer. Cultures that exhibit growth are Gram stained and then inoculated onto specialized media for isolation and identification.

The most common pathogens found in blood include species of *Staphylococcus* and *Enterococcus*, but many other bacteria may cause blood infections. Computer databases are used to unambiguously identify clinical isolates by matching their metabolic reactions in various differential media to the biochemical patterns of known pathogens. The biochemical tests incorporated into differential media evaluate the presence or absence of enzymes that catabolize a specific substrate or substrates. Although hundreds of different biochemical tests are known, just a few key tests may be sufficient to identify some pathogens.

Urinary Tract and Fecal Cultures

Urinary tract infections (UTIs) are common, especially in women. In most cases, microorganisms infect the urinary tract by ascending into the bladder from the urethra. UTIs, often introduced through the use of urinary catheters (**Figure 29.9**), are among the most common healthcare-associated infections. Direct microscopic examination of urine from a UTI patient usually shows the presence of abnormal numbers of bacteria in the urine. A Gram stain may be done directly on urine samples to identify the morphology of urinary tract pathogens, such as gram-negative rods (various enteric bacteria), gram-negative cocci (species of *Neisseria*), and gram-positive cocci (especially species of *Enterococcus*).

(a)

Matthew Sattley

(b)

Figure 29.8 Growth-dependent diagnostic testing for blood infections. *(a)* Cultures to assay both aerobic (left vial) and anaerobic (right vial) bacterial growth are inoculated with an aseptically drawn patient blood sample. *(b)* After inoculation, both vials in part *a* are incubated in an automated system that measures growth, for example, by turbidity, production of CO_2, or fluorescence. Photos courtesy of Marion General Hospital, Marion, Indiana, USA.

Matthew Sattley

Figure 29.9 Urinary catheter preparation. A healthcare professional prepares a urinary catheter. Although the apparatus is pre-sterilized, insertion of the catheter moves microorganisms from the urethra to the bladder and may result in a urinary tract infection, a common HAI.

A significant UTI typically results in bacterial counts of 10^5 or more cells per milliliter of urine. The most common causative agents of UTIs are enteric bacteria, with *E. coli* accounting for about 90% of cases. Blood agar is often used for primary enrichment and isolation of urinary tract pathogens. Selective and differential enteric media, such as EMB or MacConkey agar, permit differentiation of gram-negative lactose fermenters from non–lactose fermenters (Figure 29.7*c*) and inhibit the growth of possible gram-positive contaminants, such as commensal staphylococci. Additional differential and/or selective media may be used to identify urinary tract (or other) pathogens via traditional culturing techniques (**Figure 29.10*a, b***) or rapid and convenient commercial media kits (Figure 29.10*c*).

Proper collection of fecal samples is important for the isolation of intestinal pathogens. Fecal specimens become more acidic during storage, so delay between sampling and processing must be minimized, especially for the isolation of acid-sensitive pathogens, such as *Shigella* and *Salmonella*. The fecal sample is placed in a sterile, sealed container for transport to the laboratory. Feces containing blood or pus, as well as feces from patients with suspected foodborne or waterborne infections, are inoculated into suitable media for isolation of potential pathogens. For example, many laboratories use selective and differential media to identify *E. coli* O157:H7 and *Campylobacter* species, important intestinal pathogens typically acquired from contaminated food or water (▶ Sections 33.11 and 33.12). Intestinal eukaryotic pathogens, such as *Giardia intestinalis* (▶ Section 34.4), are identified by direct microscopic observation of parasite cysts in diluted feces or through antigen-detection assays (see Figure 29.19*c*) rather than by culturing.

Wounds and Abscesses

Infections associated with injuries such as animal bites, burns, or cuts are sampled to recover the relevant pathogen. The results must be interpreted carefully to differentiate between infection and contamination. Wound infections and abscesses often harbor a variety of normal microbiota, and swab samples from such lesions are frequently misleading. For abscesses and other purulent lesions, pus is aspirated with a sterile syringe and needle following disinfection of the skin surface. Internal purulent lesions are sampled by biopsy or from tissues removed in surgery. Gram stains are prepared directly from these specimens and examined microscopically.

Pathogens commonly associated with wound infections are *Staphylococcus aureus*, enteric bacteria, *P. aeruginosa*, and anaerobes, such as species of *Bacteroides* and *Clostridium*. Because of the varied oxygen requirements of these bacteria, samples must be obtained, transported, and cultured under anoxic as well as oxic conditions. The major isolation media are blood agar, selective media for enteric bacteria, and enrichment media containing additional supplements and reducing agents for obligate anaerobes. Widely used tools for the detection of MRSA in skin infections are *chromogenic agar media*. These selective and differential media contain a chromogenic substrate that, when metabolized, causes MRSA to produce distinctly colored colonies (see Explore the Microbial World, "MRSA—A Formidable Clinical Challenge").

Genital Specimens and Culture for Gonorrhea

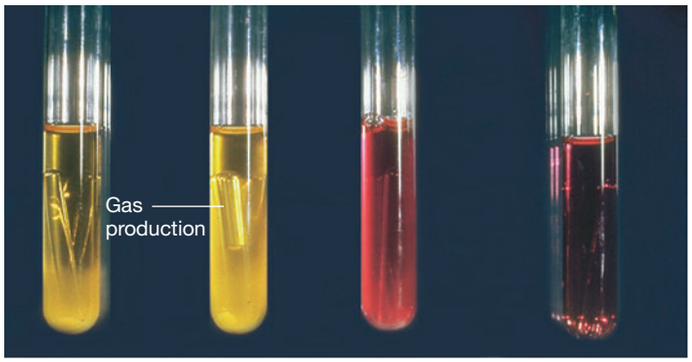
Sexually transmitted infections (STIs) that cause a purulent urethral discharge, especially in males, are classified as either nongonococcal or gonococcal urethritis. Nongonococcal urethritis is usually caused by *Chlamydia trachomatis* (▶ Section 31.13), *Ureaplasma urealyticum*, or *Trichomonas vaginalis* (▶ Section 34.4). Gonococcal urethritis is caused by *Neisseria gonorrhoeae* (▶ Section 31.13).

Cells of *N. gonorrhoeae* are gram-negative diplococci, a morphology not normally found in microbiota of the urogenital tract. Therefore, a Gram stain of a urethral, vaginal, or cervical smear revealing such cells, often surviving inside neutrophils (Figure 29.6*a*), is diagnostic for gonorrhea. Chocolate agar, a nonselective enriched medium, is often used for specimens suspected to contain *N. gonorrhoeae*. A selective medium used for isolation of *N. gonorrhoeae* is modified Thayer–Martin (MTM) agar (Figure 29.6*b*). This medium incorporates the antibiotics vancomycin, nystatin, trimethoprim, and colistin to suppress the growth of normal microbiota. These antibiotics have no effect on *N. gonorrhoeae* or *Neisseria meningitidis*, a cause of bacterial meningitis (▶ Section 31.5).

Inoculated plates are incubated in a humid atmosphere containing 3–7% CO_2 for 24–48 hours and then tested for their oxidase reaction (Figure 29.6*b*). Oxidase-positive, gram-negative diplococci growing on MTM or chocolate agar are presumed to be gonococci if the inoculum was derived from the urogenital tract. Definitive identification of *N. gonorrhoeae* requires determination of carbohydrate utilization patterns and immunological or nucleic acid probe tests. Laboratory testing of urogenital samples for *N. gonorrhoeae* (and the often-associated *C. trachomatis*) is often done using DNA amplification via polymerase chain reaction (PCR) or other molecular methods.

Culture of Anaerobic Pathogens

The identification of obligately anaerobic bacteria from patient specimens requires special isolation and culture methods (◀ Section 4.16). In general, media for anaerobes do not differ greatly from those used for aerobes, except that they (1) are usually richer in



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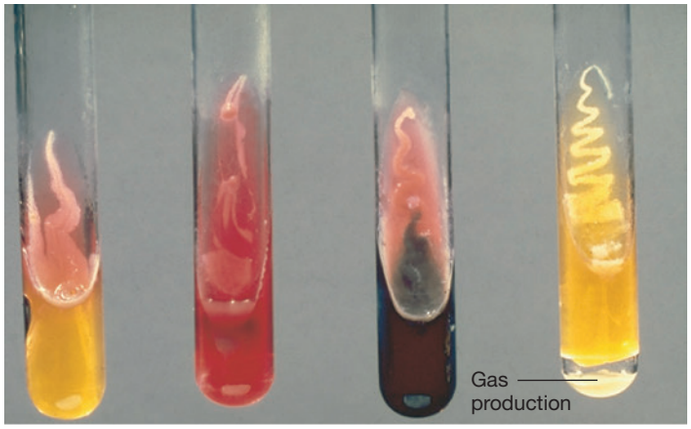

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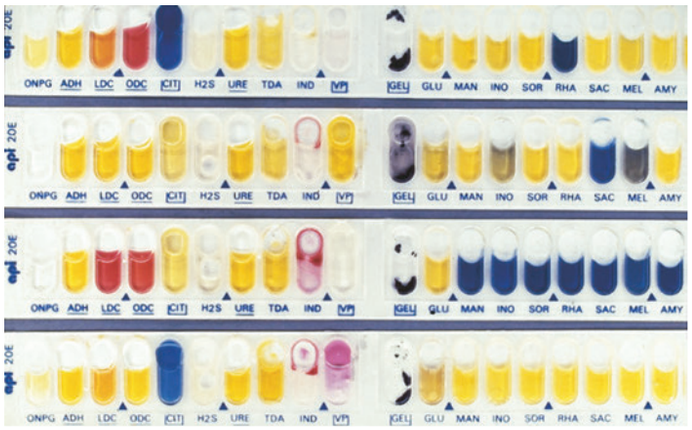


Leon J. LeBeau

(c)

Figure 29.10 Growth-dependent diagnostic tests for clinical isolates. *(a)* Differential media to assess sugar fermentation. Acid production is indicated by a color change (from red to yellow) of the pH-indicating dye in the medium. The appearance of a bubble in the small, inverted inner tube indicates gas production from fermentation. *(b)* Diagnostic test for enteric bacteria using triple-sugar iron (TSI) agar. The medium contains glucose, lactose, and sucrose. Organisms able to ferment only glucose cause acid formation only in the bottom of the tube, whereas lactose- or sucrose-fermenting organisms cause acid formation throughout the slant. The breaking up of the agar in the bottom of the tube indicates gas formation. Blackening of the agar is due to the reaction of hydrogen sulfide (from either protein degradation or thiosulfate reduction) with ferrous iron in the medium. From left to right: Fermentation of glucose only (typical of *Shigella*); growth but no fermentation (typical of *Pseudomonas*); hydrogen sulfide formation (typical of *Salmonella*); fermentation of sugars with gas production (typical of *Escherichia coli*). *(c)* Miniaturized media kits allow rapid identification of clinical isolates by running many biochemical tests on specimen samples at the same time. Four separate strips, each with a different isolate, are shown.

organic constituents, (2) contain reducing agents (usually cysteine or thioglycolate) to remove O_2, and (3) contain a redox indicator to show that conditions are anoxic. Collection, handling, and processing of specimens must exclude exposure to air because O_2 is toxic to obligate anaerobes. Samples collected by syringe aspiration or biopsy must be immediately transferred to a sealed tube containing O_2-free gas, usually with a dilute salt solution containing a reducing agent and a redox indicator to monitor O_2 contamination. Specimens are then used to inoculate anoxic media in an automated culture system or in an anoxic "glove box" filled with O_2-free gases, usually a mixture of N_2 and H_2 (◀ Figure 4.29).

Several habitats in the body, including portions of the oral cavity and the lower intestinal tract, are anoxic and support the growth of anaerobic normal microbiota. Other parts of the body may also become anoxic if injury or disease reduces the blood supply to certain tissues, a condition called *ischemia*. These anoxic sites can then be colonized by obligate anaerobes. Although potentially pathogenic anaerobic bacteria are part of the normal microbiota, their numbers are kept in check through competition with other members of the microbial community. Under certain conditions, however, normally benign anaerobes may become opportunistic pathogens. A key example is *Clostridioides* (*Clostridium*) *difficile* (Table 29.3); this usually harmless member of the normal microbiota of the lower intestinal tract commonly emerges as a healthcare-associated pathogen when extended antibiotic therapy destroys competing microbes (◀ Section 24.11 and ▶ Section 30.7).

Check Your Understanding

- What are the key requirements for proper collection of clinical specimens, and why is it important that diagnostic tests for these specimens are both highly specific and sensitive?
- Identify culture methods and conditions used for blood, wound, urine, fecal, and genital specimens. Of what importance are selective and differential growth media in pathogen detection, and what special conditions must be maintained for the isolation of anaerobic pathogens?

29.4 Choosing the Right Treatment

Pathogens isolated from clinical specimens are identified to confirm medical diagnoses and to guide antimicrobial therapy. Appropriate and effective treatment for many pathogens is based on current experience and practices. For some pathogens, however, decisions about appropriate antimicrobial therapy must be made on a case-by-case basis. Such pathogens include those for which antimicrobial drug resistance is common (for example, gram-negative enteric bacteria), those that cause life-threatening disease (for example, meningitis caused by *Neisseria meningitidis*), and those that require bactericidal rather than bacteriostatic drugs (◀ Section 4.19) to prevent disease progression and tissue damage. Bactericidal agents are indicated, for example, for bacterial endocarditis (infection of the inner tissues of the heart, such as the heart valves), where total and rapid killing of the pathogen is critical for patient survival.

Minimum Inhibitory Concentration

Antimicrobial susceptibility is measured by determining the smallest amount of agent needed to completely inhibit the growth of the

tested organism in vitro (in laboratory culture), a value called the **minimum inhibitory concentration (MIC)**. The traditional way to determine the MIC for a given agent against a given organism is to prepare a series of culture tubes inoculated with the same number of microorganisms. Each tube contains the growth medium with an increasing concentration of the antimicrobial agent. After incubation, the tubes are checked for visible growth (turbidity), and the MIC is the lowest concentration of the agent that completely inhibits the growth of the test organism (◀ Section 4.19 and Figures 4.40 and 4.41).

In modern practice, the MIC of a given antimicrobial agent is typically determined using microliter amounts of media and reagents. For example, a miniaturized version of the MIC test uses a microtiter method with twofold dilutions of several antibiotics in medium inoculated with a standard amount of the test organism (**Figure 29.11*a***). In clinical microbiology laboratories, tests for routine MIC determinations are usually automated using instruments that also allow for species identification of pure cultures obtained from patient specimens (Figure 29.11*b, c*).

Measuring Antimicrobial Susceptibility

The standard assay for antimicrobial activity is the *disk diffusion test* (Figure 29.11*d–g*). A Petri plate containing an agar medium is inoculated by evenly spreading a suspension of a pure culture of the suspected pathogen on the agar surface. Known amounts of different antimicrobial agents infused into filter-paper disks are then placed on the surface of the agar. The agents then diffuse from the disks into the agar during incubation, establishing a gradient; the farther the chemicals diffuse away from each disk, the lower is the concentration of the agent. At some distance from each disk, the effective MIC is reached. Beyond this point the microorganism is able to grow, but closer to the disk, growth is absent. A *zone of inhibition* forms with a diameter proportional to the concentration, solubility, diffusion coefficient, and overall effectiveness of the antimicrobial agent in the disk.

In addition to the disk diffusion test, Figure 29.11*g* depicts antibiotic susceptibility using the *epsilometer test* (Etest®). This assay uses a plastic strip infused with a predefined concentration gradient of

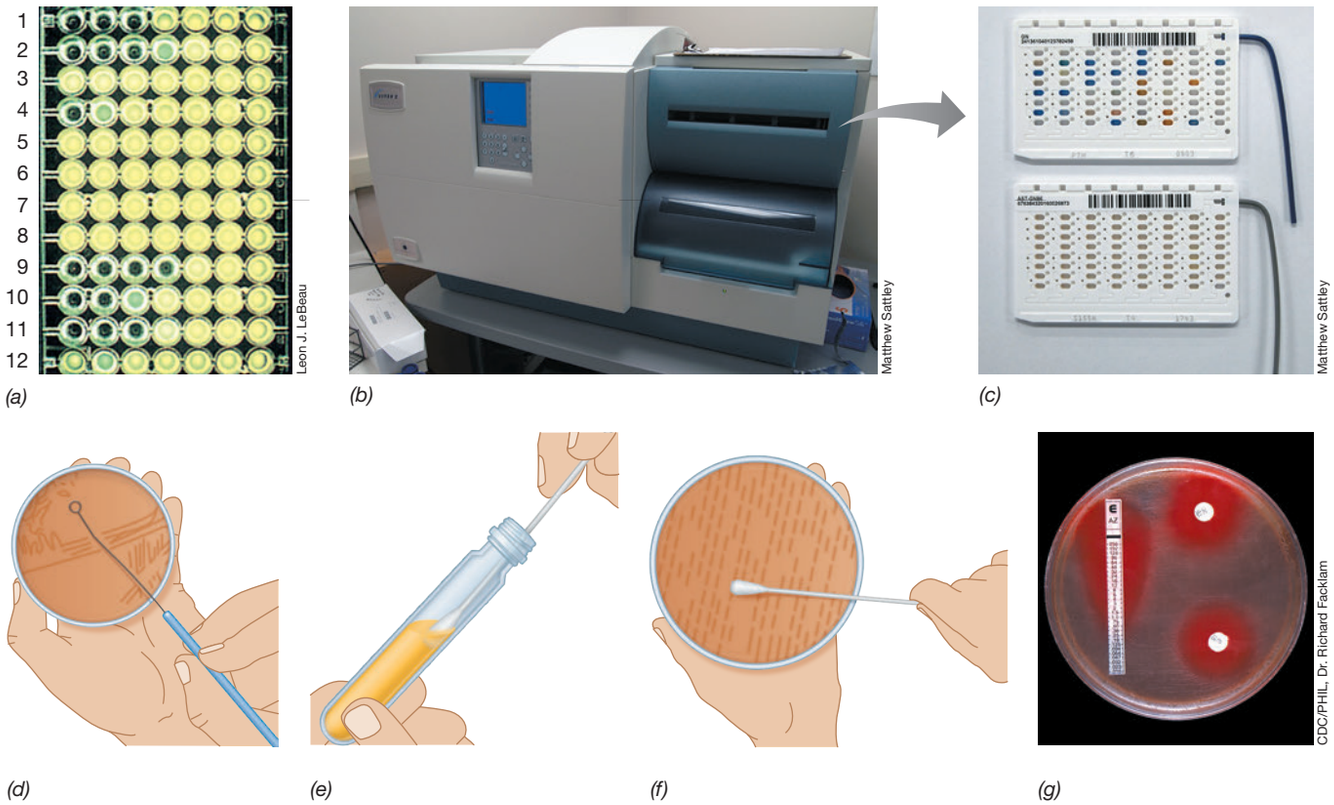


Figure 29.11 Antibiotic susceptibility testing. *(a)* Antibiotic susceptibility of a pathogen as determined by the broth dilution method in a microtiter plate. The organism is *Pseudomonas aeruginosa*. Each row has a different antibiotic in a series of concentrations. The highest concentration of antibiotic is in the well at the left; serial twofold dilutions are made in the wells to the right. In rows 1 and 2, the third well has the lowest concentration of antibiotic that shows no visible growth. In row 3, the antibiotic is ineffective at the concentrations tested because there is growth in all wells. *(b, c)* An automated system for identifying clinical isolates and determining their antibiotic susceptibility. Card wells (panel *c*) are inoculated using the attached capillary tube. Following internal incubation, computer-scanned results are available in less than 24 hours. Photos courtesy of Marion General Hospital, Marion, Indiana (USA). *(d)* For the disk diffusion test, colonies from a pure culture of the pathogen are transferred to a liquid medium and mixed. *(e, f)* A sterile swab is dipped into the bacterial suspension and streaked evenly over the entire surface of a suitable agar medium. *(g)* Disks containing known amounts of different antibiotics are placed on the inoculated agar surface. After incubation, zones of inhibition are measured, and antibiotic susceptibility is determined using a standardized chart of zone sizes. For the epsilometer test (Etest®, AB BIODISK, Solna, Sweden), a plastic strip containing an antibiotic gradient (in µg/ml) indicates the MIC at the point where the elliptical zone of inhibition meets the strip. In this example, the MIC for azithromycin (AZ) is 1.0 µg/ml.

an antimicrobial agent. When applied to the surface of an inoculated agar plate, the gradient transfers from the strip to the agar and remains stable throughout the incubation period, during which an elliptical zone of inhibition centered along the axis of the strip develops. The concentration of the antimicrobial agent (in µg/ml) is read at the point where the ellipse edge intersects the precalibrated test strip, providing a precise MIC (Figure 29.11*g*).

Assuming culture conditions are standardized, different antimicrobial agents can be compared to determine which is most effective against the isolated pathogen. The Clinical and Laboratory Standards Institute (www.clsi.org) is responsible for developing, establishing, and constantly updating consensus standards for antimicrobial testing. Hospital infection-control microbiologists produce and examine susceptibility data to generate periodic reports called *antibiograms*. These reports define the susceptibility of clinically isolated organisms to the antibiotics in current use. Antibiograms are used to monitor control of known pathogens, to track the emergence of new pathogens, and to identify the emergence of antibiotic resistance at the local level.

Check Your Understanding

Mastering Microbiology
Art Activity: Figure 29.12 Pattern of infection and immunity in untreated typhoid fever

- Describe the disk diffusion test and the Etest for antimicrobial susceptibility. For an individual organism and an antimicrobial agent, what do the results signify?
- What is the value of antimicrobial drug susceptibility testing for the microbiologist, the physician, and the patient?

III • Immunological and Molecular Tools for Disease Diagnosis

Culture-independent clinical diagnostics, including immunological and molecular-based techniques, are critical for providing rapid and effective healthcare. The quicker an infectious disease is diagnosed, the quicker treatment can begin, and in some cases, prompt treatment is essential to save the patient.

Culture methods for some pathogens, including many viruses and some pathogenic bacteria, are not routinely available, are unreliable, or are prohibitively difficult or expensive to perform. In such cases, *growth-independent* diagnostic methods are used in clinical, reference, and research laboratories to detect specific pathogens or their products. These include a variety of immunological and molecular assays that can yield a relatively quick and reliable means of identifying individual pathogens or host exposure to pathogens in the absence of cultured organisms.

29.5 Immunoassays and Disease

Many immunoassays use antibodies specific for pathogens or their products for in vitro tests designed to detect specific infectious agents. Patient immune responses, discussed in Chapters 26 and 27, can also be monitored to obtain evidence of pathogen exposure and infection.

Serology and Antibody Titers

The study of antigen–antibody reactions in vitro is called **serology**. Serological assays detect pathogen-induced antibodies in patient serum and are the basis for a number of diagnostic tests. The *specificity* of the antibody–antigen reactions associated with serological tests allows one to pinpoint an exposure to a single pathogen, assuming the antigen used to detect antibodies is unique to the pathogen in question. Moreover, serological tests vary considerably in their *sensitivity*, that is, in the amount of antibody necessary to detect antigen. For example, passive *agglutination* reactions (see Section 29.6) are fast and easy to perform but require antibody concentrations of up to 6 nanograms (ng, 10^{-9} g) per ml. By contrast, the very sensitive but more technically demanding *enzyme immunoassay* (*EIA*) tests require as little as 0.1 ng of antibody per ml and can detect as little as 0.1 ng of antigen (Section 29.7).

If an individual is infected with a suspected pathogen, the immune response—antibodies to that pathogen—should become elevated. Strong evidence for infection can therefore be obtained by determining the *antibody titer* directed against antigens produced by the suspected pathogen. The **titer** is a quantitative measure of antibody level and is defined as the highest dilution (lowest concentration) of serum at which an antigen–antibody reaction is observed (**Figure 29.12**).

A positive antibody titer indicates previous infection or exposure to a pathogen. For pathogens rarely found in a population, such as the life-threatening hantaviruses (▶ Section 32.2), a single positive test for a pathogen-specific antibody may indicate active infection. In most cases, however, the mere presence of antibody does not indicate active infection. Antibody titers typically remain detectable for long periods after a previous infection has been resolved. To link an acute illness to a particular pathogen, it is essential to show a *rise*

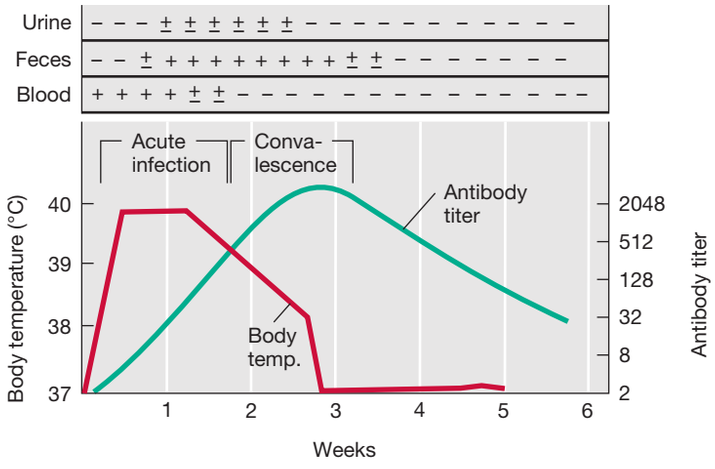


Figure 29.12 Pattern of infection and immunity in untreated typhoid fever patients. Body temperature indicates acute disease progression over time. Antibody titer is shown as the reciprocal of the highest serial dilution causing agglutination of *Salmonella enterica* (*typhi*) (▶ Section 33.5). The presence of bacteria in blood, feces, and urine was determined from cultures (−, no bacteria; ±, low numbers of bacteria; +, high numbers of bacteria). Bacteria clear from the blood as the antibody titer rises, whereas clearance from feces and urine requires more time. Body temperature drops to normal as the antibody titer rises.

in antibody titer in serum samples taken from a patient during the acute disease and later during the convalescent phase of the disease. Frequently, the antibody titer is low during the acute stage of the infection and rises during convalescence (Figure 29.12). A rise in antibody titer is strong circumstantial evidence that the illness is due to the suspected pathogen.

Skin Tests

A number of pathogens induce a delayed-type hypersensitivity (DTH) response mediated by Th1 cells (◀ Section 28.1). For these pathogens, skin testing may be useful to determine exposure. As an example, a commonly used skin test is the *tuberculin test*, which consists of an intradermal injection of a soluble extract from cells of *Mycobacterium tuberculosis*. A positive inflammatory reaction at the site of injection within 48 hours indicates current infection or previous exposure to (or vaccination against) *M. tuberculosis* (◀ Figure 28.4). Skin tests are routinely used to aid in diagnosis of tuberculosis, Hansen's disease (leprosy), and some fungal diseases because, as opposed to responses caused by pathogen-specific inflammatory Th1 cells, antibody responses for intracellular and fungal infections are often weak or undetectable.

If a pathogen is extremely localized, there may be little induction of a systemic immune response and no rise in antibody titer or skin test reactivity, even if the pathogen is proliferating profusely at the site of infection. A good example is the infection of urogenital mucosal surfaces with the bacterium *Neisseria gonorrhoeae*. Gonorrhea does not elicit a systemic or protective immune response, there is no serum antibody titer or skin test reactivity, and reinfection of individuals is common (▶ Section 31.13).

Monoclonal Antibodies

An expanding area of research with broad application in the diagnosis and treatment of disease is the development and use of **monoclonal antibodies (mAbs)**. In contrast to *polyclonal antibodies*, which occur as a mixture of immunoglobulins produced by many individual B cells and directed at numerous antigenic determinants on a pathogen, mAbs are derived from a B cell clone sensitized to a single antigenic determinant. Thus, an in vitro B cell clone culture produces monospecific mAbs that can be collected for diagnostic or therapeutic purposes. However, antibody-producing B cells are relatively short-lived and normally die within several weeks in cell culture. To produce long-lived B cell clones for commercial mAb production, antibody-producing B cells are fused with *myelomas*, tumorigenic B cells that divide and grow indefinitely (**Figure 29.13**). The "immortal" cell lines that result from this fusion are hybrid cells, appropriately called *hybridomas*. The hybrid cell lines share the biological properties of both fusion partners; they grow indefinitely in vitro and produce antibodies.

To produce a particular mAb, a mouse is immunized with the antigen of interest. Antigen-specific B cells then proliferate over several weeks and begin to produce antibodies in the mouse. B cells are then removed from the mouse and mixed with myeloma cells (Figure 29.13), but only a small number of these fuse into antibody-producing hybridomas. The hybridomas are isolated from unfused myeloma cells using a selective *HAT* medium, so called because it contains the metabolites hypoxanthine (H) and thymidine (T), as well as the cell poison aminopterin (A). The myelomas are unable to grow in this medium because they lack an enzyme that allows them to use the H and T metabolites to circumvent the poisonous effect of aminopterin. Unfused B cells can produce the necessary enzyme, but they die after several days because they cannot divide in culture. Only the fused hybridomas, which combine the properties of both cell types, are able to both produce the necessary enzyme and grow indefinitely.

An enzyme immunoassay (Section 29.7) can be used to identify hybridomas that produce the desired mAb. From a typical fusion, several distinct clones are isolated, each making a different mAb. Once the clones of interest are identified, they can be grown either in the mouse as an antibody-producing tumor or in continuous cell culture. Antibodies can then be harvested from either source.

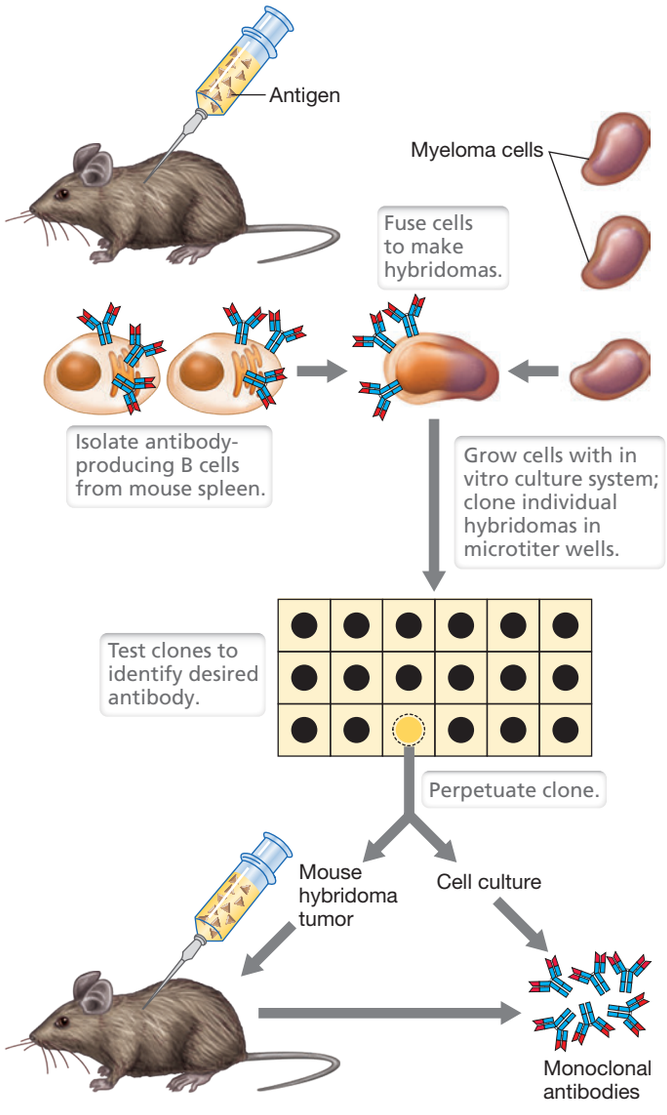


Figure 29.13 Production of monoclonal antibodies (mAbs). The hybridoma can be indefinitely cultured or passed through animals as a tumor. The hybridoma cells are stored as frozen tumor cells that can be thawed and grown in tissue culture or in a suitable animal host.

Commercial production of mAbs has replaced polyclonal antibodies for many immunodiagnostic applications because mAbs are highly specific bioreagents that can be generated with a high degree of reproducibility. Clinical diagnostic tests that use mAbs include immunological typing of bacterial pathogens, identification of cells containing foreign surface antigens (for example, a virus-infected cell), and highly specific blood and tissue typing. Because of their remarkable specificity, mAbs are also used to detect and treat human cancers (◀ Section 28.4). Malignant cells often contain tumor-specific neoantigens on their surfaces (◀ Figure 28.10), and therefore mAbs prepared against these antigens specifically target the cancer cells and can be used to deliver toxins directly to them. As discussed in Chapter 28, mAbs may also be used as *checkpoint inhibitors* to neutralize immunosuppression within the tumor microenvironment. The use of anticancer mAbs has great potential as an alternative to nonspecific chemical and radiation treatments that damage healthy cells as well as cancer cells.

Check Your Understanding

- Explain the reasons for changes in antibody titer for a single infectious agent, from the acute phase through the convalescent phase of the infection.
- Describe the method, time frame, and rationale for the tuberculin skin test. What component of the immune response does this test detect?
- What advantages do monoclonal antibodies have compared to polyclonal antibodies? How are mAbs produced?

29.6 Precipitation, Agglutination, and Immunofluorescence

Many clinically useful immunological reactions yield a product visible to the naked eye, such as precipitation or agglutination. Some other reactions are visualized microscopically when fluorescent dyes attached to specific antigens react with their specific antibody. We consider examples of these now.

Precipitation

Precipitation results from the interaction of a soluble antibody with a soluble antigen to form an insoluble complex. Tests can be done in liquid test tubes (or capillary tubes) or, as shown in **Figure 29.14**, in agarose gel. Antigens that have more than one antibody-binding epitope (◀ Section 27.2) can cross-link the bivalent antibodies that recognize them, causing a precipitate to develop from the aggregated antibody–antigen complexes. Precipitation occurs maximally when there are optimal proportions of the two reacting substances. The presence of either excess antigen or excess antibody results in the formation of soluble immune complexes (Figure 29.14).

Precipitation reactions carried out in agarose gels, called *immunodiffusion tests* (Figure 29.14 inset), are especially useful for diagnosing fungal infections, including coccidioidomycosis, histoplasmosis, blastomycosis, and paracoccidioidomycosis (▶ Section 34.2). For these tests, prepared antigen and patient antisera containing antibodies are loaded into separate wells cut into the agarose gel. The reagents diffuse outward from the wells and form precipitation bands where antibody interacts with antigen in optimal proportions (Figure 29.14). Unfortunately, precipitation reactions are not very sensitive; visible precipitation requires microgram quantities of specific antibody rather than the nanogram quantities of more sensitive diagnostic tests. Consequently, with the exception of clinical diagnostic testing for fungal infections, precipitation assays are typically used only in research and reference laboratories.

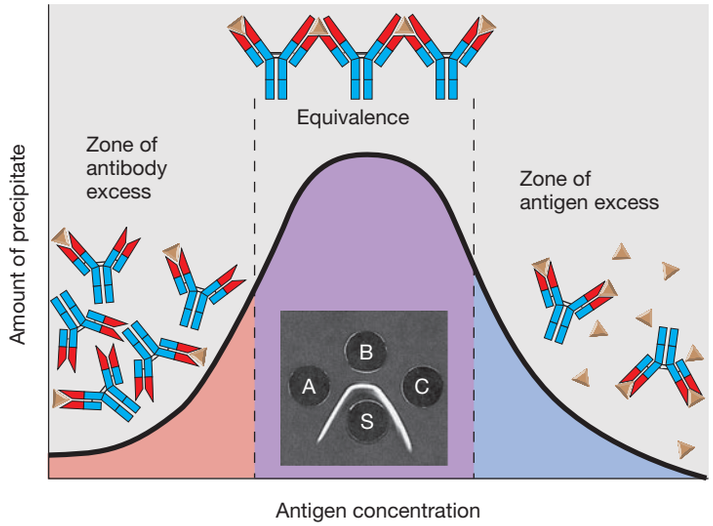
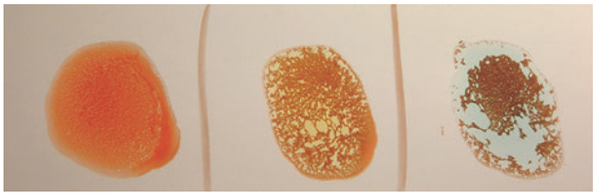


Figure 29.14 Precipitation reactions between soluble antigen and antibody. The extent of precipitation is a function of antigen and antibody concentration. Inset photo: Precipitation in agarose gel (immunodiffusion). Well *S* contains antibodies to cells of *Proteus mirabilis*. Wells *A, B,* and *C* contain soluble extracts of *P. mirabilis*. An insoluble precipitation band forms where antibody and antigen concentrations are equivalent. Immunoprecipitation photo courtesy of C. Weibull, W.D. Bickel, W.T. Hashius, K.C. Milner, and E. Ribi.

Agglutination

Agglutination is a reaction between antibody and particle-bound antigen resulting in visible clumping of the particles. Agglutination tests can be done in test tubes, in microtiter plates, or by mixing reagents on glass or coated paper slides. Agglutination tests are quick to perform, inexpensive, highly specific, and reasonably sensitive, making them suitable for large-scale use in clinical applications. Standardized agglutination tests are used for the identification of blood-group antigens (on red blood cells) (**Figure 29.15*a***), as well as pathogens and pathogen products. To determine blood groups, blood samples are mixed with either anti-A antisera or anti-B antisera and the agglutination of red blood cells, called *hemagglutination*, is assessed (Figure 29.15).

Agglutination is often assessed using rapid assays that employ small (0.8-μm diameter) latex beads coated with a specific antigen. The beads are mixed with patient serum on a slide and incubated for a short period. If patient antibody binds the antigen on the bead surface, the milky white latex suspension will become visibly clumped, indicating a positive agglutination reaction and exposure to the pathogen.



Norman L. Morris

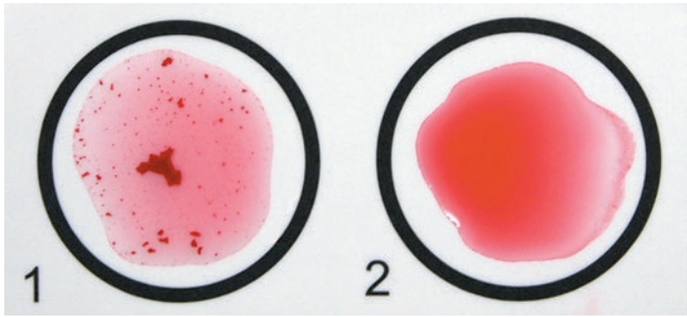
(a)

Blood type	Percentage of U.S. population	Serum	
		Anti A	Anti B
O	48	No aggl.	No aggl.
A	32	Aggl.	No aggl.
B	16	No aggl.	Aggl.
AB	4	Aggl.	Aggl.

(b)

Figure 29.15 Direct agglutination of human red blood cells: ABO blood typing. *(a)* A drop of whole blood was mixed with antigen-specific antisera for each reaction. The reaction on the left shows no agglutination with antibody, typical of blood type O. The reaction in the center shows the diffuse agglutination pattern indicative of blood type B. The reaction on the right shows the strong agglutination pattern with large, clumped aggregates typical of blood type A. *(b)* Table of expected blood typing results for people in the United States.

Latex agglutination is also used to detect bacterial surface antigens by mixing cells from a bacterial colony with antibody-coated latex beads. For example, a commercially available suspension of latex beads coated with antibodies to protein A and clumping factor, two proteins found exclusively on the surface of *Staphylococcus aureus* cells, is specific for identification of clinical isolates of *S. aureus* (**Figure 29.16**). Latex bead assays take less than a minute and can be used directly on clinical samples, such as the exudate from a purulent infection, often seen in cases of *S. aureus* infections. Latex bead agglutination assays have also been developed to identify other common pathogens, such as *Streptococcus pyogenes*, *Neisseria gonorrhoeae*, *Escherichia coli* O157:H7, and the fungus *Candida albicans*.



Matthew Sattley

Figure 29.16 Latex bead agglutination test for *Staphylococcus aureus*. In circle 1, a loopful of material from a bacterial colony was mixed in a suspension of microscopic, red latex beads coated with antibodies to two antigens found exclusively on the surface of *Staphylococcus aureus* cells. The bright red clumps indicate positive agglutination, confirming the colony as *S. aureus*. Circle 2 is a negative control showing the uniform red color expected in the absence of agglutination.

Immunofluorescence

Antibodies conjugated to fluorescent dyes can be used to detect antigens on intact cells. Such **fluorescent antibodies** are widely used for diagnostic and research applications. Fluorescent antibody staining methods can be either direct or indirect (**Figure 29.17**). In the *direct method*, the antibody that interacts with the surface antigen is itself covalently linked to the fluorescent dye. In the *indirect method*, the presence of a nonfluorescent primary antibody on the surface of a cell is detected by the use of a fluorescent secondary antibody directed against the nonfluorescent antibody.

Mastering Microbiology
Art Activity: Figure 29.17 Fluorescent antibody methods for detection of microbial surface antigens

Fluorescent dyes typically conjugated to antibodies include rhodamine B, which fluoresces red-orange, and fluorescein isothiocyanate, which fluoresces yellow-green (**Figure 29.18**). Once the fluorescent antibodies have bound to cell surface antigens, the complex can be visualized using a fluorescence microscope (◀ Figure 1.25). The cell-bound fluorescent antibodies emit their characteristic fluorescent

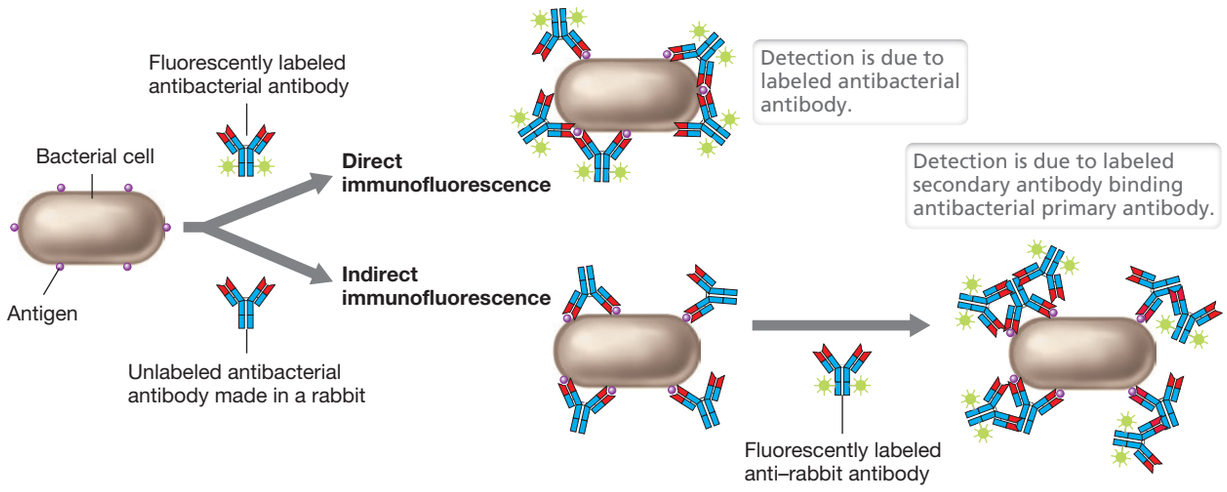
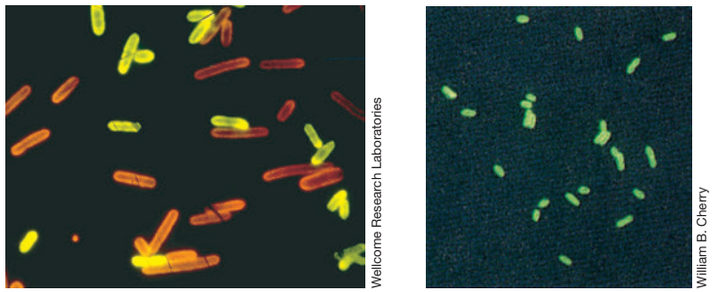


Figure 29.17 Fluorescent antibody methods for detection of microbial surface antigens. Note how indirect immunofluorescence requires a labeled secondary antibody that binds to the primary antibody.



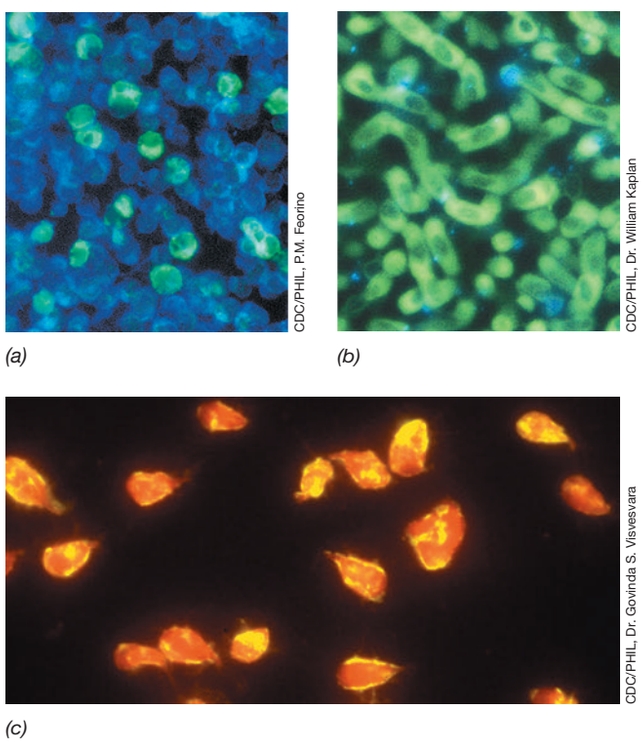
(a) (b)

Figure 29.18 Fluorescent antibody identification of bacteria. *(a)* Cells of *Clostridium septicum* were stained with antibody conjugated with fluorescein isothiocyanate, which fluoresces yellow-green. Cells of *Clostridium chauvoei* were stained with antibody conjugated with rhodamine B, which fluoresces red-orange. *(b)* Immunofluorescently stained cells of *Legionella pneumophila* (legionellosis) from biopsied lung tissue. Individual cells are 2–5 μm in length and were stained green with antibodies coupled to fluorescein isothiocyanate.

Mastering Microbiology
Art Activity: Figure 29.20 Enzyme immunoassays (EIAs)

color when excited with light of particular wavelengths. Fluorescent antibodies can be used for tasks as varied as identifying a microorganism directly in a patient specimen (Figure 29.18) to enumerating T cells in the blood of patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS).

Fluorescent antibodies applied directly to infected host tissues may permit disease diagnosis long before culture methods yield a suspected pathogen (**Figure 29.19**). For example, a presumptive diagnosis of legionellosis (Legionnaires' disease), a form of infectious pneumonia (▶ Section 33.4), can be confirmed by staining biopsied lung tissue directly with fluorescent antibodies specific for cell wall antigens of *Legionella pneumophila* (Figure 29.18*b*), the causative agent of the disease. Immunofluorescence assays are also used to help diagnose infections from viral pathogens, such as Epstein–Barr virus (EBV) (Figure 29.19*a*); fungal pathogens, such as *Aspergillus* (Figure 29.19*b*); and gastrointestinal parasitic protozoa, such as *Giardia intestinalis* (Figure 29.19*c*).



(a) (b)

(c)

Figure 29.19 Fluorescent antibody identification of pathogens. *(a)* Detection of cells infected with Epstein–Barr virus (EBV) using indirect immunofluorescence. The green-stained cells are infected with EBV, which causes mononucleosis and lymphoma. *(b)* Detection of *Aspergillus* mold in a case of aspergillosis (▶ Section 34.1 and Table 34.1) using fluorescein-conjugated antibodies. *(c)* Detection of the waterborne intestinal parasite *Giardia intestinalis* (▶ Section 34.4) using indirect immunofluorescence.

Check Your Understanding

- How is the bivalence of antibodies significant for a precipitation reaction, and under what conditions does precipitation occur maximally?
- What are the advantages and disadvantages of agglutination tests versus fluorescent antibody assays? How are the latter used to identify specific cells in complex mixtures, such as blood?

29.7 Enzyme Immunoassays, Rapid Tests, and Immunoblots

Enzyme immunoassays (EIAs), which include *enzyme-linked immunosorbent assays* (*ELISAs*), are immunodiagnostic tools widely used in clinical microbiology and research. EIAs are particularly useful because they are inexpensive, produce no hazardous waste, and are highly specific and sensitive; they can detect as little as 0.1 nanograms of antigen or antibody. *Rapid tests* are similar to EIAs except that results can often be reported within minutes instead of hours. Many rapid tests provide **point-of-care diagnostics** but are generally not as specific or sensitive as EIAs.

The comparatively complex and time-consuming **immunoblot (Western blot)** uses immobilized pathogen proteins as antigens to bind antibodies from patient specimens, providing highly specific evidence for pathogen exposure (see Figure 29.22). Immunoblots are often used to confirm results obtained from other serological tests, including rapid tests and EIAs.

EIAs

In EIA tests, an enzyme is covalently attached to an antigen or antibody molecule, creating an immunological tool with high specificity and sensitivity. Enzymes typically bound to antigen or antibody include peroxidase, alkaline phosphatase, and β-galactosidase, all of which interact with specific substrates to form colored reaction products that can be detected in low amounts. Four EIA formats are commonly used for evaluation of specimens for infectious disease: *direct EIA* (detects antigen), *indirect EIA* (detects antibodies), *antigen sandwich EIA* (detects antibodies using a sandwich technique), and *combination EIA* (detects both antigen and antibodies). The principal features of each platform are illustrated in **Figure 29.20**.

Direct EIAs are designed to detect antigens, such as virus particles in a blood or fecal sample (Figure 29.20*a*). Antibodies to a pathogen antigen are coated onto a support, such as a plastic microtiter plate, and the patient sample is then added. After antigen in the sample binds to the antibody, a second antibody, specific to the same antigen and coupled to an enzyme, is added. Finally, enzyme substrate

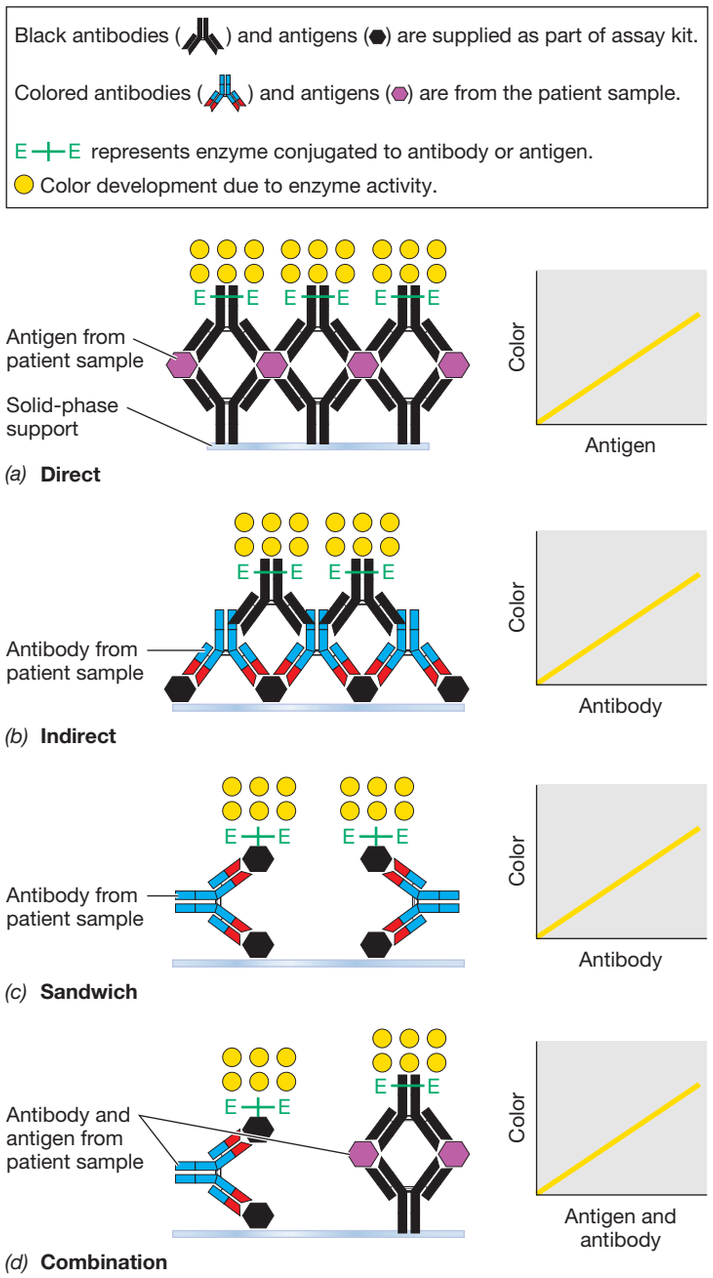


Figure 29.20 Enzyme immunoassays (EIAs). Patient samples are in color. Assay reagents are shown in black. All assays are fixed to a solid-phase support (light blue). Enzymes bound to antigen or antibody convert substrate to a colored product, shown in yellow. In each assay, the amount of colored product is proportional to the amount of pathogen-specific antibody or antigen derived from a patient sample. *(a)* The *direct EIA* uses immobilized pathogen-specific antibody and enzyme-labeled pathogen-specific antibody to detect pathogen antigen in patient samples such as blood. *(b)* The *indirect EIA* uses immobilized pathogen antigen and enzyme-labeled antibody directed to immunoglobulin to detect pathogen-specific antibodies in patient samples such as blood. *(c)* The *sandwich EIA* uses immobilized pathogen antigen and enzyme-labeled pathogen antigen to detect pathogen-specific antibodies in patient samples such as blood. The sandwich EIA is more sensitive than the direct or indirect EIA methods. *(d)* The *combination EIA* uses both the sandwich and direct assays in one platform to identify antibody and antigen in patient samples, maximizing sensitivity. In all cases, it is the enzymatic conversion of a colorless substrate to a colored product, measured spectrophotometrically, that enables quantification of specific antigen and/or antibody.

is added, and the enzyme converts the substrate to its colored product in proportion to the amount of patient antigen bound by the enzyme–antibody complex. Direct EIAs are useful for detecting bacterial exotoxins, such as those produced by *Vibrio cholerae* and *Staphylococcus aureus* (◀ Section 25.6), as well as a variety of viruses, including those that cause influenza and hepatitis.

Indirect EIAs are used to detect antibodies to pathogens in body fluids (Figure 29.20*b*). The indirect test starts with pathogen antigen immobilized on a supportive matrix. Patient serum is added, and antibodies (if present) bind to the antigen. Next, an antibody–enzyme complex specific for the patient antibodies is added. Finally, the addition of the enzyme substrate results in the development of a colored product that is proportional to the concentration of patient antibody in the sample. Indirect EIAs are used to detect serum antibodies to a wide variety of bacterial, viral, and eukaryotic pathogens.

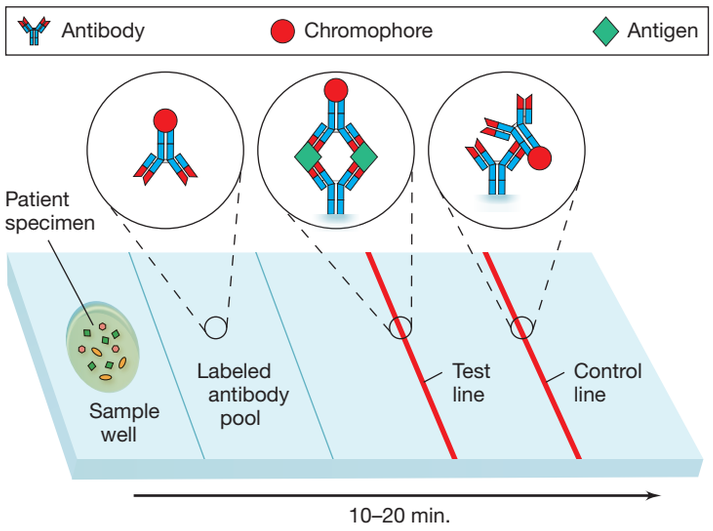
The antigen sandwich EIA also detects antibodies to pathogens in body fluids (Figure 29.20*c*). The sandwich test starts with pathogen antigen immobilized on a support. Patient serum is added, and antibodies (if present) bind to the antigen. Next, the same antigen coupled to enzyme is added, followed by the addition of the enzyme substrate, resulting in the development of colored product that is proportional to the concentration of patient antibody in the sample. This method is especially sensitive because it detects pathogen-specific antibody irrespective of antibody class. This method is often used for HIV screening (the third-generation HIV test) because it can detect IgM produced during the primary immune response to HIV as soon as four weeks after infection. By contrast, most indirect EIAs use anti-IgG as the enzyme-conjugated antibody, delaying observation of antibodies to HIV until the secondary antibody response at least five weeks after infection (◀ Figure 27.10).

The combination EIA, shown in Figure 29.20*d*, makes use of a direct EIA to detect pathogen antigen and the sandwich method to detect pathogen-specific antibodies, both on a single matrix. This method, used for a fourth-generation HIV test, is more sensitive than the third-generation sandwich test; antigen can be detected as little as 2.5 weeks after HIV infection, reducing the time to treatment.

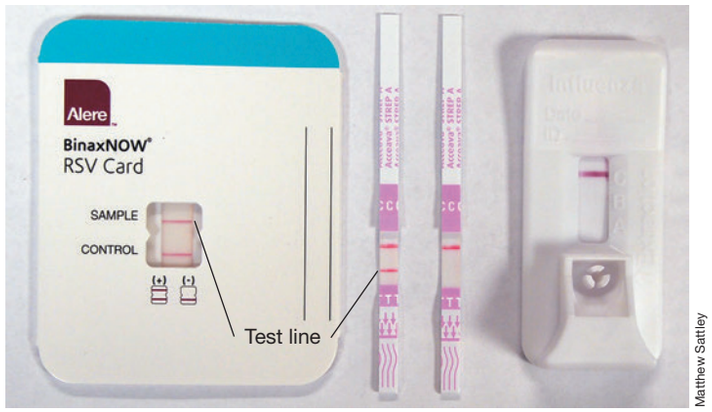
Rapid Tests

Rapid immunoassay procedures use reagents adsorbed to a fixed support material, such as paper strips or plastic membranes. These point-of-care tests cause a color change on the strip within minutes and serve as rapid diagnostic aids for a variety of infectious diseases, including HIV/AIDS.

For most rapid tests, a body fluid (generally urine, blood, saliva, or sputum) is applied to a sample well in a reagent–support matrix. To detect antigen in patient samples, for example to determine infection by *Streptococcus pyogenes* (strep throat), the matrix contains soluble antibodies that are specific to the antigen in question and conjugated to a colored molecule called a *chromophore* (**Figure 29.21**). As the liquid sample diffuses through the matrix, patient antigens (if present) bind the chromophore-labeled antibodies. Capillary action pulls each labeled antigen–antibody complex through the matrix, where it contacts a single line of fixed antibodies. The labeled antigen–antibody complex binds a fixed antibody and becomes immobilized. As the concentration of labeled complex increases, the chromophore becomes visible as a line of color along the fixed antibody, indicating



(a)



(b)

Figure 29.21 Rapid tests. *(a)* A patient specimen containing a mixture of antigens is applied to the sample well of a support matrix. Capillary action pulls the liquid sample through the matrix, and specific antigen (if present) binds soluble, chromophore-labeled antibodies. The labeled antigen–antibody complexes diffuse through the matrix and bind a line of fixed antibodies. A colored line becomes visible as the concentration of labeled complex builds, indicating a positive test for the antigen. Unbound labeled antibodies bind a second line of fixed antibody as a control. *(b)* From left to right, rapid tests for respiratory syncytial virus (RSV), group A streptococci (GAS), and influenza A/B. The RSV test and the left GAS test show a test line, indicating positive reactions. The right GAS test and the influenza A/B test show only a control line, indicating negative reactions. Photo courtesy of Marion General Hospital, Marion, Indiana (USA).

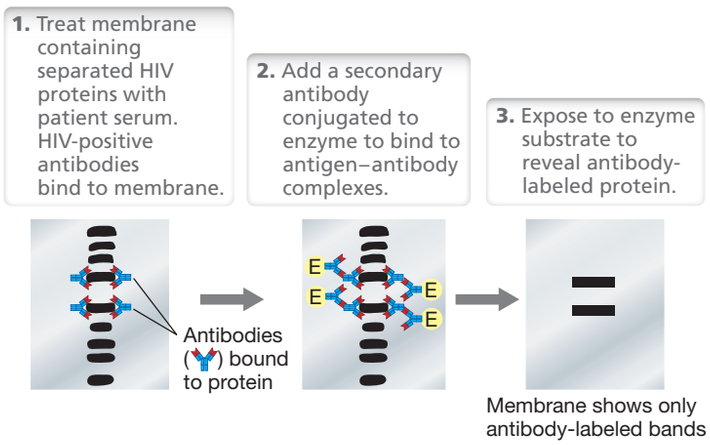
a positive test for the antigen. Labeled antibodies not bound by antigen concentrate at a second line of fixed antibodies that are specific to the labeled antibody rather than the antigen, thus forming a second colored line that serves as the control.

These tests are valuable for point-of-care analysis and provide rapid diagnostic results that can be reported almost immediately, avoiding the need for delays in patient care or for follow-up visits to obtain test results. The drawback to rapid tests, however, is that they are often less specific or less sensitive than more elaborate assays for the same pathogens. As a result, rapid tests often need to be confirmed by EIA or other tests, such as the immunoblot, discussed next.

Immunoblots

Immunoblot methodology requires the *separation* of proteins on a polyacrylamide gel, the *transfer* (blotting) of the proteins from the gel to a nitrocellulose or nylon membrane, and finally, the *identification* of the proteins using specific antibodies (**Figure 29.22*a***). The HIV immunoblot (Figure 29.22*b*) can be used to accurately diagnose HIV infection, but although it is highly specific, it is generally not used as a screening tool because it is less sensitive, more time consuming, and more expensive than the HIV EIA. However, because the HIV EIA occasionally yields false-positive results, the immunoblot method is often used to confirm positive EIA tests. The HIV immunoblot procedure is similar to immunoblot methods used to diagnose infection by other pathogens. In general, immunoblots are used to detect pathogen-specific antibody in patient samples.

To perform the HIV immunoblot, membrane strips containing fixed HIV proteins are incubated with the patient serum sample. If the sample is HIV-positive, patient antibodies will bind to the HIV proteins on the membrane. To detect whether antibodies from the serum sample have bound to HIV antigens, a detecting antibody, anti–human IgG conjugated to an enzyme, is added to the strips. If



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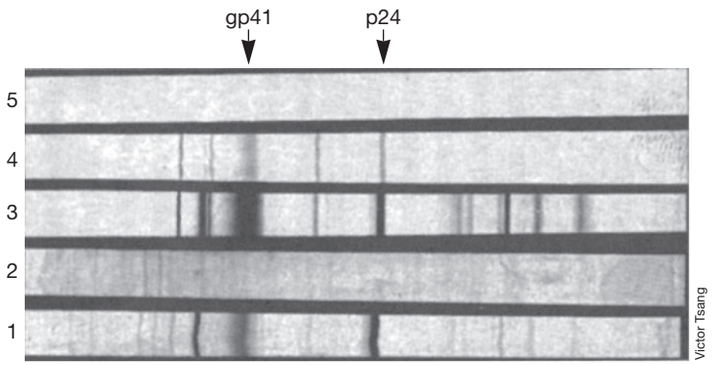


(b)

Figure 29.22 The immunoblot (Western blot) and its use in the diagnosis of human immunodeficiency virus (HIV) infection. *(a)* Protocol for an immunoblot. *(b)* The molecules p24 (capsid protein) and gp41 (envelope glycoprotein) are diagnostic for HIV. Lane 1, positive control serum (from known AIDS patients); lane 2, negative control serum (from healthy volunteer); lane 3, strong positive from patient sample; lane 4, weak positive from patient sample; lane 5, reagent blank to check for background binding.

the detecting antibody binds, the activity of the conjugated enzyme, after addition of substrate, will form a colored band on the strip at the site of antibody binding. The patient is HIV-positive if the positions of the bands in the patient sample match those of a positive control; a negative control serum is also analyzed and must show no bands (Figure 29.22*b*). As the test is mostly used to confirm positive EIA results for HIV (or correct false positives), variations in band intensity do not affect interpretation of the results.

Check Your Understanding

- Compare direct, indirect, sandwich, and combination EIAs with respect to their ability to identify infection with a particular pathogen.
- Compare the advantages and disadvantages of EIA, rapid tests, and immunoblots with respect to speed, sensitivity, and specificity.

29.8 Nucleic Acid–Based Clinical Assays

In Section 12.1 we discussed how the polymerase chain reaction (PCR) amplifies nucleic acids, forming multiple copies of target sequences. PCR techniques can employ primers for a pathogen-specific gene to examine DNA derived from suspected infected tissue, even in the absence of an observable or culturable pathogen. As a result, PCR-based tests are widely used in the clinical lab for pathogen identification, and they are particularly useful for identifying viruses and other intracellular pathogens that are difficult or impossible to culture using current techniques. PCR methods are extremely sensitive and do not depend on pathogen isolation or growth, and no detection of an immune response to the pathogen is required. Instead, microbe-specific nucleic acid sequences are detected in the assays.

Nucleic Acid Hybridization and Amplification

Nucleic acid hybridization (◀ Section 12.1) is the central theme of nucleic acid–based molecular methods. In clinical medicine, hybridization methods are employed to identify specific pathogens in patient samples by using unique nucleic acid *probes* to detect the presence of specific DNA sequences. Nucleic acid probes are single-stranded DNA molecules having a sequence complementary to that of a gene of interest. A DNA probe oligonucleotide may be less than 100 base pairs or up to several kilobases in length. If a microbe from a clinical specimen contains DNA or RNA sequences complementary to the probe, the probe will hybridize (following appropriate sample preparation to yield single-stranded target molecules), forming a double-stranded molecule (**Figure 29.23**). To detect the hybridization reaction, the probe is labeled with a reporter molecule, which is usually a fluorescent compound, but radioisotopes or enzymes may also be used.

To carry out a probe assay, samples are treated with strong alkali, usually sodium hydroxide (NaOH), to lyse the cells and partially denature the pathogen DNA, forming single-stranded DNA

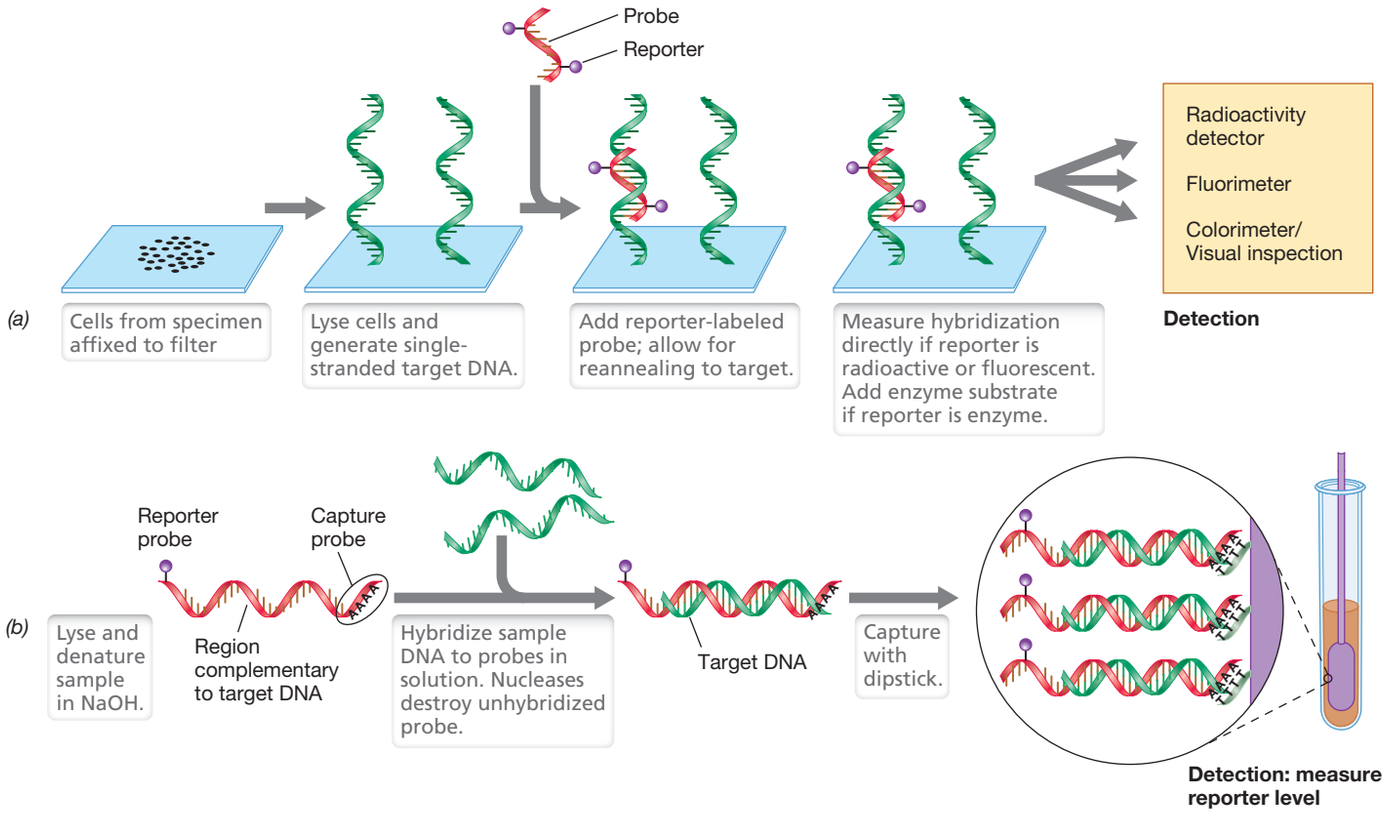


Figure 29.23 Nucleic acid probe methodology in clinical diagnostics. *(a)* Membrane filter assay. The reporter can be a radioisotope, a fluorescent dye, or an enzyme. *(b)* Dipstick assay. Dual reporter and capture probes are used. The capture probe contains a poly(A) tail that hybridizes to a poly(T) oligonucleotide affixed to the dipstick. Binding of the target DNA–reporter complex is usually detected as a visible color change.

molecules (Figure 29.23). Incubation at an appropriate temperature facilitates formation of a stable duplex between target DNA and probe DNA. The extent of hybridization is measured using the reporter molecule attached to the probe. Some assays use two-component probes that function as both a *reporter* probe and a *capture* probe; the addition of a sequence tag allows the hybridized molecule to be affixed to a matrix, usually a dipstick, for detection purposes (Figure 29.23*b*).

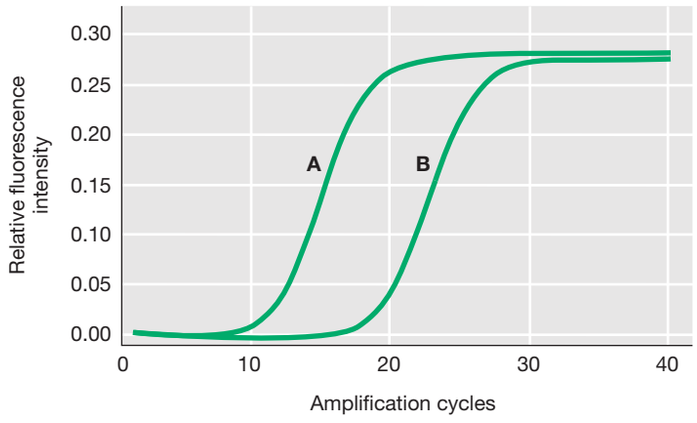
Nucleic acid hybridization also plays a critical role in the various PCR-based techniques used to amplify target DNA or RNA molecules. PCR analysis begins with the extraction of DNA or RNA from the sample to be tested. Next, the nucleic acid must be amplified using appropriate gene-specific nucleic acid *primers*. These short oligonucleotides (typically 15–27 base pairs in length) are not used as probes but instead function to jump-start DNA polymerase during PCR amplification of pathogen-specific genes. Lastly, the amplified nucleic acid product (the *amplicon*) is visualized, a procedure that may involve gel electrophoresis or, more often in clinical laboratories, fluorescence. The presence of the appropriate amplified gene segment confirms the presence of the pathogen.

Quantitative PCR and Reverse Transcription PCR

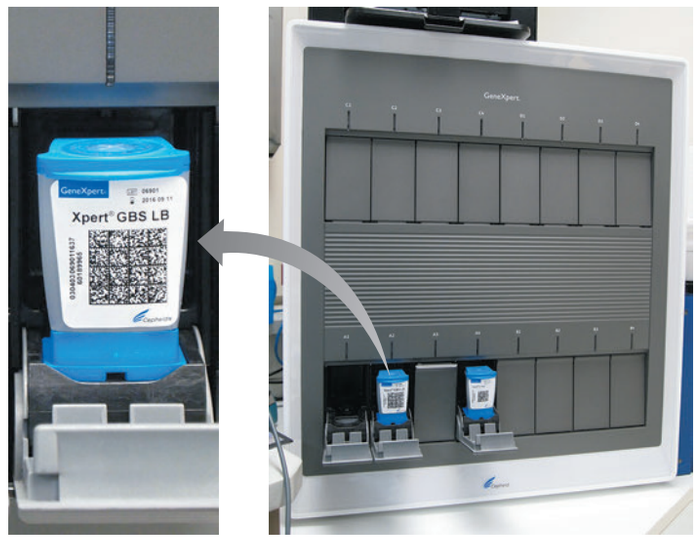
Many clinical PCR tests employ *quantitative real-time PCR* (*qPCR*). This process uses fluorescent probes to label PCR amplicons, thereby allowing the accumulation of target DNA to be visualized. Because probe fluorescence increases upon binding to DNA, the level of fluorescence increases proportionally as the target DNA is amplified. The fluorescent probes may be either nonspecific or specific for the target DNA. For example, the dye SYBR Green binds *nonspecifically* to double-stranded DNA and fluoresces only when bound. When added to the PCR mixture, SYBR Green fluorescence indicates the presence of double-stranded DNA produced by the amplification process (**Figure 29.24*a***). By contrast, *gene-specific* fluorescent probes, made by attaching a fluorescent dye to a short DNA probe specific to a target sequence, fluoresce only when double-stranded DNA of the correct sequence accumulates.

Because qPCR amplification can be monitored continuously via fluorescence, visualization by gel electrophoresis is not necessary to confirm amplification. Using modern instrumentation, such as that shown in Figure 29.24*b*, detection of a gene diagnostic for a particular pathogen in a clinical sample may be performed in about 2 hours. Moreover, by monitoring the *rate* of fluorescence increase in the PCR reaction, it is possible to accurately determine the amount of target DNA present in the original sample (Figure 29.24*a*). Thus, qPCR can be used to assess the abundance of a pathogen in a sample by quantifying a gene characteristic for that particular organism.

Another variation of basic PCR is *reverse transcription PCR* (*RT-PCR*), which uses pathogen-specific *RNA* to produce complementary *DNA* (cDNA) directly from patient samples (◀ Section 12.1). This technology is especially useful for the detection of RNA viruses, including retroviruses such as HIV. The first step in RT-PCR is to use the enzyme reverse transcriptase to make a cDNA copy of an RNA sample. PCR is then used to amplify the cDNA. By isolating RNA in a sample and making cDNA copies of the corresponding gene(s), one can employ qPCR to monitor the expression of a particular gene from a pathogen. The amplified DNA can then be sequenced or probed for identification.



(a)



(b)

Figure 29.24 Quantitative real-time polymerase chain reaction (qPCR) for clinical diagnostics. *(a)* DNA extracted from a gram-negative bacterial culture was monitored for expression of 16S rRNA (curve A) and *npt* (curve B), a kanamycin resistance marker, using gene-specific primers. The fluorescent dye SYBR Green was added to the PCR mixture and used to visualize amplified DNA as it formed. The curve on the left (A) had 0.15 fluorescence units after 15 cycles, while the curve on the right (B) had 0.15 fluorescence units after 22 cycles, indicating that the 16S rRNA had a higher abundance of template DNA than *npt* in this strain. *(b)* qPCR instrumentation in a clinical laboratory. The single-use cartridges contain all necessary components for the qPCR reaction, including group-specific primers and fluorescent dyes, and pathogens can be identified in less than 2 hours. The cartridge on the left is specific for detection of group B streptococci (*Streptococcus agalactiae*). Photos courtesy of Marion General Hospital, Marion, Indiana (USA).

Qualitative PCR

Some diagnostic tests based on the qPCR format use a slightly different amplification protocol and an additional step to identify pathogen-associated genes. This method, called *qualitative PCR*, uses labeled hybridization primers that are incorporated into an amplicon product of a qPCR reaction.

In the example shown in **Figure 29.25**, the hybridization probes are targeted to the DNA *pol* gene of herpes simplex 1 and 2 viruses (HSV-1 and HSV-2)(◀ Section 11.7). The amplicon is detected using two distinct hybridization probes labeled with fluorescent dyes. The

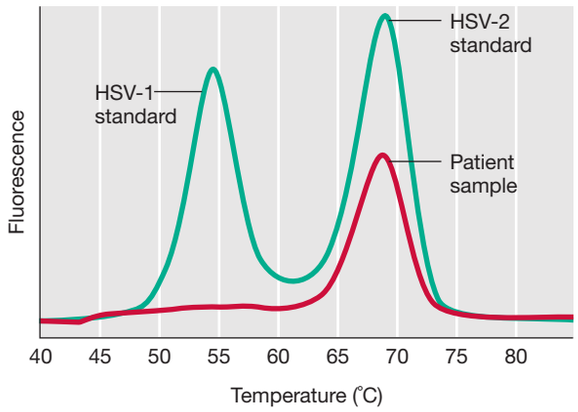


Figure 29.25 Qualitative PCR for the *pol* gene of HSV-1 (herpes simplex 1 virus) and HSV-2. DNA from a patient sample was assayed for the *pol* gene of both HSV-1 and HSV-2 following quantitative PCR (qPCR). Two fluorescent-labeled probes hybridize with an internal sequence of the amplified fragment of each viral genome during the PCR cycle. After hybridization to the template DNA, the probes are excited by a light source, and their fluorescence is measured. After the PCR cycle, each virus shows a distinct DNA melting curve. The melting profile in the patient sample (red) corresponds to the HSV-2 standard (green), indicating infection with HSV-2.

probes hybridize to an internal sequence of the amplified fragment during the annealing phase of the PCR cycle. After hybridization to the template DNA, the probes are excited by a light source in the PCR instrument. The emitted fluorescence is then measured, and, after the PCR cycle, a melting curve analysis is performed to differentiate between samples positive for HSV-1 and HSV-2. Because of nucleotide polymorphisms between the DNA *pol* genes of the two virus subtypes, the melting curve for HSV-1 is distinct from that of HSV-2 (Figure 29.25). The results are compared to internal assay control reactions, and an unambiguous diagnosis of viral infection can be obtained within hours.

With a solid foundation of clinical diagnostics in hand, we move on in Chapter 30 to explore how infectious diseases are transmitted, tracked, and controlled in the fast-moving field of *epidemiology*.

Check Your Understanding

- What advantage(s) does nucleic acid amplification have over standard culture methods for identification of microorganisms? What are the disadvantages?
- How do quantitative PCR (qPCR) and qualitative PCR differ?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Microbiology and the Healthcare Environment

29.1 Clinical laboratory safety requires training and planning to prevent contamination and possible infection of laboratory workers. Specific precautions and procedures proportional to the risk of infection by a given agent, designated by biosafety levels (BSL), must be in place to handle contaminated materials and patient specimens.

Q How are most laboratory-associated infections contracted? What action can be taken to prevent laboratory infections?

29.2 Patients in healthcare facilities are unusually susceptible to infectious disease because of their compromised health and potential exposure to various pathogens in the facilities. Many healthcare-associated infections are caused by drug-resistant pathogens.

Q What are healthcare-associated infections (HAI) and how are they acquired? What problems can they cause?

II • Isolating and Characterizing Infectious Microorganisms

29.3 Appropriate sampling, observation, and culture techniques are necessary to isolate and identify potential pathogens. The selection of techniques requires knowledge of the ecology, physiology, and metabolism of suspected pathogens. Most pathogens exhibit unique metabolic patterns when grown on specialized selective and differential media. Growth-dependent patterns provide information helpful for unambiguous pathogen identification.

Q In order to ensure accurate diagnosis, what measures should be taken during the collection of specimens and while detecting and culturing pathogens?

29.4 Pathogens isolated from clinical samples are often tested for antibiotic susceptibility to ensure appropriate antibiotic therapy. Testing is based on the minimum inhibitory concentration of an agent necessary to completely inhibit growth of a pathogen.

Q How does the traditional method of determining minimum inhibitory concentration (MIC) compare to current practice?

III • Immunological and Molecular Tools for Disease Diagnosis

29.5 An immune response is a natural outcome of infection. Specific immune responses characterized by a rise in antibody titers and positive T cell–mediated skin tests can be used to provide evidence of infections and to monitor convalescence. Monoclonal antibodies reproducibly provide specificity for a wide range of diagnostic and therapeutic purposes.

Q Why does antibody titer rise after infection? Is a high antibody titer indicative of an ongoing infection? Explain. Why is it necessary to obtain an acute and a convalescent blood sample to monitor infections?

29.6 Precipitation and agglutination reactions produce visible results involving antigen–antibody interactions. Fluorescent antibodies are used for quick, accurate identification of pathogens and other antigenic substances in tissue samples, blood, and other complex mixtures. Fluorescent antibody–based methods can be used for identification of a variety of microbial cell types.

Q Why are agglutination tests so widely used in clinical diagnostics? How are fluorescent antibodies used to diagnose diseases? What advantages do immunofluorescent techniques have over traditional culturing?

29.7 Enzyme immunoassays (EIAs), rapid tests, and immunoblots are sensitive and specific immunological assays. These tests can be engineered to detect either antibody or antigen for diagnosis of infections by any of a large number of pathogens.

Q EIAs are extremely sensitive diagnostic tools. Why, then, is the immunoblot (Western blot) procedure used to confirm screening tests that are positive for human immunodeficiency virus (HIV)?

29.8 Nucleic acid amplification (PCR) methods are applied as extremely specific diagnostic tools used for a large number of pathogens. Quantitative PCR (qPCR) and qualitative PCR techniques provide quantification and identification of pathogens, and reverse transcription PCR (RT-PCR) is especially useful for detecting RNA viruses.

Q Distinguish between quantitative and qualitative PCR. How does qualitative PCR expand upon the qPCR technique?

APPLICATION QUESTIONS

1. Define the procedures you would use to isolate and identify a new pathogen. Keep in mind Koch's postulates (◀ Figure 1.33) as you form your answer. Be sure to include growth-dependent assays, immunoassays, and molecular assays. Which of your assays could be adapted to be used as a routine test for rapid clinical diagnosis?
2. Imagine yourself as a clinical microbiologist with all of the diagnostic tools described in this chapter available for your analyses. Which tool(s) would you use (and why) for the following cases? (1) A patient has a life-threatening infection caused by a difficult-to-culture bacterium, and treatment of the infection is absolutely dependent on an extremely rapid identification of the pathogen. (2) A patient has a less severe bacterial infection with a pathogen that is easily culturable and treatable.

CHAPTER GLOSSARY

Agglutination a reaction between antibody and particle-bound antigen resulting in visible clumping of the particles

Differential media growth media that allow identification of microorganisms based on phenotypic properties

Enzyme immunoassay (EIA) a test that uses antibodies or antigens linked to enzymes to detect antigens or antibodies in body fluids

Fluorescent antibody an antibody molecule covalently modified with a fluorescent dye that makes the antibody visible under fluorescent light

Healthcare-associated infection (HAI) an infection acquired by a patient in a healthcare facility, particularly during a stay in the facility. Also called a *nosocomial infection*.

Immunoblot (Western blot) the use of labeled antibodies to detect specific proteins after separation by electrophoresis and transfer to a membrane

Minimum inhibitory concentration (MIC) the smallest amount of an antimicrobial substance that prevents growth of the target microorganism

Monoclonal antibody (mAb) a single type of antibody made by a single B cell hybridoma clone

Point-of-care diagnostics clinical testing that produces a clear medical result and a treatment plan within the same clinical visit

Precipitation a reaction between antibody and a soluble antigen resulting in a visible, insoluble complex

Selective media culture media that allow the growth of certain organisms while inhibiting the growth of others through one or more added media components

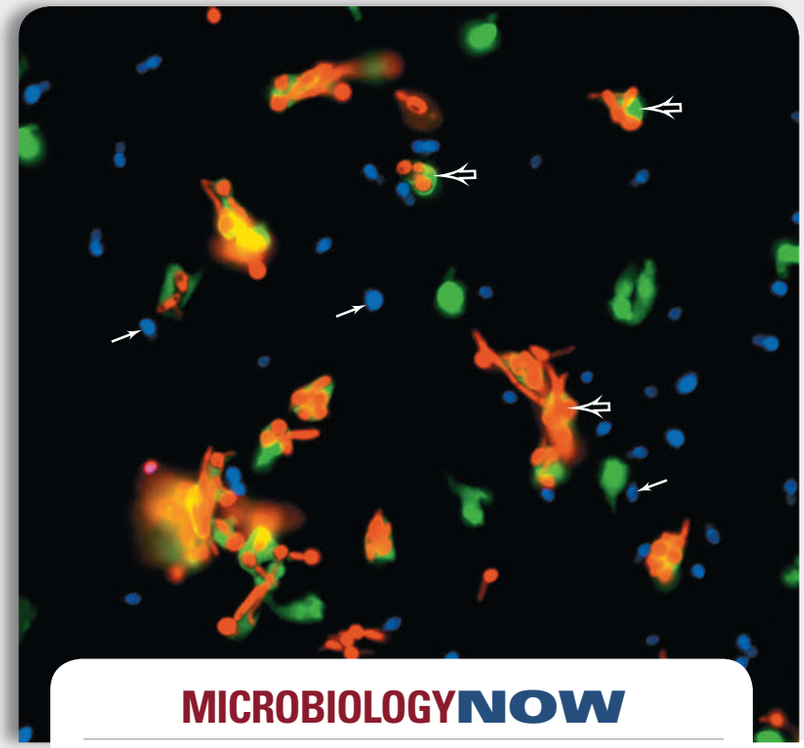
Sensitivity the lowest amount of antigen that can be detected by a diagnostic test

Serology the study of antigen–antibody reactions in vitro

Specificity the ability of a diagnostic test to identify a specific pathogen

Titer the number of individual particles of a substance, such as antibodies or infectious virions, per unit volume of a solution

Epidemiology and Public Health 30



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III Emerging Infectious Diseases, Pandemics, and Other Threats 976

MICROBIOLOGYNOW

A New Urgent Threat Is Emerging in Public Health Microbiology

For several years now, epidemiologists have been tracking the emergence of a new, highly invasive and deadly pathogen, but it is neither a bacterium nor a virus. This pathogenic microbe is a nosocomial (healthcare-associated) fungus, and the U.S. Centers for Disease Control and Prevention (CDC) has recently added it to a list of microorganisms considered to be "urgent threats." The new fungal pathogen, first described in 2009, is a yeast called *Candida auris*, a relative of the opportunistic yeast *Candida albicans*, which causes vaginal candidiasis and oral thrush.

Since its initial description, cases of *C. auris* infection have been documented in hospitals on every continent but Antarctica. Systemic infection by *C. auris* results in a mortality rate approaching 60%, with immunocompromised patients comprising the largest fraction of these fatalities. One major *C. auris* outbreak in the United Kingdom occurred among intensive care unit patients. In this incident, the yeast was transmitted between patients through multiuse thermometers that measure temperature in the armpit. Despite staff's adherence to established protocols to disinfect the equipment between uses, a shocking 86% of patients that had contact with the thermometers subsequently developed *C. auris* infection.

Invasive infections by *C. albicans* often result from neutropenia (decreased numbers of neutrophils). However, studies have shown that *C. auris* infection frequently occurs even in the presence of normal numbers of neutrophils, suggesting that innate defenses do not adequately contain the pathogen. In the presence of both *C. albicans* (red cells, photo) and *C. auris* (blue), neutrophils (green) readily engaged and killed *C. albicans* (open arrows) but ignored *C. auris* (closed arrows). This fascinating but disconcerting finding suggests that *C. auris* possesses an efficient mechanism of phagocyte evasion. Considering that *C. auris* is also resistant to several antifungal drugs, healthcare providers and epidemiologists may have a long struggle ahead of them to control this emerging pathogen.

Source: Nett, J.E. 2019. *Candida auris*: An emerging pathogen "incognito"? *PLoS Pathog. 15*(4): e1007638. doi:10.1371/journal.ppat.1007638.

Beginning with this chapter and to the end of the book, we focus on the nature and diversity of infectious diseases. Everything we have learned up to this point—cell structure, metabolism, growth, genetics, and genomics; microbial evolution, diversity, and ecology; and host–microbe relationships and the immune response—will help us better understand the disease strategies and exploitable weaknesses of infectious microbial agents.

As a prelude to our coverage of the clinical aspects of infectious diseases in the following four chapters, we explore the "big picture" of how infectious diseases flow through populations. **Epidemiology** is the study of the occurrence, distribution, and determinants of health and disease in populations and is intertwined with **public health**, the health of the population as a whole. Although in developed countries infectious diseases are not leading causes of death, in developing countries infectious diseases can account for nearly half of all deaths. Hence, identifying and solving problems associated with infectious disease transmission is a major goal of the epidemiologist.

I • Principles of Epidemiology

Focused on the distribution, prevalence, and transmission of disease, epidemiology is foundational to the health and prosperity of the human population on a global scale. Moreover, disease tracking often reveals patterns that improve disease treatment.

In this first part of the chapter we consider the principles of epidemiology and define key terms in the lexicon of the epidemiologist.

30.1 The Language of Epidemiology

The epidemiologist traces the spread of a disease to identify its origin and mode of transmission in a population. The population might be all people in a certain city, country, or region, or it could be the entire human population. Alternatively, the population under study could be a particular demographic within a larger population, such as only males or only those of a specific race or age group. Raw data are gathered from disease-reporting networks such as city, county, state, and national public health departments, clinical records, and patient interviews.

Mastering Microbiology
Art Activity: Figure 30.1 The concepts of disease incidence and disease prevalence

A major job of the epidemiologist is to carry out **disease surveillance**—the observation, recognition, and reporting of diseases as they occur—and then analyze the data provided by local and national health authorities to reveal trends and signals of disease outbreaks. The epidemiologist thus stands in contrast to the clinical health provider—the one who actually treats the infected patient. However, in order to both track a disease and predict its spread in a population, the epidemiologist must integrate clinical results and surveillance data to formulate effective public health measures for disease control.

Disease Incidence and Prevalence

The epidemiologist often uses the words *incidence* and *prevalence* when discussing infectious diseases. The **incidence** of a particular disease is the *number of new cases* in a population in a given time period (Figure 30.1). For example, in 2016 there were 38,700 new cases of HIV infection in the United States, for an incidence of 12 new cases per 100,000 people per year. The **prevalence** of a given disease is the *total number of new and existing disease cases* in a population in a given time period (Figure 30.1). For example, within the United States there were 1,008,929 persons living with HIV/AIDS at the end of 2016. Expressed another way, the prevalence of HIV/AIDS in the United States was about 317 cases per 100,000 persons in 2016.

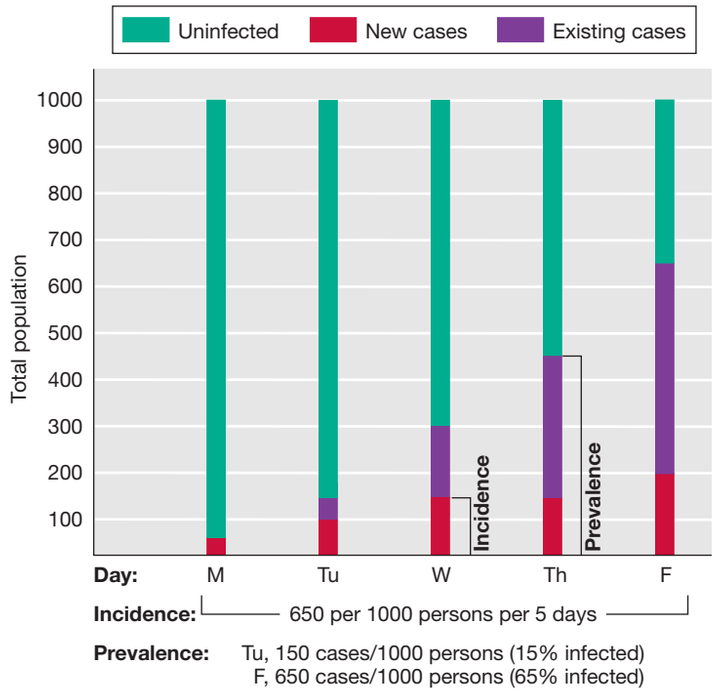


Figure 30.1 The concepts of disease incidence and disease prevalence. Disease incidence is a rate function and is defined as the number of new cases over a given time period (day[s], week, month, etc.); incidence is an indicator of infection risk. Disease prevalence is the total number of diseased individuals at some time point and is a snapshot of the extent of a disease in a population at any given time.

Essentially a *rate* measurement, disease incidence can be used to predict the *risk* of disease for an individual in a defined population within a specific time period. By contrast, prevalence measures the total disease burden in a population and can be thought of as a "snapshot" of the disease at a specific instant (Figure 30.1). The incidence and prevalence of disease are also major indicators of the public health of a population.

The Scope of Disease

Other common epidemiological terms speak to the scope of a disease. A disease is an **epidemic** when it simultaneously infects an unusually high number of individuals in a population; a **pandemic** is a widespread, usually global epidemic. By contrast, an **endemic disease** is one that is constantly present—typically in low numbers—in a population (Figure 30.2). An endemic disease implies that the pathogen may not be highly virulent or that the majority of individuals in the population may be immune, resulting in low but persistent numbers of cases. Individuals infected with a pathogen that causes an endemic disease are **reservoirs** of infection, a source of infectious agents from which susceptible individuals may be infected.

Sporadic cases of a disease occur one at a time in geographically separated areas, suggesting that the cases are not related. A disease

(a) **Endemic disease** *(b)* **Epidemic disease** *(c)* **Pandemic disease**

Figure 30.2 Endemic, epidemic, and pandemic disease. Each dot represents a disease case or outbreak. *(a)* Endemic diseases are present in the population in specific geographical areas. *(b)* Epidemic diseases show high incidence in a wider area, usually developing from an endemic focus. *(c)* Pandemic diseases are distributed intercontinentally.

outbreak, on the other hand, is the appearance of a large number of cases in a short time in an area previously experiencing only sporadic or endemic disease. Diseased individuals that show no symptoms or only mild symptoms are said to have *subclinical infections*. Subclinically infected individuals are frequently **carriers** of the particular pathogen, with the pathogen reproducing within them and being shed into the environment where it can infect others. Finally, the term **virulence**, often used in epidemiological parlance, is a measure of the *relative ability* of a pathogen to cause disease. Some pathogens are highly virulent while others are only weakly so (◀ Section 25.3).

Stages of Disease

A well-adapted pathogen lives in balance with its host, taking what it needs for existence and causing only minimal harm. Such pathogens may cause **chronic infections** (long-term infections) in the host. When there is a balance between host and pathogen, both host and pathogen survive. Tuberculosis (▶ Section 31.4) is a good example of a chronic infection. On the other hand, a host whose resistance is compromised because of factors such as poor diet, age, and other stressors can be harmed or even killed; for example, a chronic tuberculosis infection can eventually kill the host.

New pathogens occasionally emerge to which specific populations or even an entire species has not developed resistance. Such emerging pathogens often cause **acute infections**, characterized by rapid and dramatic disease onset and a relatively quick return to health. Influenza caused by a new strain of influenza virus (▶ Section 31.8) would be an example of an acute infection, as would many other infectious diseases that show a rapid onset and recovery, such as various food infections and food poisonings (Chapter 33), or even the common cold (▶ Section 31.7). The progression of clinical symptoms for an acute infectious disease can be divided into stages, and the terms used to describe these stages are also part of the epidemiologist's lexicon:

1. *Infection:* The organism invades, colonizes, and grows in the host.
2. *Incubation period:* Some time always passes between infection and the appearance of disease signs and symptoms. Some diseases, like influenza, have very short incubation periods, measured in days; others, like AIDS, have longer incubations, sometimes extending for years. The incubation period for a given disease is determined by the inoculum size, the virulence and life cycle of the pathogen, and the resistance of the host. At the end of the incubation period, the first signs and symptoms, for example, a mild cough and a feeling of general fatigue in the case of an ensuing cold, usually appear.
3. *Acute period:* The disease is at its height, with overt symptoms and signs such as fever and chills.
4. *Decline period:* Disease signs and symptoms subside. As fever subsides, usually following a period of intense sweating, a feeling of well-being develops. The decline period may be rapid (within one day), in which case decline occurs by *crisis*, or it may be slower, extending over several days, in which case decline occurs by *lysis*.
5. *Convalescent period:* The patient regains strength and returns to the normal healthy state.

After the acute period, the immune mechanisms of the host (Chapters 26 and 27) become increasingly important for complete recovery from the disease.

Mortality, Morbidity, and DALY

The terms *morbidity* and *mortality* are commonly used in epidemiology. **Mortality** is the incidence of *death* in a population. Infectious diseases were the major causes of death worldwide in 1900, but they are now less prevalent in developed countries. Noninfectious "lifestyle" diseases such as heart disease and cancer are now much more prevalent in developed regions and cause higher mortality than do infectious diseases (◀ Figure 1.13). However, this could change rapidly if public health measures were to break down. Worldwide, and especially in developing countries, infectious diseases are still major causes of mortality (**Table 30.1** and see Figure 30.9).

Morbidity is the incidence of *disease* in a population and includes both fatal and nonfatal diseases. Morbidity statistics indicate the public health of a population more precisely than mortality statistics because many diseases have relatively low mortality. Put another way, the major causes of *illness* are quite different from the major causes of *death*. For example, high-morbidity infectious diseases include acute respiratory diseases such as the common cold and acute digestive disorders. However, seldom do these diseases cause death in populations living in developed countries. Thus, both of these diseases have high morbidity, but low mortality. On the other hand, Ebola virus infects relatively few people worldwide every year, but the mortality in some outbreaks approaches 70% and averaged 40% in the West African Ebola outbreak of 2013–2015. Thus, Ebola has low morbidity, but high mortality.

Epidemiologists tend to focus on morbidity and mortality statistics as a means of ranking the severity of pathogens and tracking disease trends. However, illness and death are not the only outcomes of an infectious disease. Lost among these statistics is the reduction in life quality and productivity due to a disease. The **disability-adjusted life year (DALY)** is a quantitative measure of disease burden and is defined as the cumulative number of years lost due to an

TABLE 30.1 Worldwide deaths due to infectious diseases[a]

Disease	Deaths (% of deaths from all infectious diseases)	Causative agent(s)
Respiratory infections[b]	26	Bacteria, viruses, fungi
Diarrheal diseases	12	Bacteria, viruses
Tuberculosis[c]	11	Bacterium
Acquired immunodeficiency syndrome (AIDS)	9	Virus
Malaria	4	Protist
Meningitis, bacterial[c]	2	Bacteria
Encephalitis	1	Bacteria, viruses, fungi
Hepatitis (all types)[d]	1	Viruses
Syphilis	1	Bacterium
Measles[c]	1	Virus
Other communicable diseases	32	Various agents

[a]Data show the ten leading causes of death due to infectious diseases in 2016. Worldwide there were nearly 57 million total deaths, and 20% of these were from infectious diseases, mostly in developing countries. In the United States in 2016, deaths from infectious diseases were about 4% of total deaths (influenza, pneumonia and septicemia were leading causes). Data adapted from data published by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA.
[b]For some acute respiratory agents, such as influenza viruses and *Streptococcus pneumoniae*, there are effective vaccines; for others, such as colds, there are no vaccines.
[c]Diseases for which effective vaccines are available.
[d]Vaccines are available for hepatitis A virus and hepatitis B virus. There are currently no vaccines for other hepatitis agents, but a drug is available that can cure hepatitis C infections.

illness itself, a disability due to an illness (whether an infectious disease or not), or premature death.

The leading causes of death are not the leading causes of disability; about one-third of all disability years lost are due to psychiatric and neurological conditions. But many infectious diseases cause chronic disability and thus such data are important measures of the overall burden of disease. This is especially true of a series of *neglected tropical diseases*, a group of infectious diseases found mainly in tropical countries that are major disablers rather than killers. These include in particular parasitic worm infections such as hookworm, filariases, and schistosomiasis (▶ Section 34.7). Hundreds of millions of people suffer from these infections worldwide, and although some die, most do not. However, life quality and longevity of survivors is often greatly diminished, and DALY numbers attempt to quantify this frequently overlooked but nevertheless important aspect of epidemiological statistics.

With some of the epidemiologist's common terminology in mind, we are now able to move on to consider how infectious diseases spread (or do not spread) in susceptible populations.

Check Your Understanding

- Why do epidemiologists acquire population-based data about infectious diseases?
- Distinguish between an endemic disease, an epidemic disease, and a pandemic disease.
- Which is more severe, a disease with a high mortality or one with a high morbidity? What is a DALY?

30.2 The Host Community

The colonization of a susceptible host population by a pathogen may lead to rapid transmission to uninfected hosts, widespread infections, and an epidemic. As the host population develops resistance, however, the spread of the pathogen is checked, and eventually a balance is reached in which host and pathogen populations reach a state of equilibrium. In an extreme case, failure to reach equilibrium could result in death and eventual extinction of the host species. If the pathogen has no other host, then the extinction of the host also results in extinction of the pathogen. The evolutionary success of a pathogen thus depends on its ability to establish an equilibrium with its host rather than destroy the host population altogether. In most cases, the evolution of the host and the pathogen affect one another; that is, the host and pathogen *coevolve*.

Coevolution of a Host and a Pathogen

A classic example of host and pathogen coevolution is a case where myxoma virus was intentionally introduced to control an exploding wild rabbit population in Australia. The virus, spread by the bite of mosquitoes and also from animal to animal by direct contact, is extremely virulent for rabbits and causes fatal infections in susceptible animals. Within several months, the introduced epidemic had spread over a large area, rising to peak incidence in the summer when the mosquito vectors were present, and then declining in the winter as mosquitoes disappeared. In this experiment, over 95% of infected rabbits died during the first year, but within six years, rabbit mortality dropped to about 30%, indicating that the resistance of the wild rabbit population to the virus had increased dramatically (**Figure 30.3**). When viruses isolated from these wild rabbits were used to infect laboratory rabbits that had not previously been exposed to the virus, the virus could be seen to have lost virulence over the

Figure 30.3 Myxoma virus and host coevolution. Myxoma virus was introduced into Australia to control the wild rabbit population. Virus virulence was measured as the average mortality in laboratory rabbits for infection with myxoma virus recovered from the field each year. Rabbit mortality was determined by removing young wild rabbits from dens and infecting them with a viral strain that killed 90–95% of control laboratory rabbits.

six-year period. This was further confirmed by the resistance observed in newborn wild rabbits exposed to the virus. Within three years, viral virulence decreased by about 80% and maintained this diminished level of virulence (Figure 30.3). Thus, within just a few years, the rabbit population had evolved to reach an equilibrium with the pathogen.

For pathogens that do not exhibit host-to-host transmission, there is no selection for decreased virulence to support mutual coexistence, as was seen in the rabbit myxoma virus experiment. An example of this is *Clostridium tetani*, a common soil bacterium that causes tetanus when accidently introduced into flesh through a penetrating wound (◀ Section 25.6 and ▶ Section 32.9). Vectorborne pathogens transmitted solely by the bite of ticks or other arthropods, such as in spotted fever rickettsiosis (Rocky Mountain spotted fever, ▶ Section 32.3), are also under no evolutionary pressure to spare the human host. As long as the vector is only a *carrier* of the pathogen and does not contract the disease itself, there is no selection for weakened strains of the pathogen and thus the pathogen can maintain a high level of virulence.

Herd Immunity

Spread of an infectious disease through a highly susceptible population is typically much different than through a population where many, or even just some, potential hosts are immune, either from a previous natural infection with the same pathogen or by artificial means through vaccination. If a high enough proportion of the individuals in a population are immune to a pathogen, then the population as a whole can be protected from an epidemic, resulting in a collective level of resistance to infection called **herd immunity** (Figure 30.4).

The concept of herd immunity is easy to understand. In essence, what herd immunity amounts to is a breakage in the chain of pathogen transmission from one susceptible host to another because most hosts in the population are immune (Figure 30.4). Herd immunity is not a fixed number, and the assessment of herd immunity is important for understanding the development of epidemics. The more highly infectious a pathogen, or the longer its period of infectivity, the greater the proportion of immune individuals necessary to prevent epidemic disease spread. For a highly infectious disease such as measles, 90–95% of the population must be immune to confer herd immunity (see Table 30.3). By contrast, a lower proportion of immune individuals can prevent an epidemic of a less infectious agent or one with only a brief period of infectivity. Mumps virus, which is less infectious than measles virus, exhibits this pattern. In the absence of immunity, even poorly infectious agents can be transmitted from person to person if susceptible hosts have repeated or constant contact with an infected individual. This is the case for the transmission of H5N1 avian influenza among humans (Section 30.8).

Check Your Understanding

- Explain coevolution of host and pathogen. Cite a specific example.
- How does herd immunity prevent a nonimmune individual from acquiring a disease? Give an example.

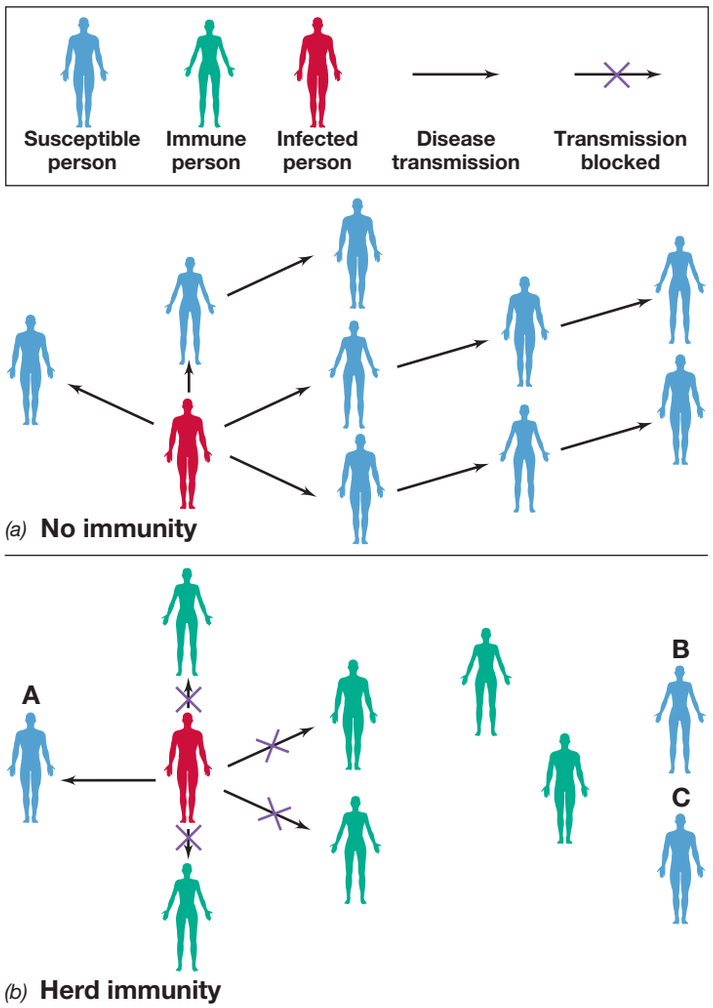


Figure 30.4 Herd immunity and transmission of infection. Immunity in some individuals protects susceptible individuals from infection. *(a)* In a population with no immunity, transfer of a pathogen from one infected individual can ultimately infect (arrows) all individuals as newly infected individuals in turn transfer the pathogen to other individuals. *(b)* In a population that is only moderately dense and that has some immunity against a moderately transmissible pathogen such as influenza, an infected individual cannot transfer the pathogen to all susceptible individuals because resistant individuals, immune due to previous exposure or immunization, break the cycle of pathogen transmission: Susceptible individual A becomes infected, but susceptible individuals B and C are protected. The proportion of a population that must be immune for herd immunity to be effective also varies with the disease; highly infectious diseases require a higher proportion of immune individuals for herd immunity to prevent transmission (see Table 30.3).

Mastering Microbiology
Art Activity: Figure 30.4 Herd immunity and transmission of infection

30.3 Infectious Disease Transmission and Reservoirs

Epidemiologists follow the transmission of a disease by correlating geographic, climatic, socioeconomic, and demographic data with disease incidence. These correlations are then used to identify possible modes of transmission and disease patterns. Epidemiologists group infectious diseases by their *mode of transmission*. This approach reflects the ecology of the organism and is the pattern we will use in Chapters 31–34.

UNIT 7

Modes of Disease Transmission

Mastering Microbiology
Art Activity: Table 30.2 Major means of human infectious disease transmission

Three major modes of infectious disease transmission are known and are summarized in **Table 30.2**. These include diseases transmitted from *person to person*, including those transmitted by touching *fomites* (inanimate objects); diseases transmitted by substances taken into the body (for example, food, water, or air), called *vehicles*; and diseases transmitted by *vectors*, that is, other organisms, especially those that access the bloodstream, such as ticks and biting insects. Each mechanism has three stages in common: (1) escape from the host or reservoir, (2) travel, and (3) entry into a new host.

Person-to-person disease transmission occurs when an infected host transmits a disease directly to a susceptible host without the assistance of an intermediate host. Upper respiratory infections such as the common cold and influenza are most often transmitted person to person by airborne droplets resulting from sneezing or coughing. Many of these droplets, however, do not remain airborne for long, and so transmission requires close, although not necessarily intimate, person-to-person contact. Some pathogens are extremely sensitive to environmental factors such as drying and heat and are unable to survive for significant periods of time away from the host. These pathogens, transmitted only by intimate person-to-person contact, such as exchange of body fluids in sexual intercourse, include those responsible for sexually transmitted diseases including syphilis (*Treponema pallidum*), gonorrhea (*Neisseria gonorrhoeae*), and HIV/AIDS (HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome). Direct person-to-person contact is also how pathogens such as staphylococci (boils and pimples) and fungi (ringworm) are transmitted. Some of these pathogens (*Staphylococcus aureus* is a good example) can spread by vehicle transmission as well because when inoculated into a vehicle such as food, they grow rapidly and produce poisonous toxins.

TABLE 30.2 Major means of human infectious disease transmission

Mode of transmission	Examples
Person to person	
Direct contact: Sexual intercourse; handshakes	Gonorrhea; *Staphylococcus aureus* infection
Indirect contact: Water glasses and other fomites	Influenza; common cold
Airborne droplets: Sneezes, coughing	Influenza; measles; tuberculosis
Vehicle	
Waterborne: Sewage-contaminated water	Cholera; giardiasis
Foodborne: Contaminated foods	Staphylococcal food poisoning; salmonellosis
Airborne: Fungal spores	Histoplasmosis; coccidioidomycosis
Soilborne: Puncture wound contaminated with soil	Tetanus
Soil aerosol or infected animal	Anthrax
Vector	
Arthropods/insects: Mites, ticks, mosquitoes	Typhus; Lyme disease; malaria

In addition to direct contact between hosts, infectious diseases may also be transmitted by contact with inanimate objects or by other organisms. When contaminated with a viable pathogen, inanimate agents, called **fomites**, can transfer the pathogen to a susceptible host. A huge variety of fomites can harbor pathogens and may include bedding, clothing, toys, books, door knobs, eating utensils, paper and coin currency, and surgical instruments—virtually any object or surface that is accessible to public contact. The term **vehicle** is used to describe nonliving sources of pathogens that may transmit disease to large numbers of individuals upon entering their bodies. Common disease vehicles include contaminated food and water (Table 30.2), but air and soil may also function as vehicles of disease transmission, such as in the transmission of infectious microbial spores. A key distinction in this terminology is that fomites are nonliving objects that are *touched or handled*, whereas vehicle-associated epidemics are typically traced to a *common contaminated source*—shared commodities consumed in large amounts by local or regional populations.

Living disease carriers are called **vectors**, and arthropods (mites, ticks, fleas, etc.) and vertebrates (usually dogs, cats, or rodents) are common disease vectors. Vectors are often not definitive hosts for the pathogen but simply carry the pathogen from one host to another. For instance, many arthropods obtain their nourishment by biting and sucking blood, and if the pathogen is present in the blood, the arthropod will ingest the pathogen and transmit it when biting another individual. In some cases, viral pathogens multiply in the arthropod vector, which is then considered an *alternate host*. Such is the case for West Nile virus (in the *Culex* mosquito) and the bacterium *Yersinia pestis* (in the rat flea), the causative agent of plague (▶ Sections 32.6 and 32.7, respectively). Such replication leads to greater pathogen abundance in the vector, and this increases the probability that a subsequent bite will lead to infection.

A marked seasonality or periodicity of a disease often signals a particular mode of transmission. Seasonal patterns of transmission can result from environmental factors, such as weather patterns, that influence the survival of the pathogen or its vector. For example, California encephalitis—a viral disease transmitted by mosquitoes—shows a pattern in which the disease peaks during the summer and fall months but disappears in the winter, coinciding with the activity of its mosquito vector (**Figure 30.5**). By contrast, human influenza occurs in an annual cyclic pattern unrelated to arthropod activities, causing person-to-person epidemics propagated among schoolchildren and other populations of susceptible individuals. Cases of influenza are often high in schools or crowded offices because the virus is transmitted through the respiratory route by airborne droplets; peak incidence occurs in midwinter and early spring when schools are in session and people are indoors much of the day.

Disease Carriers and Disease Reservoirs and Control

As described earlier, a disease *carrier* is a pathogen-infected individual who has a subclinical infection and shows either no symptoms or only mild symptoms of the disease; carriers are thus potential sources of infection for others. Carriers may be in the incubation period of the disease, in which case the carrier state precedes the development of actual symptoms (Section 30.1). Respiratory infections such as colds and influenza, for example, are often spread via carriers who are unaware of their infection and so are not taking

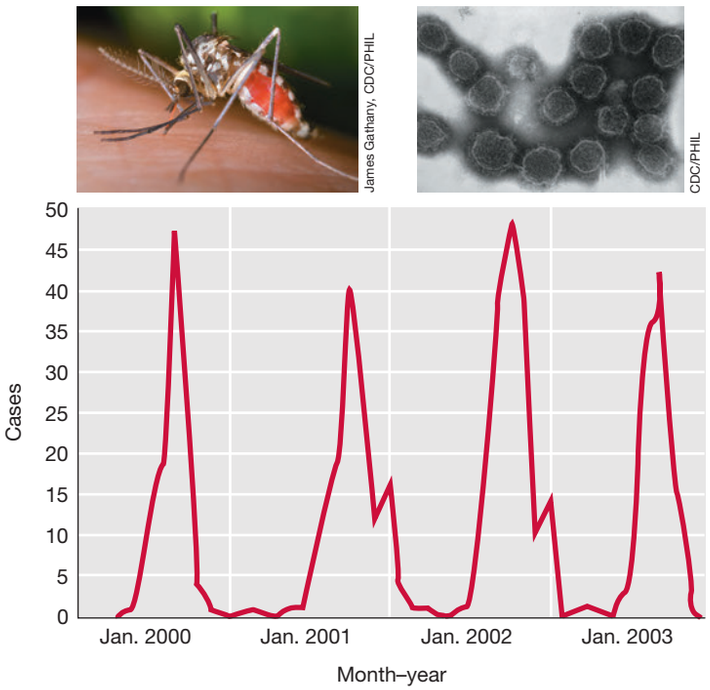


Figure 30.5 Cyclical nature of California encephalitis in the United States. California encephalitis is transmitted by the mosquito *Ochlerotatus triseriatus* (left photo) and is caused by the La Crosse encephalitis virus, a negative-strand and enveloped RNA virus (right photo). Because it depends on a seasonally available vector, disease incidence shows a sharp rise in late summer, followed by a complete decline in winter. Data are from the CDC, Atlanta, Georgia, USA.

any precautions against infecting others. The carrier state lasts only a short time for carriers who develop acute disease. However, *chronic carriers* usually appear healthy and may spread disease for extended periods of time. Some examples here include carriers of hepatitis B, typhoid fever, HIV/AIDS, tuberculosis, and upper respiratory *Staphylococcus aureus* infections.

Disease reservoirs are sites at which infectious agents remain viable and from which susceptible individuals may become infected. Reservoirs may be either animate or inanimate. Some pathogens whose reservoirs are not in animals only incidentally infect humans and cause disease. For example, some species of *Clostridium*, common soil bacteria, occasionally infect humans, causing life-threatening diseases such as tetanus, botulism, and gangrene. In these cases, the pathogen is not dependent on the host for survival, so host–pathogen balance is not required. For many pathogens (including many human pathogens), however, living organisms are the only reservoirs. In these cases, the host is essential for the life cycle of the infectious agent; maintenance of human pathogens of this kind requires host-to-host transmission. Many viral and bacterial respiratory pathogens and sexually transmitted pathogens fall into this category. When humans are the main or only disease reservoir, implementing infection controls may or may not be easy and depends to a great extent on the nature of the disease and its mode of transmission. With diphtheria, for example, confirmed cases are isolated and quarantined to prevent spread of the pathogen (Section 30.5). However, for a disease like gonorrhea, where symptoms are often inapparent in females, tracking down and treating disease carriers can be difficult if not impossible.

Some infectious diseases are caused by pathogens that reproduce in both humans and animals. A disease that primarily infects animals and is only occasionally transmitted to humans is called a **zoonosis**; rabies is a good example. The reservoir for rabies is wild mammals, primarily skunks, raccoons, foxes, and certain bats. Although person-to-person transmission of zoonoses is rare, control of zoonoses in humans is nearly impossible because of the frequent contact some humans have with wild animals and the fact that the animal reservoir can probably never be effectively controlled. Certain other infectious diseases are caused by organisms such as protists and helminths (parasitic worms) that undergo complex life cycles requiring an obligate transfer from a nonhuman host to a human host and back to the nonhuman host; the diseases malaria and schistosomiasis (Chapter 34) are good examples here. In the case of malaria, the major reservoir other than humans is the mosquito *Anopheles gambiae*, and some control of the disease can be achieved by chemical or physical controls on the insect reservoir. In schistosomiasis, by contrast, the reservoir is an aquatic snail, and so eliminating the reservoir is impossible, although treatments for the disease are possible. Interestingly, "reverse zoonoses" (transmission from humans to animals) are also possible, and for an example, see MicrobiologyNow in Chapter 33.

Check Your Understanding

- What is a zoonotic disease? A disease reservoir?
- What is the difference between a disease vehicle and a disease vector?
- An effective vaccine is available for human rabies, yet cases continue to occur every year around the world. What aspect of rabies makes it such a challenge to completely eradicate?

30.4 Characteristics of Disease Epidemics

Endemic infectious diseases are constantly present over long periods of time but typically occur at only low incidence in the population. In tropical Africa, for example, malaria is endemic; both morbidity and mortality from malaria have remained relatively constant on a long-term basis. By contrast, the 2014 outbreak of Ebola hemorrhagic fever in West Africa ran to epidemic proportions in major districts of Sierra Leone, Liberia, and Guinea. The 2019 Ebola outbreak in the Democratic Republic of Congo (along with a few cases in Uganda) infected over 3150 people, two-thirds of whom died. The prompt implementation of strict public health measures (Section 30.5) is required to contain outbreaks and limit the spread of Ebola. As a highly transmissible disease (▶ Section 31.12), Ebola has the potential to become pandemic if those infected but not yet showing symptoms travel away from the epidemic area. However, a highly effective Ebola vaccine, now available, may temper this prediction.

Epidemics

Disease epidemics show characteristic features and require that rapid epidemiological conclusions be reached and clinical treatment instituted if the epidemic is to be contained. The characteristic features of epidemics include distinct patterns in the disease cycle and inherent properties of the pathogen that affect its virulence and herd immunity. Major epidemics are usually classified as either *common-source epidemics* or *host-to-host epidemics*. The patterns of disease

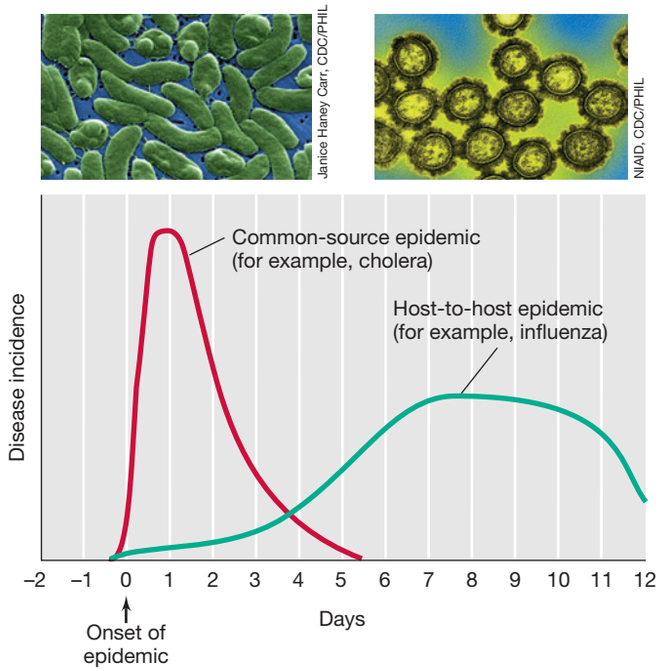


Figure 30.6 Types of epidemics. The shape of the curve that plots incidence of an epidemic disease against time identifies the likely type of the epidemic. For a common-source epidemic, such as cholera resulting from contaminated water shared by many people, the curve rises sharply to a peak and then declines rapidly. Host-to-host infectious disease incidence rises relatively slowly as new cases accumulate. Inset photos: left, scanning electron micrograph of a *Vibrio* sp. closely related to *Vibrio cholerae*, the cholera agent; right, transmission electron micrograph of virions of H1N1 influenza virus.

incidence observed in these two types of epidemics are contrasted in Figure 30.6.

Mastering Microbiology
Art Activity: Figure 30.6 Types of epidemics

A **common-source epidemic** results from an infection (or intoxication) of a large number of people from a contaminated source, such as food or water, that all infected individuals have ingested. Such epidemics are often caused by a breakdown in the sanitation of a central food or water distribution system, but they can also be more local, such as contaminated food in a particular restaurant. Foodborne and waterborne common-source epidemics are primarily intestinal diseases; the pathogen leaves the body in fecal material, contaminates food or water supplies as a result of improper sanitation, and then enters the intestinal tract of the recipient during ingestion of the food or water (Chapter 33).

Common-source disease outbreaks are characterized by a rapid rise to a peak incidence because a large number of individuals become ill within a relatively brief period of time (Figure 30.6). Moreover, assuming that epidemiological surveillance quickly identifies the disease vehicle, cases of a common-source disease decline fairly rapidly, as well. Cholera is the classic example of a common-source epidemic as the disease is almost exclusively waterborne; if a sanitation breakdown occurs (or if sanitation is lacking, as is often the case in developing countries), the cholera bacterium can be shed from a carrier or an active infection into a water source used by many other people and quickly trigger an epidemic (Figure 30.6).

In contrast to the common-source disease pattern, in a **host-to-host epidemic** the disease incidence shows a relatively slow, progressive rise (Figure 30.6) and a gradual decline. Cases continue to be reported over a period of time equivalent to several incubation periods of the disease. A host-to-host epidemic can be initiated by the introduction of a single infected individual into a susceptible population, with this individual infecting one or more people depending on the extent of herd immunity (Figure 30.4) in that population. In a host-to-host epidemic, the pathogen replicates in susceptible individuals, reaches a communicable stage, is transferred to other susceptible individuals, and again replicates and becomes communicable; such epidemics are often controlled by effective herd immunity due to previous infection or vaccination. Influenza and chicken pox (Chapter 31) are examples of diseases that can spread in host-to-host epidemics.

Basic Reproduction Number (R_0)

The infectivity of a pathogen can be predicted using mathematical models that estimate the **basic reproduction number** (R_0) that the pathogen may trigger. The R_0 is defined as the number of expected secondary transmissions from each single case of a disease in an entirely susceptible population, and Table 30.3 lists the R_0 of selected infectious diseases. R_0 directly correlates with the herd immunity necessary to prevent spread of infection; the higher the R_0 value, the greater the herd immunity required to stop infection (Table 30.3). Unfortunately, conditions are not always ideal and the mathematical models that predict R_0 may not take into account such factors as numbers of recovered individuals, population density (close contact), length of contact time, populations of high-risk individuals, ease of travel, and other variables that may affect disease spread. As a result, R_0 is a theoretical construct and can only estimate infectivity. Nevertheless, R_0 is still a useful gauge of the relative infectivity of a pathogen and helps to establish targets for immunization coverage to prevent spread of a particular infectious disease.

We move on now to consider public health on a global basis. In the era of "globalization," disease transmission must be considered in a global context if public health personnel are to keep epidemics from becoming pandemics.

TABLE 30.3 Basic reproduction number (R_0) and herd immunity necessary for community protection from selected infectious diseases

Disease	[a]R_0	Herd immunity[a]
Diphtheria	7	85%
Influenza[b]	1.6	29%
Measles	18	94%
Mumps	7	86%
Pertussis	17	94%
Polio	7	86%
Rubella	7	85%
Smallpox	7	85%

[a] R_0 and herd immunity values are the highest estimates for each disease.
[b] Values shown are for the pandemic (H1N1) 2009 influenza. Each influenza epidemic has a different R_0 and herd immunity value. Herd immunity values assume a 100% effective vaccine. Vaccine efficacy for influenza is about 60% and observed herd immunity values are 40% or greater depending on the susceptible host populations.

Check Your Understanding

- Distinguish between common-source and host-to-host epidemics. Cite at least one example of each. Describe how common-source epidemics can be recognized using epidemiological surveillance data.
- Define the basic reproduction number for a pathogen. How does this value correlate to immunization requirements for herd immunity?

II • Public and Global Health

Establishing and maintaining protocols to control the vehicles and reservoirs of infectious disease is a universal goal of public health programs around the world. International cooperation is key to effectively controlling both localized outbreaks and proliferation of infectious disease.

In Part II, we focus on public health issues including some of the methods and tools used to identify, track, contain, and eradicate infectious diseases within populations. We also draw a stark contrast between the causes of mortality in developed versus developing countries. Although noninfectious diseases are the major killer in developed countries, infectious disease remains the leading cause of mortality in other countries.

30.5 Public Health and Infectious Disease

Public health refers to the health of the general population and to the activities of public health authorities in the control of disease. The incidence and prevalence of many infectious diseases dropped dramatically during the twentieth century, especially in developed countries, because of universal improvements in public health due to advances in basic living conditions. Access to safe water and food, improved public sewage treatment, less crowded living conditions, and lighter workloads have all contributed immeasurably to disease control. Several historically important diseases, including smallpox, typhoid fever, diphtheria, brucellosis, and poliomyelitis, have been controlled (and in the case of smallpox, even eliminated) by active, disease-specific public health measures, and we review these here.

Controls Directed Against Common Vehicles and Major Reservoirs

Common vehicles for pathogen dispersal include food, water, and air. The control of foodborne and waterborne pathogens (Chapter 33) has seen the greatest successes through improved methods of preventing microbial contamination of food and water. For example, water purification procedures in the United States, first instituted early in the twentieth century, dramatically reduced the incidence of typhoid fever (Figure 30.7), and laws controlling food purity, preparation, and storage, coupled with strict monitoring of the food and water distribution network, have greatly decreased the incidence of common-source diseases. However, in contrast to food and water, controlling transmission of respiratory (airborne) pathogens is much more difficult. Other than wearing personal protection such as face masks and avoiding individuals you know are infected, few effective measures of airborne infection control are possible except in specialized environments such as hospital operating rooms where chemical and physical agents can treat the rather small amount of circulating air.

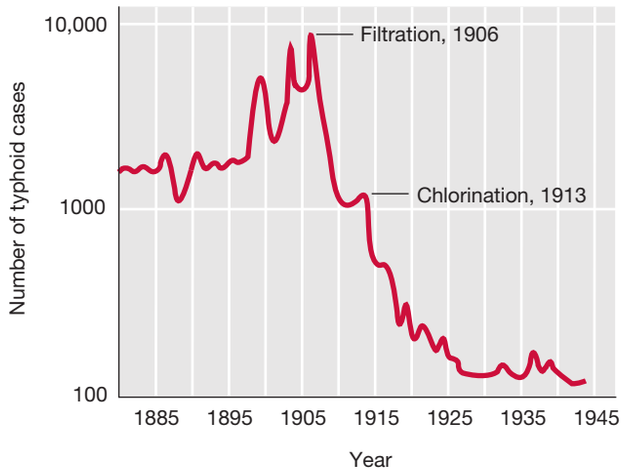


Figure 30.7 Historical progression of typhoid fever in Philadelphia. The introduction of filtration and chlorination eliminated typhoid fever in Philadelphia and other cities with well-regulated water supplies. The risk of typhoid in the United States today is very low but occasional cases are reported.

When the disease reservoir is primarily *domestic* animals, infection of humans can be prevented if the disease is eliminated from the infected animal population by vaccinating herds and removing diseased individuals. However, as we have seen (Section 30.3), when the disease reservoir is a *wild* animal, eradication is much more difficult. Eradication of rabies, for example, would require the immunization or destruction of all wild animal reservoirs, an impossible task. When insect vectors are involved, effective control can often be accomplished with insecticides. However, the use of chemicals must be balanced with health and environmental concerns because in some cases, the elimination of one public health problem (the disease vector) simply creates another (toxic chemical exposure).

When humans are the disease reservoir—as, for example, in HIV/AIDS—control and eradication can be difficult, especially, as mentioned previously in reference to gonorrhea, if there are asymptomatic carriers. By contrast, certain diseases that are limited to humans and have no asymptomatic phase can be prevented through immunization or treatment with antimicrobial or other drugs. However, the disease can be eradicated only if those who have contracted the disease and all possible contacts are immunized, treated, or if necessary, quarantined. Such a strategy was successfully employed by the World Health Organization to eradicate smallpox worldwide (see later) and is currently being used to eradicate polio.

Immunization

Smallpox, diphtheria, tetanus, pertussis (whooping cough), measles, mumps, rubella, and poliomyelitis have been controlled primarily by immunization. Diphtheria, for example, is no longer considered even endemic in the United States. Vaccines are routinely administered in childhood for a number of other infectious diseases (◀ Figure 28.8). As we emphasized in Section 30.4, 100% immunization is not necessary for effective disease control in a population because of herd immunity, although the percentage needed to

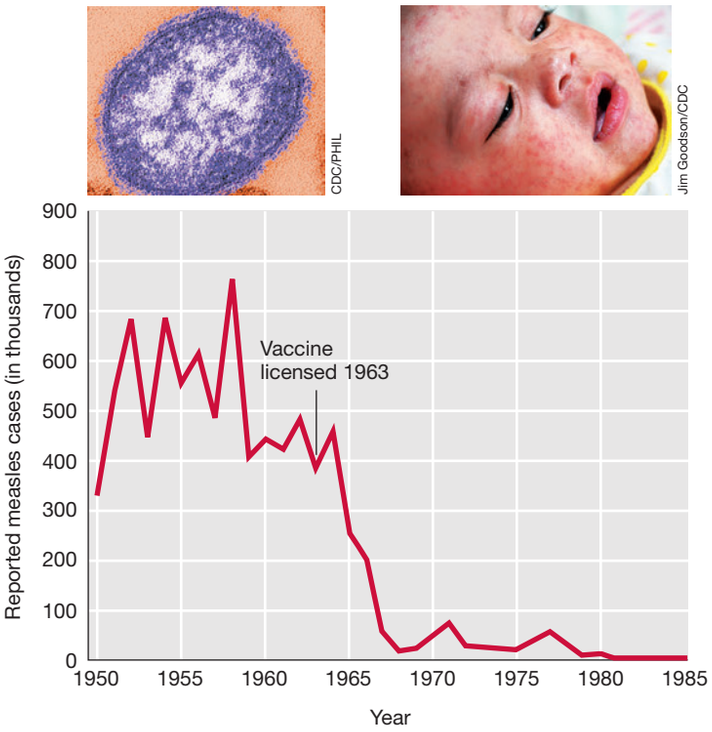


Figure 30.8 Measles immunization in the United States. The introduction of a measles vaccine eliminated measles as a common childhood infection within 20 years. Inset photos: left, transmission electron micrograph of a measles virion (a negative-strand enveloped RNA virus); right, photo of an infant showing the spotted rash characteristic of measles. With an extremely high R_0 (Table 30.3), measles outbreaks can spike quickly in unvaccinated populations.

ensure disease control is still quite high and varies with the infectivity and virulence of the pathogen (Table 30.3).

Measles epidemics offer an example of the power of herd immunity. The occasional resurgence of the highly contagious measles virus ($R_0 = 18$, Table 30.3) emphasizes the importance of maintaining appropriate immunization levels for a given pathogen. Until 1963, the year an effective measles vaccine was licensed, nearly every child in the United States acquired measles through natural infections, resulting in over 300,000 annual cases. However, after introduction of the vaccine, the number of annual measles infections decreased rapidly (Figure 30.8). Case numbers reached a low of 1497 by 1983. However, by 1990, the percentage of children immunized against measles fell to 70%, and the number of new cases rose to 27,786. A concerted effort to increase measles immunization levels to above 90% (about that needed for effective herd immunity, Table 30.3) virtually eliminated measles in the United States by the early 2000s. However, in the past several years measles incidence is once again rising due to immunization levels insufficient to maintain effective herd immunity.

Isolation, Quarantine, and Surveillance

Isolation and quarantine are effective public health measures. **Isolation** is the separation of persons who have an infectious disease from those who are healthy. **Quarantine** is the separation and restriction of well persons who may have been exposed to an infectious disease to see if they develop the disease. The length of isolation or quarantine for a given disease varies and is typically the longest period of communicability for that disease. To be effective, these measures must prevent infected or potentially infected individuals from contacting uninfected susceptible individuals. By international agreement, six infectious diseases require isolation and quarantine: *smallpox, cholera, plague, yellow fever, typhoid fever*, and *relapsing fever*. Each is a very serious, particularly communicable disease. Spread of certain other highly contagious diseases, such as Ebola hemorrhagic fever, SARS, H5N1 influenza, and meningitis, may also be subject to quarantine or isolation as outbreaks emerge in particular regions.

As mentioned earlier (Section 30.1), disease surveillance is a major job of the epidemiologist. Table 30.4 lists the infectious diseases currently under surveillance (referred to as *reportable diseases*) in the United States. The **Centers for Disease Control and Prevention (CDC)** is the agency of the United States Public Health Service that tracks disease trends reported by physicians and other health professionals, provides the latest disease information, and forms public policy regarding disease prevention. The CDC operates a number of infectious disease surveillance programs and also carries out surveillance of major noninfectious diseases, such as

TABLE 30.4 Reportable infectious agents and diseases in the United States

Diseases caused by bacteria
- Anthrax
- Botulism
- Brucellosis
- Chancroid
- *Chlamydia trachomatis* infection
- Cholera
- Diphtheria
- Ehrlichiosis/Anaplasmosis
- Gonorrhea
- *Haemophilus influenzae*, invasive disease
- Hansen's disease (leprosy)
- Hemolytic uremic syndrome
- Legionellosis
- Listeriosis
- Lyme disease
- Meningococcal disease (*Neisseria meningitidis*)
- Pertussis
- Plague
- Psittacosis
- Q fever
- Salmonellosis
- Shiga toxin–producing *Escherichia coli* (STEC)
- Shigellosis
- Spotted fever rickettsiosis
- Streptococcal toxic shock syndrome
- *Streptococcus pneumoniae*, invasive disease
- Syphilis, all stages
- Tetanus
- Toxic shock syndrome (staphylococcal)
- Tuberculosis
- Tularemia
- Typhoid fever
- Vancomycin-intermediate *Staphylococcus aureus* (VISA)
- Vancomycin-resistant *Staphylococcus aureus* (VRSA)
- Vibriosis (non-cholera *Vibrio* infections)

Diseases caused by viruses
- Arboviruses (encephalitis, non-neuroinvasive disease, and Zika)
- Dengue
- Hantavirus pulmonary syndrome
- Hepatitis A, B, C
- HIV infection/AIDS
- Novel influenza A
- Measles
- Mumps
- Polio
- Rabies
- Rubella
- Severe acute respiratory syndrome (SARS-CoV)
- Smallpox
- Varicella (chicken pox)
- Viral hemorrhagic fevers
- West Nile virus
- Yellow fever

Diseases caused by protists
- Babesiosis
- Cryptosporidiosis
- Cyclosporiasis
- Malaria
- Giardiasis

Disease caused by a helminth
- Trichinellosis (trichinosis)

Disease caused by a fungus
- Coccidioidomycosis/Valley fever

cancers, heart disease, and stroke. The overall practical goal of disease surveillance is to formulate and implement plans for diagnosis and treatment of infections.

Pathogen Eradication

Concerted disease eradication programs can sometimes completely eradicate an infectious disease, and such was the case with naturally occurring smallpox, eradicated worldwide in 1980. Smallpox was a viral disease with a virus reservoir consisting solely of the individuals with acute smallpox infections, and transmission was exclusively person-to-person through direct contact. Although smallpox cannot be treated once acquired, immunization practices have been very effective. The World Health Organization (WHO) implemented a smallpox eradication plan in 1967. Because of the success of previous vaccination programs, smallpox had already been confined to endemic status in parts of Africa, the Middle East, and the Indian subcontinent. WHO field health workers proceeded to vaccinate everyone in these areas they could locate with the goal of providing either direct or herd immunity (Section 30.2) to the entire population. Each subsequent outbreak or suspected outbreak was targeted by WHO teams that quickly traveled to the outbreak site, isolated individuals with active disease, and vaccinated all contacts. To break the chain of possible infection, they then immunized everyone who had contact with the contacts, and this aggressive vaccination policy eventually eliminated smallpox.

Several other communicable diseases are candidates for global eradication. Poliomyelitis, like smallpox a viral disease with a human-only reservoir, is on its way to elimination using the same vaccination strategy used against smallpox; in 2018, a total of only 33 cases of wild polio were reported worldwide. Diseases caused by parasites have also been targeted, including Chagas disease (by treating active cases and destroying the insect vector) and dracunculiasis (by treating drinking water to prevent transmission of *Dracunculus medinensis*, the Guinea helminth parasite). Eradication of certain bacterial diseases is also on the horizon. For example, syphilis is a candidate because the disease is found only in humans and is readily treatable with antibiotics. Diphtheria, caused by the bacterium *Corynebacterium diphtheriae*, could also be eradicated worldwide by application of the strict immunization protocols that have virtually eliminated diphtheria from North America.

Check Your Understanding

- Compare public measures for controlling infectious disease caused by insect vectors and human carriers.
- Outline the steps taken to eradicate smallpox.
- Describe some of the public health activities of the U.S. Centers for Disease Control and Prevention.

30.6 Global Health Comparisons

The World Health Organization (WHO) has divided the world into six geographic regions for the purpose of collecting and reporting health information, such as causes of morbidity and mortality. These geographic regions are Africa, the Americas (North America, the Caribbean, Central America, and South America), the eastern Mediterranean, Europe, Southeast Asia, and the western Pacific. Here we compare mortality data from a relatively developed region, the Americas, to those from a developing region, Africa, to emphasize the fact that infectious diseases are still major causes of morbidity and mortality in many regions of the world.

Infectious Disease in the Americas and Africa

Mortality statistics in developed and developing countries are significantly different, as illustrated by a comparison of data from the Americas and from Africa in 2016 when the worldwide population was nearly 7.5 billion. Worldwide, 56.9 million individuals died, giving a mortality rate of 7.6 deaths per 1000 inhabitants per year, and 11.4 million (20%) of these deaths were attributable to infectious diseases. There were 992 million people in the Americas in 2016, and there were 6.9 million deaths, or 6.9 deaths per 1000 persons per year. In Africa, there were 1.02 billion people in 2016 and 8.8 million deaths, or 8.7 deaths per 1000 persons per year. These statistics show differences in overall mortality between developed and developing regions, but a comparative examination of the *causes* of mortality is even more instructive.

Figure 30.9 indicates that infectious diseases caused the most deaths in Africa, whereas in the Americas, noninfectious diseases,

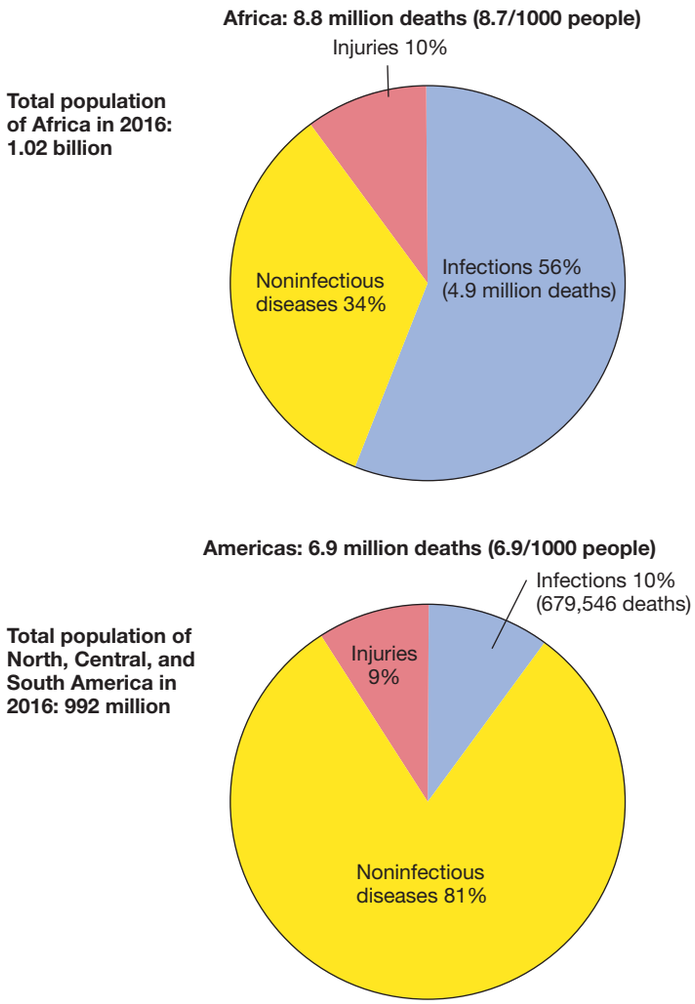


Figure 30.9 Causes of death in Africa and the Americas, 2016. Noninfectious diseases include cancer, cardiovascular diseases, and diabetes. Injuries include accidents, murder, suicide, and war. Data are from the World Health Organization, Geneva.

such as cancer and cardiovascular disease, were the leading causes of mortality. In Africa, there were about 4.9 million deaths due to infectious diseases, and the life expectancy was 61 years of age. The African death toll due to infectious diseases was nearly 9% of the total deaths in the world. In stark contrast, only 679,546 died of infectious disease in the Americas, and the life expectancy was 75 years of age.

The higher life expectancy in developed countries is a direct consequence of the reduction in death rates from infection over the last century, and most of these gains are due to advances in public health. By contrast, lack of resources in developing countries limits access to adequate sanitation, safe food and water, immunizations, healthcare, and medicines, leading to increases in infectious diseases and, as a consequence, to significantly shorter life expectancy. Interestingly, some health professionals predict that life expectancies in certain developed regions may soon begin to decrease, but this is not attributed to infectious diseases. On the contrary, lifestyle diseases are emerging as a major health factor in the Americas, and obesity-related health complications, such as type 2 diabetes, coronary heart disease, and hypertension, are becoming more prevalent.

Travel to Endemic Areas

The high incidence of disease in many parts of the world is a concern for people traveling to such areas. However, travelers can be immunized against many of the diseases that are endemic in foreign countries. Specific recommendations for immunization for those traveling abroad are updated biannually and published by the CDC (http://www.cdc.gov/).

For many countries, immunization certificates for yellow fever are required for entry from areas with endemic yellow fever. These areas include much of equatorial South America and Africa. Most other nonstandard immunizations, such as those for rabies and plague, are recommended only for people who are expected to be at high risk, such as veterinary healthcare providers. The CDC summarizes current information for the potential for infectious disease transmission throughout the world, including diseases for which currently there are no effective (or only experimental) vaccines (for example, HIV/AIDS, malaria, Ebola hemorrhagic fever, dengue fever, amebiasis, encephalitis, and typhus). Travelers should take precautions, such as avoiding insect and animal bites, drinking only water that has been properly treated to kill all microorganisms, eating properly stored and prepared food (and avoiding fresh uncooked foods), and undergoing antibiotic and chemotherapeutic programs for prophylaxis or for suspected exposures. Although these precautions do not guarantee that one will remain disease-free, adhering to them greatly reduces the risk of infection.

Sometimes diseases appear to "come out of nowhere" or, alternatively, reappear after a long absence. In the final part of this chapter, we explore these interesting cases of infectious disease.

Check Your Understanding

- Contrast mortality due to infectious diseases in Africa and the Americas.
- Do you know what vaccinations you have received? List infectious diseases for which you have not been immunized and with which you could come into contact next year.

III • Emerging Infectious Diseases, Pandemics, and Other Threats

Emerging diseases, infectious disease pandemics, and the malicious use of bioweapons present a constant threat to human welfare. Vigilant surveillance is necessary to detect these threats to global health and to respond in an effective way.

In recent years, new infectious diseases have emerged and established diseases have reemerged with alarming frequency. In Part III of this chapter, we discuss some of these diseases and the reasons for their sudden emergence or reemergence. We also investigate the potential for the purposeful use of infectious microbes as agents of war or civilian terror.

30.7 Emerging and Reemerging Infectious Diseases

Infectious diseases are global, dynamic health problems. In this section we examine some recent patterns of infectious disease, some reasons for the changing patterns, and the methods used by epidemiologists to identify and deal with new threats to public health.

Emerging and Reemerging Diseases

The worldwide distribution of diseases can change dramatically and rapidly. Alterations in the pathogen, the environment, or the host population contribute to the spread of new diseases, with potential for high morbidity and mortality. Diseases that suddenly become prevalent are called **emerging diseases** and are not limited to "new" diseases; they also include **reemerging diseases**, diseases that were previously under control but suddenly appear as a new epidemic. Examples of global emerging and reemerging disease are shown in **Figure 30.10**, and select diseases with high potential for emergence or reemergence are described in **Table 30.5**. Occasionally, new diseases emerge unexpectedly and for unknown reasons. For example, within just a few years of its discovery, the drug-resistant yeast *Candida auris* is an emerging pathogen that is becoming a significant concern as a causative agent of serious healthcare-associated infections (see MicrobiologyNow at the opening of this chapter).

Emerging epidemic diseases are not a new phenomenon. Among the diseases that rapidly and sometimes catastrophically emerged in the past are plague (caused by the bacterium *Yersinia pestis*) and influenza. For example, in the Middle Ages, up to one-third of all humans were killed by the periodic plague epidemics that swept across Europe, Asia, and Africa. Influenza caused a devastating worldwide pandemic in 1918–1919, claiming up to 100 million lives, and the pandemic H1N1 influenza virus that emerged in 2009 killed up to a half million people in its first year. In the 1980s, HIV/AIDS and Lyme disease emerged as new diseases, and health officials worldwide are paying particular attention to the potential for rapid emergence of pandemic influenza developing from H5N1 avian influenza. In 2014 and again in 2019, isolation of patients and extra protections for their caregivers were practiced during the West African and central African Ebola hemorrhagic fever epidemics (▶ Section 31.12 and Figure 31.34*b*) to prevent the spread of this extremely dangerous viral disease.

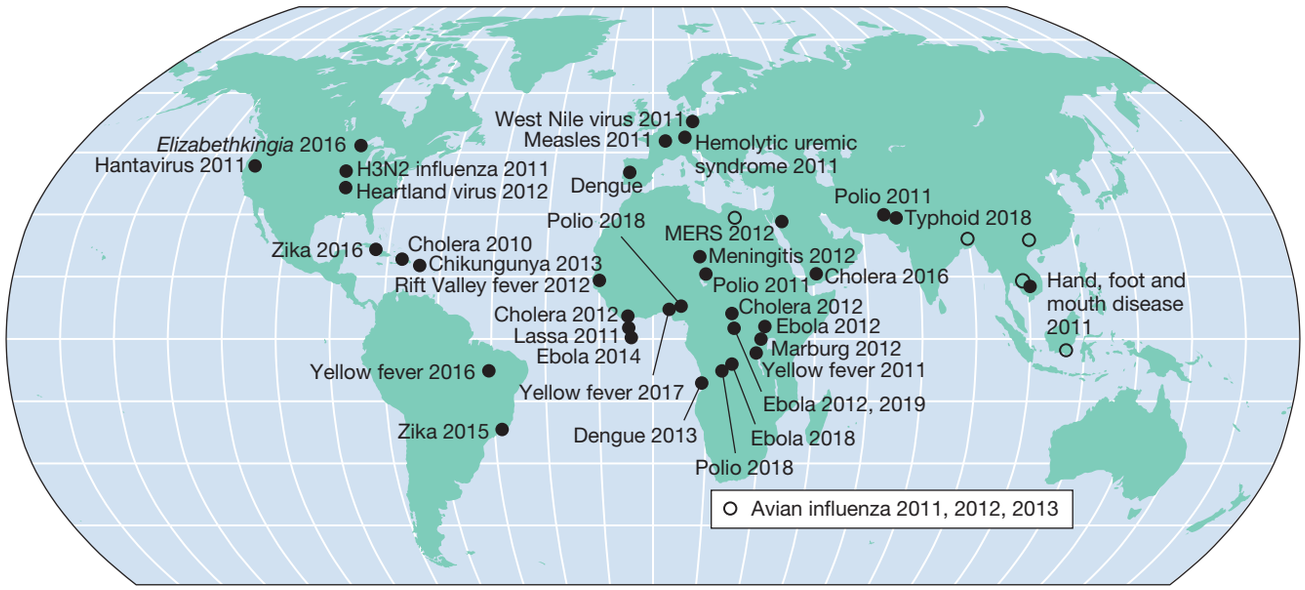


Figure 30.10 **Recent outbreaks of emerging and reemerging infectious diseases.** The diseases shown are local outbreaks capable of producing widespread epidemics and pandemics. Not shown are established pandemic diseases such as HIV/AIDS and predictable annual epidemic diseases such as seasonal epidemic human influenza. MERS, Middle East respiratory syndrome. Avian influenza is caused by influenza A H5N1 (Section 30.8).

TABLE 30.5 Emerging and reemerging epidemic infectious diseases

Agent	Disease and symptoms	Mode of transmission	Cause of emergence
Bacteria			
Borrelia burgdorferi	Lyme disease: rash, fever, neurological and cardiac abnormalities, arthritis	Bite of infective *Ixodes* tick	Increase in deer and human populations in wooded areas
Mycobacterium tuberculosis	Tuberculosis: cough, weight loss, lung lesions	Sputum droplets (exhaled through a cough or sneeze) from a person with active disease	Antimicrobial drug resistance as multidrug-resistant and extensively drug-resistant tuberculosis
Clostridioides difficile	Antibiotic-associated enterocolitis	Person-to-person contact and through fomites in the healthcare setting	Gut dysbiosis due to long-term use of antibiotics
Vibrio cholerae	Cholera: severe diarrhea, rapid dehydration	Water contaminated with the feces of infected persons; food exposed to contaminated water	Poor sanitation and hygiene; carried to non-endemic areas via infected travelers and commerce
Viruses			
Dengue	Hemorrhagic fever	Bite of an infected mosquito (primarily *Aedes aegypti*)	Poor mosquito control; increased urbanization in tropics; increased travel and shipping
Filoviruses (Marburg, Ebola)	Fulminant, high mortality, hemorrhagic fevers	Direct contact with infected blood, organs, secretions, and semen	Contact with vertebrate reservoirs
Influenza H5N1 (avian influenza)	Fever, headache, cough, pneumonia, high mortality	Direct contact with infected animals or humans, not easily spread via respiratory aerosols	Danger of animal–human virus reassortment; antigenic shift
Zika	Asymptomatic, or fever, rash, muscle and joint pain, headache	Bite of an infected mosquito (primarily *Aedes aegypti*), mother to fetus, sexual contact, blood transfusion	Poor mosquito control; increased urbanization in tropics
Fungi			
Candida	Candidiasis: fungal infections of the gastrointestinal tract, vagina, and oral cavity	Member of endogenous microbiota becomes an opportunistic pathogen; contact with secretions or excretions from infected persons	Immunosuppression; medical devices (catheters); antibiotic use

Emergence Factors

Many factors play into the emergence of new pathogens, including human demographics and behavior, economic development, global travel, public health breakdowns, and other factors. The movement of humans from rural to urban areas facilitates disease transmission. For example, high population densities in cities have facilitated transmission of dengue fever (▶ Section 32.5), a serious mosquito-borne viral disease that infects about 400,000 people yearly, mostly in tropical and subtropical urban areas, including far southern reaches of the United States (**Figure 30.11**). Human behavior in large population centers, such as sexually promiscuous practices that cause the spread of hepatitis and HIV/AIDS, also contributes to disease emergence. Changes in land use may also promote the spread of disease. For example, Lyme disease, the most common vectorborne disease in the United States, is on the rise largely due to residential reforestation efforts, which increase contact between Lyme-infected deer ticks and humans, consequently increasing disease incidence.

Common-source foodborne disease epidemics occur when sanitation measures in the food industry fail. In 2009, a single U.S. meat-processing plant spread *Escherichia coli* O157:H7 to people in eight states. The contaminated food source, ground beef, was recalled and the epidemic was eventually stopped, but not before several people died. International travel and commerce also affect the spread of pathogens. For example, a single person harboring Ebola virus on an international flight could infect many other passengers because of the ease with which the Ebola virus spreads (▶ Section 31.12). In such cases, the disease can rapidly spread to major population centers if healthy passengers who had contact with the diseased passenger become infected and then disembark and continue their travels.

Pathogen adaptation can contribute to disease emergence. For example, most RNA viruses, including influenza and HIV, mutate rapidly. These mutant RNA viruses present major epidemiological problems because their altered genomes often affect their antigens, making immunity to old viral antigens ineffective for neutralizing the mutant viruses. Bacterial genetic mechanisms are also capable of enhancing virulence and promoting emergence of new epidemics. Virulence-enhancing factors are often carried by mobile genetic elements (Chapter 13) that can be transferred between members of the same species, and sometimes to other species, as well. Such transfers can quickly generate emerging pathogens, and multidrug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* are good examples of this.

A breakdown of public health measures is sometimes responsible for the emergence or reemergence of diseases. For instance, cholera (caused by *Vibrio cholerae*) can be adequately controlled, even in endemic areas, by providing proper sewage disposal and water sanitation. As discussed in Section 30.8, any compromise to water treatment systems, especially as a result of natural disasters such as earthquakes and flooding, can cause local outbreaks. Inadequate public vaccination programs can also lead to the resurgence of previously controlled diseases. For example, pertussis, a serious but preventable childhood respiratory disease, has increased recently in Eastern Europe and in the United States, partly because of inadequate immunization among adults and children.

Finally, weather patterns can also upset the usual host–pathogen balance. Disease vectors such as mosquitoes have been moving northward in response to climate change. Even a single seasonal weather abnormality can have an effect, as evidenced by the 1993 hantavirus hemorrhagic fever outbreak in the American Southwest (▶ Section 32.2). A very mild winter coupled with record rainfall led to an explosive increase in the population of rodents that can host hantavirus. This increased exposures for susceptible human hosts and led to the spread of this lethal zoonotic disease.

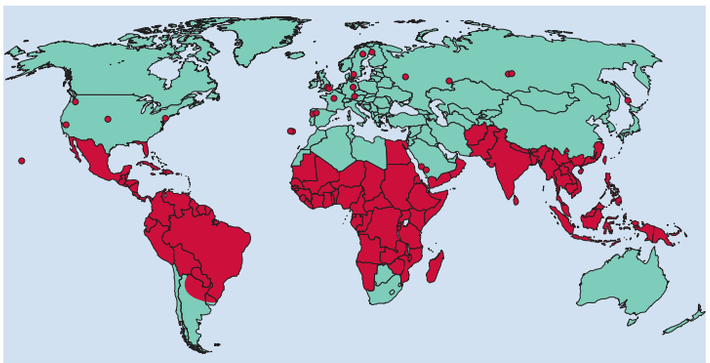
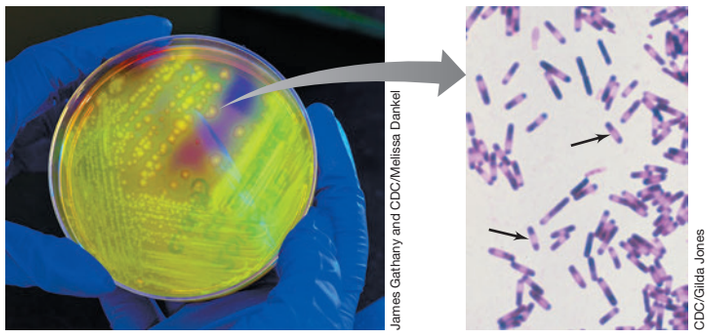


Figure 30.11 Dengue virus 2018. Dengue virus is now found in all tropical and subtropical countries as a result of the spread of its *Aedes aegypti* mosquito vector. The red areas are now endemic for the virus and mosquito vector. The red dots indicate outbreaks outside the known endemic areas. Prior to 1981, dengue virus was unknown in the Americas. Data are from the CDC, Atlanta, Georgia, USA.

The Emergence of Toxigenic *Clostridioides difficile*

In many cases, antibiotic use itself can be a driver of pathogen emergence. As discussed in Chapter 24, prolonged use of antibiotics to treat infections can lead to enteric *dysbiosis*, a disruption to the composition of the normal gut microbiota. Opportunistic pathogens that are resistant to these antibiotics can then flourish, causing potentially serious secondary infections. *Clostridioides* (formerly *Clostridium*) *difficile* is one of these pathogens (**Figure 30.12**), and it has rapidly emerged over the past two decades as a major cause of life-threatening, antibiotic-associated enterocolitis (inflammation of the digestive tract).

Causing nearly half a million cases of illness and about 30,000 deaths every year in the United States alone, mostly among the elderly and immunocompromised, *C. difficile* has become a considerable medical challenge. About 80% of cases of *C. difficile* infection (CDI) are healthcare-associated (nosocomial, ◀ Section 29.2) and occur in those age 65 and older. In addition to being multidrug resistant, *C. difficile* is able to form endospores (Figure 30.12*b*; ◀ Section 2.8). These highly resistant structures can remain viable following standard disinfection and hygiene protocols used in the healthcare setting, including resistance to alcohol-based hand sanitizers, and therefore the bacterium tends to persist on fomites and surfaces. Subsequent transfer of the endospores, often through healthcare personnel that have come into contact with contaminated materials, to patients undergoing long-term antibiotic treatment can lead to the onset of life-threatening diarrheal colitis.



(a) (b)

Figure 30.12 ***Clostridioides difficile*, an emerging pathogen and causative agent of antibiotic-associated enterocolitis.** *(a)* Plate culture of *C. difficile* grown on cycloserine–cefoxitin–fructose agar. The culture is illuminated with ultraviolet light, resulting in a chartreuse fluorescence from the colonies. *(b)* Photomicrograph showing cells of *C. difficile*. Note the developing endospores (arrows) inside the rod-shaped cells.

The symptoms of CDI are caused by the production of two exotoxins: toxin A (TcdA) and toxin B (TcdB). These toxins are glucosyltransferases that inactivate guanosine triphosphatases in colonic epithelial cells. This activity disrupts actin dynamics in the cytoskeleton and eventually kills the cells. Antibiotic treatment of CDI using metronidazole, vancomycin, or the more recently approved fidaxomicin, a bactericidal antibiotic that inhibits RNA synthesis, is not universally effective. There is evidence that implementing a high-fiber diet can aid in recovery from CDI, but by far the most effective treatment to date has been *fecal transplant*, the infusion of fecal matter and its associated gut microbiota from a healthy donor into the diseased patient (◀ Section 24.11). This treatment has shown remarkable success in allowing full recovery from *C. difficile* infection in up to 95% of cases.

Addressing Emerging Diseases

The keys for addressing emerging diseases are prompt recognition of the disease and intervention to prevent pathogen transmission. Emerging diseases have, at least at first, low incidence and are usually absent from the official notifiable disease list for the United States prepared by the CDC (Table 30.4). Emerging diseases are first recognized from their unique epidemic incidence, clusters and other epidemiological patterns, and clinical symptoms unrelated to known pathogens. Such disease patterns trigger intensive public health surveillance followed by specific interventions designed to control further outbreaks. Methods such as isolation, quarantine, immunization, and drug treatment can be applied to contain outbreaks. For vectorborne and zoonotic diseases, the nonhuman host or vector must be identified to intervene in the life cycle of the pathogen and stop human infection.

International public health surveillance and intervention programs were instrumental in controlling the emergence of severe acute respiratory syndrome (SARS), a disease that appeared suddenly in Asia and unpredictably from a zoonotic source. On the other hand, even a rapid and focused response was unsuccessful in containing the spread of pandemic (H1N1) 2009 influenza, as we will see in the next section.

Check Your Understanding

- What is the difference between an emerging and a reemerging infectious disease?
- What factors are important in the emergence or reemergence of potential pathogens, such as *Clostridioides difficile*?
- Indicate general and specific methods that would be useful for identifying emerging infectious diseases.

30.8 Examples of Pandemics: HIV/AIDS, Cholera, and Influenza

Through the centuries, several diseases have reached pandemic proportions. Here we consider three—HIV/AIDS, cholera, and influenza—for which epidemiological studies have been extensive.

HIV/AIDS

HIV/AIDS is a continuum of disease, starting with the infection of an individual with the human immunodeficiency virus (HIV). Eventually, infection results in acquired immunodeficiency syndrome (AIDS), a disease which, if not treated, cripples the immune system, leading to opportunistic infections that can be fatal (▶ Section 31.15). The first reported cases of AIDS were diagnosed in the United States in 1981. Since then, more than 1.2 million cases have been reported in the United States with over 650,000 deaths (**Figure 30.13**); worldwide, about 35 million AIDS deaths have occurred.

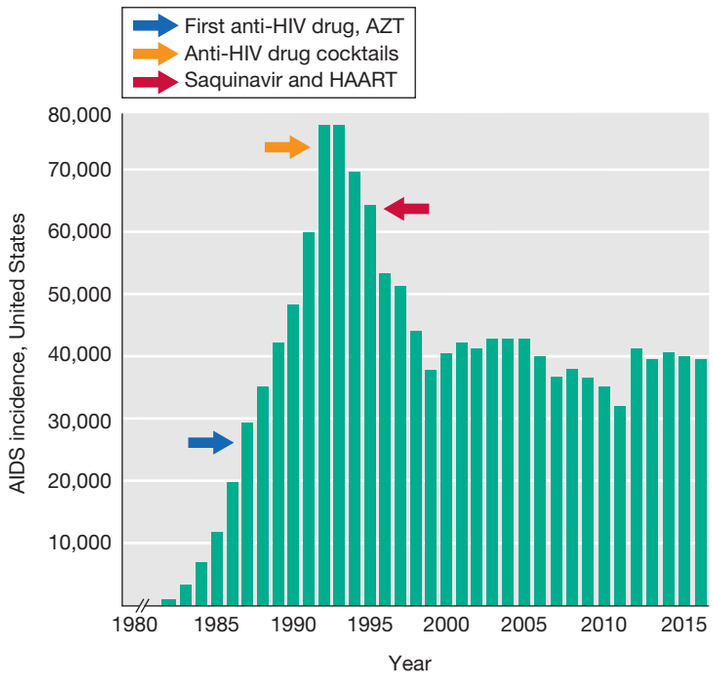


Figure 30.13 **Annual new cases of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) in the United States.** Cumulatively, there were about 1.3 million cases of HIV/AIDS through 2016. In 2009, the HIV/AIDS case definition changed to include all new HIV infections and AIDS diagnoses (▶ Section 31.15). Colored arrows indicate the introduction of different anti-HIV drugs. See Sections 28.6 and 31.15 for a discussion of anti-HIV therapies. Data are from the HIV Surveillance Report: Diagnosis of HIV Infection in the United States and Dependent Areas, 2017, CDC, Atlanta, Georgia, USA.

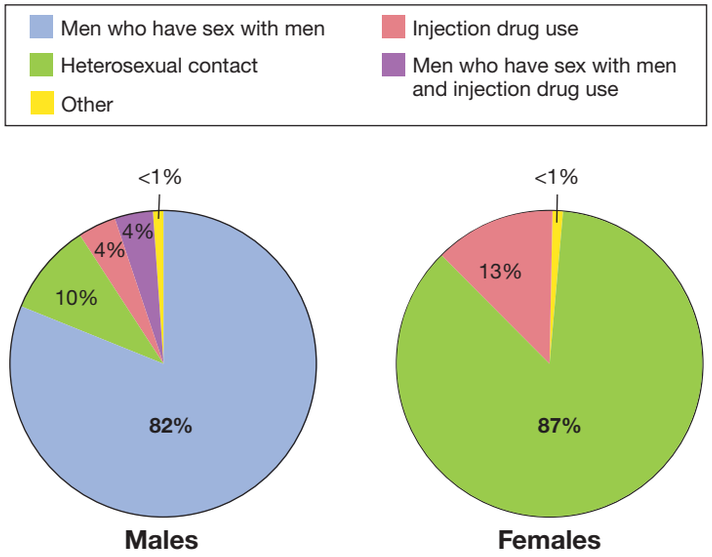


Figure 30.14 Distribution of AIDS cases by risk group and gender in the United States, 2016. Data from the CDC, Atlanta, Georgia, USA, were gathered from 31,931 males and 7,528 females diagnosed with HIV/AIDS in 2016.

Epidemiological studies in the United States in the 1980s suggested a high AIDS prevalence among men who have sex with men and among intravenous drug abusers. Individuals receiving blood or blood products were also at high risk. Collectively, these epidemiological data indicated a transmissible agent transferred during sexual activity or by contaminated blood. Soon after the discovery of HIV in 1983, laboratory tests were developed to detect antibodies to the virus in blood. With this tool in hand, surveys of HIV incidence and prevalence defined the spread of HIV and showed conclusively that body fluids, primarily blood and semen, were the vehicles for transmission of the virus (**Figure 30.14**).

The HIV/AIDS data showed that in the United States, the number of AIDS cases was disproportionately high in men who have sex with men, but among women, heterosexuals were the largest risk group (Figure 30.14). Further analyses of the epidemiological data showed that the new infection rate for African American men was seven times that of Caucasian males, indicating that social and economic factors may also influence infection risk. However, regardless of gender or racial specifics, AIDS epidemiology provided a clear picture of HIV transmission: Virtually all who acquired HIV engaged in sex or intravenous drug use in which body fluids—semen or blood—were transferred and commonly had sex or exchanged syringe needles with multiple partners. We discuss the pathology and therapy of HIV/AIDS in Section 31.15.

Cholera

Cholera is primarily a waterborne infection (▶ Section 33.3 and Figure 33.2) that is normally kept in check by appropriate public health measures for water treatment (Chapter 22). Cholera is caused by ingestion of contaminated water containing *Vibrio cholerae*, a gram-negative, curved rod–shaped species of *Proteobacteria* that produces a powerful enterotoxin that triggers severe diarrhea (◀ Section 25.6 and Figure 25.16). Cholera is endemic in Africa, Southeast Asia, the Indian subcontinent, and Central and South America. Epidemic cholera occurs frequently in areas where sewage treatment either suffers a major breakdown, as can result from a flood or an earthquake, or is inadequate or altogether absent. Indeed, it is estimated that a greater percentage of the world's population has access to cellular phones than to water sanitation facilities. In 2016, the World Health Organization (WHO) reported over 130,000 cases of cholera that led to 2420 deaths. However, the WHO estimates that only 5–10% of cholera cases are actually reported because diarrheal diseases from various pathogens are so common (Table 30.1); thus, total worldwide incidence of cholera likely exceeds 1 million cases per year.

Epidemic cholera may develop into pandemics when travelers from endemic areas carry the pathogen to new locations with susceptible populations and poor sanitation. Since 1817, cholera has swept the world in seven major pandemics (**Figure 30.15**). All but one of these originated on the Indian subcontinent, where cholera is endemic. Two distinct pandemic strains of *V. cholerae* are recognized, known as the *classic* and the *El Tor* biotypes. The *V. cholerae* O1 El Tor biotype started the seventh pandemic in Indonesia in 1961, and its spread continues to the present day. This pandemic has caused over 5 million cases of cholera and at least 250,000 deaths and continues to be a major cause of morbidity and mortality, especially in developing countries (Figure 30.15).

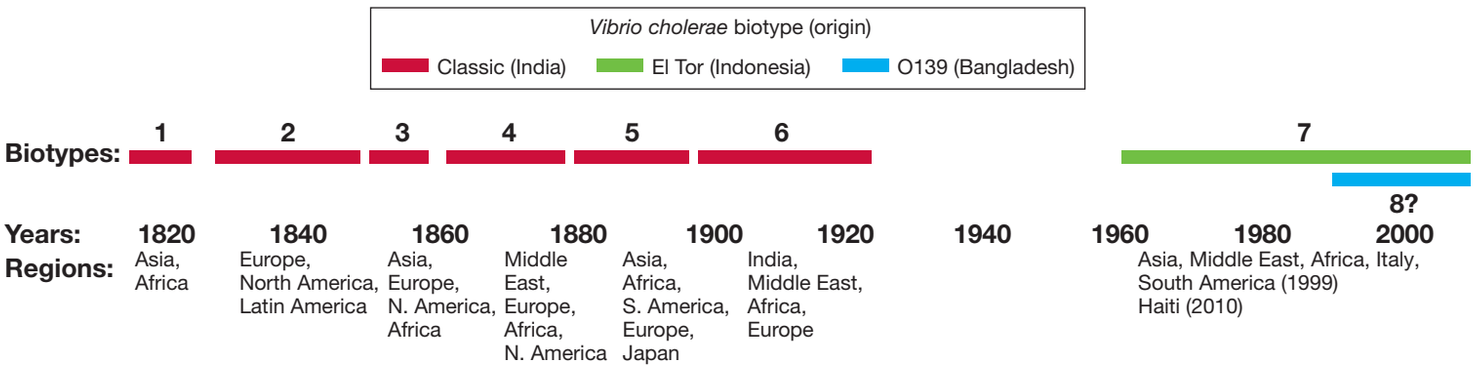


Figure 30.15 Cholera pandemic timeline. Seven cholera pandemics have been nearly consecutive for over 200 years. The seventh pandemic started in 1961 and is ongoing. The O139 strain that appeared in 1991 is endemic to Bangladesh and the Bay of Bengal and is causing epidemics that may be the prelude to an eighth pandemic.

In October 2010 Haiti experienced its first cholera in over 100 years, and in just two years experienced nearly 600,000 cases and 8000 deaths. The outbreak began in the aftermath of the catastrophic 2010 earthquake. There were likely two triggers of this cholera outbreak, the first being a classic scenario of poor sanitation following a disaster and the second an accidental importation from an outside source. *Vibrio cholerae* is present in marine waters, and as a result of the earthquake, cells of this pathogen may have washed into coastal freshwaters where they contaminated drinking water sources. But in addition, United Nations aid workers that arrived from Nepal, where a recent cholera outbreak had occurred, inadvertently shed *V. cholerae* into sanitation streams that found their way into Haitian drinking water sources.

Pandemic (H1N1) 2009 and Future Influenza Pandemics

Human influenza pandemics occur every 10 to 40 years as a result of major genetic changes in the influenza A virus genome that affect the virus's immune status (*antigenic drift* and *antigenic shift*, ▶ Section 31.8 and Figure 31.26). The most devastating influenza pandemic of all time occurred in 1918; this flu infected over half a *billion* people worldwide and killed approximately 50 *million* people before it ran its course. The 1918 pandemic was caused by a strain of influenza termed H1N1.

A more recent influenza pandemic began in March 2009 with the outbreak of epidemics in Mexico. The culprit virus, a strain designated (H1N1) 2009, was a hybrid of the 1918 strain and a later strain that caused a pandemic in 1957; (H1N1) 2009 contained genes from bird, swine, and human influenza viruses. Such *reassortant viruses*, as they are called (▶ Section 31.8), can be highly virulent, as they tend to produce antigens to which humans have no prior exposure and thus no immunity.

Without prior exposure and with no effective vaccine at the ready, the stage was set for the reassortant (H1N1) 2009 flu to spread rapidly and reach pandemic proportions. Within six months of its emergence, (H1N1) 2009 had spread to almost every country in the world, qualifying it as a true pandemic (**Figure 30.16**). Although official numbers range widely, it is estimated that more than a quarter of the world's population was infected in the pandemic. In the United States, about 60 million persons were infected, with mortality confirmed as due to (H1N1) 2009 numbering about 3400 persons. By late 2010 the (H1N1) 2009 pandemic was fading, and today few cases are observed because antigens from this strain of virus are typically included in seasonal influenza vaccines (◀ Table 28.3).

Could new influenza pandemics sweep across the world? Perhaps the greatest threat to global stability would be another influenza pandemic that has the virulence and infectivity of the 1918 pandemic. Because epidemiological surveillance is currently so extensive, this possibility is unlikely, but the risk can never be zero. In recent years public health officials worldwide have been following the emergence and reemergence of a potentially devastating strain of influenza virus designated influenza A H5N1, originally found in birds. This virus first appeared in Hong Kong in 1997, jumping directly from chickens and ducks to humans. Since then H5N1 has reemerged several times in small outbreaks, with the most recent occurring in Egypt, Indonesia, Cambodia, Bangladesh, and China (Figure 30.10). Through 2018, 860 cases of human H5N1 infection have been confirmed, resulting in 454 deaths, for a mortality rate of 53%. This high mortality rate underscores the lethal potential of this virus.

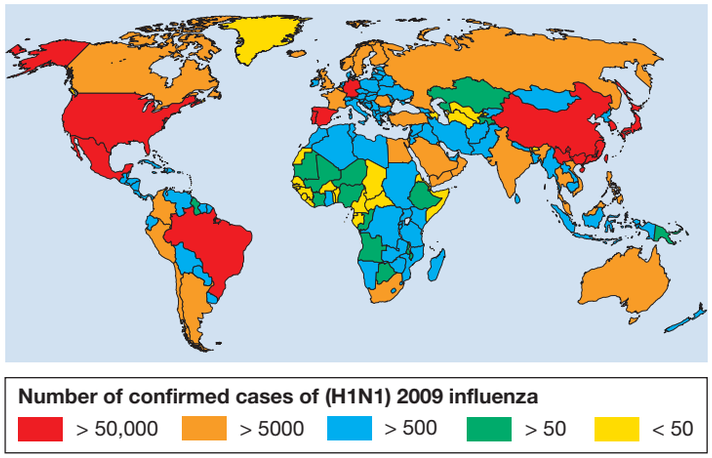


Figure 30.16 Pandemic (H1N1) 2009 influenza incidence. Data show minimal estimates of cases worldwide by country. It is estimated that approximately 1.7 billion people were infected by the (H1N1) 2009 pandemic flu (24% of the total population) and that deaths were between 150,000 and 575,000 [the large range for mortality estimates is because many deaths that were likely due to (H1N1) 2009 were not confirmed as such].

Besides poultry and humans, H5N1 has also infected swine. If a reassortant strain were to emerge from pigs and had the capacity to spread from person to person, such a virus could trigger an influenza pandemic of unprecedented mortality. Because of this, plans are in place nationally and internationally to provide appropriate vaccines and support for potential pandemics initiated by this and other emergent influenza strains. We discuss the disease influenza in detail in Section 31.8.

Check Your Understanding

- Describe the major risk factors for acquiring HIV infection in the United States. How do these differ in males and females?
- Identify the most likely means of acquiring cholera. Why do cholera epidemics keep occurring?
- What is a reassortant influenza virus and why can such viruses be so dangerous?

30.9 Public Health Threats from Microbial Weapons

As if avoiding the wrath of pathogenic microbes that can infect us naturally is not enough, humans have researched the use of certain pathogens as weapons to be intentionally deployed on others. **Biological (microbial) warfare** is the use of microbial agents to incapacitate or kill a military or civilian population in an act of war or terrorism. Although the use and development of microbial weapons are forbidden by international law, microbial weapons have already seen use, and facilities for their production likely exist in rogue countries and perhaps also in avowed terrorist groups. Because of this, microbial weapons research continues in many peaceful nations so as to best understand the most serious threats and learn how to counter them.

Characteristics of Microbial Weapons

Effective microbial weapons are pathogens, or in a few cases toxins, that are (1) relatively easy to produce and deliver, (2) safe for use by the offensive forces, and (3) able to incapacitate or kill people in a systematic and consistent manner. Although microbial weapons are potentially useful in the hands of conventional military forces, the greatest likelihood of microbial weapons use is by terrorists because of the ready availability and low cost of producing and propagating many of the organisms.

Virtually all pathogenic bacteria or viruses can potentially be used for biological warfare, and *select agents* that have significant potential for use as microbial weapons are listed in **Table 30.6**. The most frequently mentioned candidates are smallpox virus and *Bacillus anthracis*, the bacterium that causes anthrax. Both of these microbes can be easily disseminated, are transmissible from person to person, and typically cause high mortality. Other agents have their advantages and disadvantages as microbial weapons and are categorized as to their potential risk from Category A to Category C in Table 30.6.

TABLE 30.6 Select agents and diseases by bioweapons threat category[a]

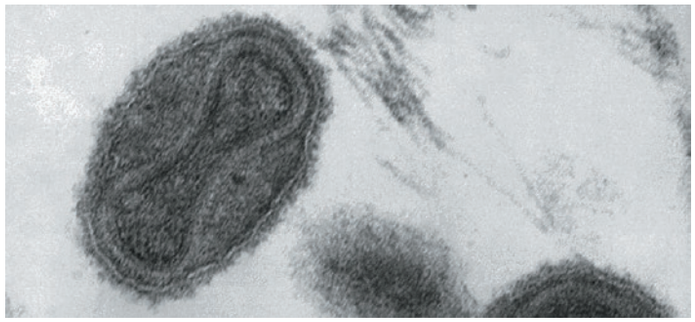
Category A
Highest-priority agents that pose a risk to national security. These agents are easily disseminated or transmitted and result in high mortality rates. They require special action for public health preparedness.
Disease/Pathogen
Anthrax (*Bacillus anthracis*) Botulism (*Clostridium botulinum* toxin) Plague (*Yersinia pestis*) Smallpox (*Variola major*) Tularemia (*Francisella tularensis*) Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg] and arenaviruses [e.g., Lassa, Machupo])
Category B
Second-highest-priority agents. These agents are moderately easy to disseminate, result in moderate morbidity and low mortality, and require specific enhancements of public health diagnostic capacity and disease surveillance.
Disease/Pathogen
Brucellosis (*Brucella* species) Epsilon toxin of *Clostridium perfringens* Food safety threats (e.g., *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella*) Glanders (*Burkholderia mallei*) Melioidosis (*Burkholderia pseudomallei*) Psittacosis (*Chlamydophila psittaci*) Q fever (*Coxiella burnetii*) Ricin toxin from *Ricinus communis* (castor beans) Staphylococcal enterotoxin B (*Staphylococcus aureus*) Typhus fever (*Rickettsia prowazekii*) Viral encephalitis (alphaviruses such as Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis) Water safety threats (*Vibrio cholerae*, *Cryptosporidium parvum*, and others)
Category C
Third-highest-priority agents are emerging pathogens that are available, easily produced and disseminated, with high potential for high morbidity and mortality.
Pathogens
Emerging infectious diseases such as hantavirus

[a]*Source:* Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

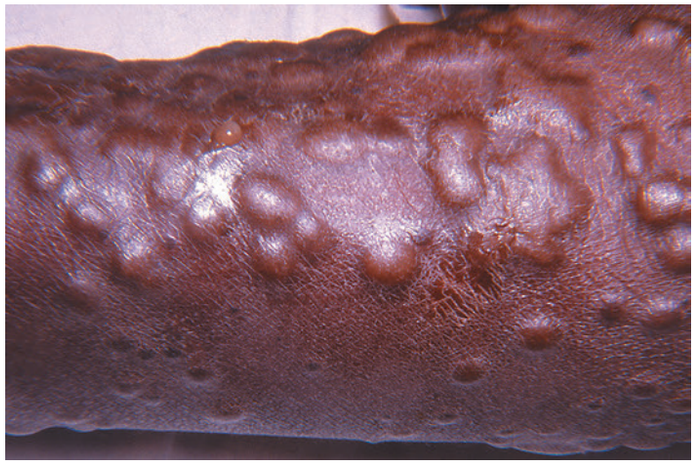
The United States government, through the Centers for Disease Control and Prevention, has developed the Select Agent Program surveillance system to monitor possession and use of potential bioterrorism agents. In addition, the CDC Laboratory Response Network and the Health Alert Network have been upgraded to enhance their diagnostic capabilities and increase the reporting abilities of local and regional healthcare centers to rapidly identify bioterrorism events as well as emerging diseases.

Smallpox and Anthrax

Smallpox virus (**Figure 30.17*a***) has intimidating potential as a microbial weapon because it can be spread easily by direct contact or by aerosol spray; is highly debilitating; causes a high fever, severe fatigue, and the eventual formation of pus-filled skin blisters (Figure 30.17*b*); and has a mortality rate of 30% or higher. Although an extremely effective smallpox vaccine is available, it has not been in use in the general population since smallpox was eradicated worldwide in 1980. Moreover, the potential of smallpox virus being deployed as a military weapon is considered low because military personnel are routinely vaccinated. Nevertheless, preparations for a potential smallpox attack on civilians in the United States have been made and would include immunization of key individuals, such as those attending smallpox patients or handling clinical specimens from smallpox patients.



(a)



(b)

Figure 30.17 Smallpox and its use as a microbial weapon. *(a)* Smallpox virus, a double-stranded DNA virus (◀ Section 11.6). *(b)* Characteristic papular smallpox rash and blisters on the arm. Smallpox was officially declared eradicated worldwide in 1980.

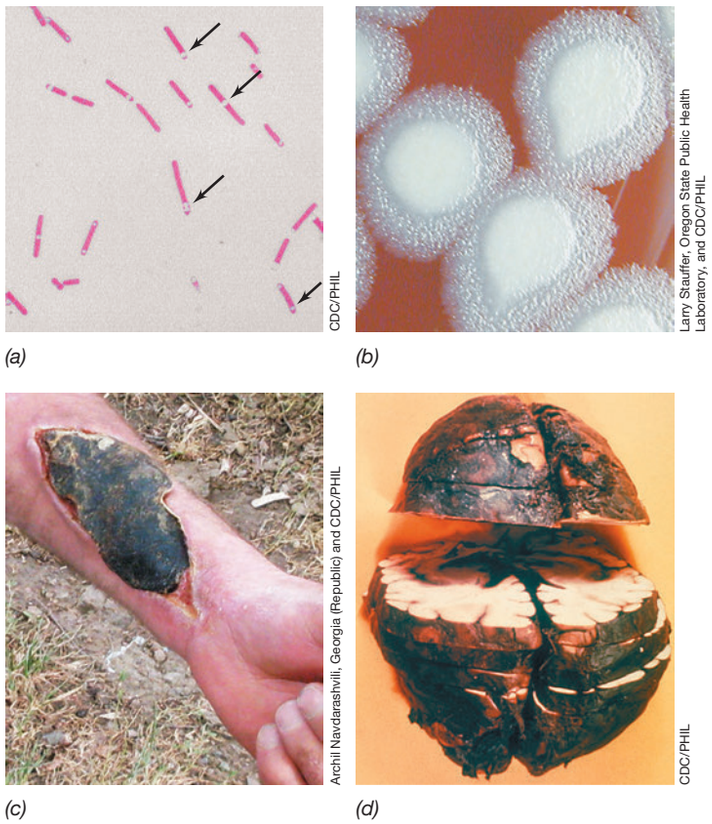


Figure 30.18 ***Bacillus anthracis*** **and anthrax.** *(a) Bacillus anthracis* is a gram-positive, endospore-forming rod approximately 1 μm in diameter and 3–4 μm in length. Note the developing endospores (arrows). *(b)* Characteristic "ground glass" appearance of colonies of *B. anthracis* on blood agar plates. *(c)* Cutaneous anthrax. The blackened lesion seen on this forearm is called an eschar, and results from tissue necrosis. *(d)* Inhalation anthrax can cause cerebral hemorrhage, as shown by the dark coloration in this fixed and sectioned human brain removed at autopsy.

Bacillus anthracis is the causative agent of anthrax, and its unique properties allow it to be easily employed as a bioweapon. Chief among these is that it is easily grown aerobically, producing distinctive colonies on enriched culture media, and it differentiates into highly resistant endospores (**Figure 30.18*a, b***; ◀ Section 2.8). Once prepared, endospores can be dried and stored indefinitely and then disseminated as a weapon by aerosol or in powdered suspension. There are three clinical forms of anthrax (▶ Section 32.8). *Cutaneous anthrax* is contracted when abraded skin is contaminated by *B. anthracis* endospores; the organism grows and kills the skin, forming a necrotic tissue lesion called an *eschar* (Figure 30.18*c*). The rarest form, *gastrointestinal anthrax*, is contracted from consumption of endospore-contaminated plants or meat from animals infected with anthrax. *Inhalation anthrax* (also called *pulmonary anthrax*) is the deadliest form and is contracted when *B. anthracis* endospores are inhaled. The symptoms of inhalation anthrax include pulmonary and cerebral hemorrhaging (Figure 30.18*d*), which makes this form of anthrax especially dangerous. All forms of anthrax have the potential to become systemic infections; however, cutaneous anthrax is easily treatable with antibiotics and is fatal in only about 20% of untreated cases. Without treatment, gastrointestinal anthrax is fatal in about half of those infected, whereas inhalation anthrax mortality approaches 100%.

Inhalation anthrax is the form of the disease that makers of microbial weapons would aim for, as has already transpired in the United States. At least 22 cases of anthrax leading to five deaths occurred in a 2001 bioterrorism attack in which *B. anthracis* endospore preparations were mailed in envelopes to certain news outlets and government officials. Of the 22 anthrax cases, 11 were inhalational and 11 cutaneous. These weaponized anthrax endospore preparations were mixed within a fine particulate material that allowed the endospores to be spread by air currents. Thus, opening an envelope containing the lethal mixture (or releasing it into a ventilation system) could contaminate surrounding areas and personnel.

Vaccination for anthrax is possible and is restricted to individuals who are considered at risk. This includes agricultural animal workers (livestock tenders and those working with animal products), laboratory personnel working with anthrax, veterinarians, and military personnel. As was discussed with smallpox, anthrax is an unlikely military weapon but could be a very effective means of terrorizing a civilian population because the vast majority of the population is unvaccinated.

We move on now from our consideration of disease tracking to exploring human infectious diseases themselves, grouped by their mode of transmission in the final four chapters.

Check Your Understanding

- What characteristics make a pathogen or its products particularly useful as a biological weapon?
- Indicate the steps you would take to identify and treat infections from smallpox virus or anthrax in a bioterror attack.

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Principles of Epidemiology

30.1 Epidemiology is the study of the occurrence, distribution, and determinants of health and disease in populations. Epidemiologists employ surveillance measures to record the incidence and prevalence of infectious diseases and track disease outbreaks that may lead to epidemics or pandemics. The morbidity and mortality of any given disease is a function of the transmissibility of the pathogen and the severity of the disease symptoms. Disease carriers may be symptom-free, or they may have chronic infections that are never resolved.

Q How does the job of an epidemiologist differ from that of a clinical healthcare provider?

30.2 Effects on populations as well as individuals must be studied to understand infectious disease. The interactions of pathogens with hosts can be dynamic, affecting the long-term evolution and survival of all species involved. Herd immunity provides disease protection for susceptible hosts.

Q Explain how the infectivity of a pathogen is correlated with the epidemic spread of a disease in various populations.

30.3 Infectious diseases can be transmitted directly from person to person, indirectly from living vectors or inanimate objects (fomites), or from common-source vehicles such as food and water. Disease reservoirs can include soil, insects, infected people before they show symptoms, chronic carriers, and many other sources. An understanding of disease reservoirs, carriers, and pathogen life cycles is critical for controlling disease epidemics.

Q Compared with a fomite, such as a contaminated cup, why is a disease vehicle such as food a much more powerful disease transmitter?

30.4 Epidemics may be of host-to-host origin or originate from a common source. The basic reproduction number (R_0) of a pathogen gives a relative picture of its seriousness and an indication of how effective herd immunity must be to prevent its spread.

Q If an emerging pathogen was found to have an R_0 of 20, would herd immunity need to be higher or lower than for an emerging pathogen with an R_0 of 5?

II • Public and Global Health

30.5 Food and water purity regulations, vector control, immunization, quarantine, isolation, and disease surveillance are all public health measures that reduce the incidence of communicable diseases. In the case of some diseases, such as smallpox, total eradication worldwide has been possible.

Q If an outbreak of yellow fever occurred, who would be isolated and who would be quarantined?

30.6 Infectious diseases account for about 20% of all mortality worldwide. Most cases of infectious diseases occur in developing countries. Control of many infectious diseases can be accomplished by public health measures that include adequate sanitation, food and water protections, and widespread vaccination programs. A high infectious disease load can significantly reduce the average life expectancy of the population in a country.

Q Contrast the leading causes of death in Africa and in the Americas.

III • Emerging Infectious Diseases, Pandemics, and Other Threats

30.7 Changes in host, vector, or pathogen conditions, whether natural or artificial, can encourage the explosive emergence or reemergence of infectious diseases. Global surveillance and intervention programs by organizations such as the World Health Organization and the U.S. Centers for Disease Control and Prevention are especially attuned to emerging and reemerging pathogens to prevent local epidemics from spreading.

Q How can bacterial genetic exchange fuel the emergence of new pathogens?

30.8 Several infectious diseases with significant mortality show pandemic characteristics. HIV/AIDS affects those who exchange bodily fluids, most often by either promiscuous unprotected sex or intravenous drug use. Cholera is primarily a waterborne infection, and control can be achieved by maintaining adequate clean water and waste sanitation measures. New pandemic influenza strains resulting from bird–swine–human influenza reassortments present the biggest infectious disease threat worldwide.

Q Why is H5N1 avian influenza considered a major threat to public health?

30.9 Bioterrorism from smallpox or anthrax is a threat in a world of rapid international travel and easily accessible technical information. Aerosols or disease vehicles are the most likely modes of delivery of microbial weapons. Prevention and containment measures rely on a well-prepared public health infrastructure.

Q Why are smallpox and anthrax more likely to be bioterrorism threats to civilians than to military personnel?

APPLICATION QUESTIONS

1. Smallpox, a disease that was limited to humans, was eradicated, whereas plague, a zoonotic disease with a reservoir in rats and related rodents, will likely never be eradicated worldwide. Explain this statement. Devise a plan to eradicate plague in a limited area such as a town or city. Be sure to consider methods that involve the reservoir, the pathogen, and the host.
2. Like smallpox, HIV/AIDS is considered to be a disease that could be eliminated worldwide because it is propagated by known means and there are no animal reservoirs. Although eliminating HIV infection is possible, why would it be much more difficult than eliminating smallpox? What would be involved in an HIV infection eradication program?
3. H5N1 avian influenza has high potential for causing a pandemic under certain circumstances. If such a highly transmissible human–avian strain were to evolve in Asia and you were a national public health official, what measures would you employ to stop the spread of the new influenza strain to other continents? If you failed to contain the new virus, where would you expect to see the first cases of such pandemic influenza, in metropolitan or rural areas?

CHAPTER GLOSSARY

Acute infection a short-term infection, usually characterized by dramatic onset

Basic reproduction number (R_0) the number of expected secondary transmissions from each single case of a disease in an entirely susceptible population

Biological (microbial) warfare the use of microbial agents to incapacitate or kill a military or civilian population in an act of war or terrorism

Carrier a subclinically infected individual who may spread a disease

Centers for Disease Control and Prevention (CDC) the agency of the United States Public Health Service that tracks disease trends, provides disease information to the public and to healthcare professionals, and forms public policy regarding disease prevention and intervention

Chronic infection a long-term infection

Common-source epidemic an infection (or intoxication) of a large number of people from a contaminated common source such as food or water

Disability-adjusted life year (DALY) a quantitative measure of disease burden defined as the cumulative number of years lost due to an illness itself, a disability due to an illness, or premature death

Disease surveillance the observation, recognition, and reporting of diseases as they occur

Emerging disease an infectious disease whose incidence recently increased or whose incidence threatens to increase in the near future

Endemic disease a disease that is constantly present, usually in low numbers, in a population

Epidemic the occurrence of a disease in unusually high numbers in a localized population

Epidemiology the study of the occurrence, distribution, and determinants of health and disease in populations

Fomite an inanimate object that when contaminated with a viable pathogen can transfer the pathogen to a host

Herd immunity the resistance of a population to a pathogen as a result of the immunity of a large portion of the population

Host-to-host epidemic an epidemic resulting from person-to-person contact, characterized by a gradual rise and fall in number of new cases

Incidence the number of new reported cases of a disease in a population in a given time period

Isolation in the context of infectious disease, the separation of persons who have an infectious disease from those who are healthy

Morbidity the incidence of disease in a population

Mortality the incidence of death in a population

Outbreak the occurrence of a large number of cases of a disease in a short period of time

Pandemic a widespread, usually worldwide epidemic

Prevalence the total number of new and existing cases of a disease reported in a population in a given time period

Public health the health of the population as a whole

Quarantine the separation and restriction of well persons who may have been exposed to an infectious disease to see if they develop the disease

Reemerging disease an infectious disease previously under control but that produces a new epidemic

Reservoir a source of infectious agents from which susceptible individuals may be infected

Vector a living agent that transfers a pathogen (differs from genetic vector, discussed in Chapter 12)

Vehicle a nonliving source of pathogens that transmits the pathogens to large numbers of individuals; common vehicles are food and water

Virulence the relative ability of a pathogen to cause disease

Zoonosis any disease that occurs primarily in animals but can be transmitted to humans

31 Person-to-Person Bacterial and Viral Diseases

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IV Sexually Transmitted Infections 1006

MICROBIOLOGYNOW

Reversing Antibiotic Resistance in a Recalcitrant Pathogen

Tuberculosis (TB) is a highly prevalent and life-threatening respiratory disease, causing nearly 1.5 million deaths annually. Here we highlight a new strategy for effective treatment of this devastating disease.

The causative agent of TB, *Mycobacterium tuberculosis* (yellow-green cells in photo shown being engulfed by a macrophage, red), is a slow-growing, gram-positive and acid-fast bacterium that is treated clinically by administration of a suite of drugs for many months. Chief among these is *isoniazid*, a growth factor analog that mimics nicotinamide, a vitamin required by *M. tuberculosis* to synthesize the waxlike mycolic acids found in its tough cell wall. In the presence of isoniazid, *M. tuberculosis* cannot construct its normal cell wall, making the cells more easily penetrated by antibiotics. Unfortunately, isoniazid does not always work, and this problem is becoming increasingly common.

Isoniazid resistance in *M. tuberculosis* has been linked in part to its ability to form a biofilm (a colony of microbial cells encased in a porous organic matrix and attached to a surface) in pulmonary tissues. The biofilm growth mode both facilitates and exacerbates the *M. tuberculosis* infection. Using a systematic screening process, researchers recently found a compound called *C10* that inhibits biofilm formation by *M. tuberculosis* in laboratory cultures. Although C10 itself showed no bactericidal activity, the bacteria were readily killed when even low concentrations of isoniazid were administered simultaneously with C10, and notably, resistance to this combination never developed. Furthermore, even strains that were already resistant to isoniazid were killed by this treatment, demonstrating that resistance to isoniazid could be *reversed* when used in combination with C10.

The next step will be to investigate the efficacy of the C10/isoniazid combination in living systems. If these tests are successful, the result may bring new potency to an old weapon against TB.

Source: Flentie, K., et al. 2019. Chemical disarming of isoniazid resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. (USA) 116*(21): 10510. doi:10.1073/pnas.1818009116.

Several million species of microorganisms likely exist in nature, but only a few hundred are known to cause disease. In this and the next three chapters we focus on this vitally important subset of the microbial world. We investigate the biology of the pathogens as well as the diseases they cause, including disease diagnosis, treatment, and prevention.

Our infectious disease coverage is ecological, being organized around each pathogen's *mode of transmission*. Although the biology of the different causative agents will certainly be highlighted, an ecological approach to infectious disease coverage emphasizes the single most important feature that links diseases caused by different microbes. Hence, tuberculosis and influenza are caused by a bacterium and a virus, respectively, but both diseases are transmitted from person to person by the airborne route.

In this chapter we explore diseases transmitted from person to person, whether through the air, by direct contact, or through sexual contact. In Chapter 32 we focus on diseases transmitted by animal and arthropod vectors or from soil, and in Chapter 33 we cover diseases transmitted from common sources, including water and food. We end our tour of the microbial world in Chapter 34 where we examine fungal and parasitic infections.

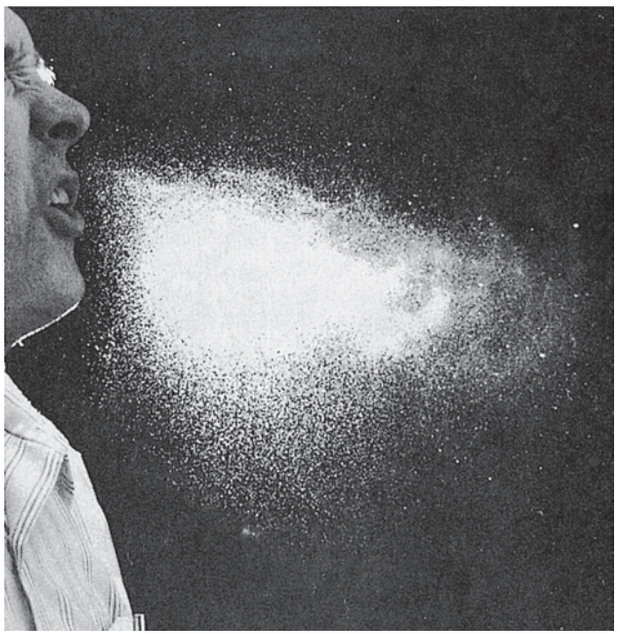

T.D. Brock

Figure 31.1 High-speed photograph of an unstifled sneeze. Effluent is expelled at over 100 m/s (200 miles/h).

I • Airborne Bacterial Diseases

A wide diversity of pathogenic bacteria are transmitted through airborne particles and cause diseases ranging from relatively mild and primarily upper respiratory tract infections to life-threatening upper or lower respiratory tract infections that can compromise a patient's very ability to breathe.

Worldwide, acute respiratory infections kill more than 4 million people a year, mainly in developing countries. Children and the elderly make up most of the fatalities, but in general, respiratory infections are the most common of all human diseases. Aerosols, such as those generated by a sneeze (**Figure 31.1**), as well as by coughing, talking, or breathing, are major vehicles for person-to-person transmission of respiratory diseases. Besides directly infecting a new host, infectious mucus from an aerosol can also contaminate objects, such as a door handle, and transmit infection well after the aerosol event. In these ways, respiratory diseases spread quickly, especially in congested areas, as airborne pathogens exploit a simple yet highly effective means of infecting new hosts.

31.1 Airborne Pathogens

Microorganisms found in air are derived from soil, water, plants, animals, people, surfaces, and other sources. Most microorganisms survive poorly in air. As a result, airborne pathogens are effectively transmitted between people only over short distances. Certain pathogens, however, survive drying well and can remain alive in dust or on fomites for long periods of time. For example, because of their thick cell walls, gram-positive bacteria (such as species of *Staphylococcus* and *Streptococcus*) are generally more resistant to drying than are gram-negative bacteria. Likewise, the waxy layer of *Mycobacterium* cell walls resists drying and promotes survival of pathogens such as *Mycobacterium tuberculosis*.

Large numbers of droplets can be expelled during a sneeze (Figure 31.1). Infectious droplets are about 10 μm in diameter and each droplet can contain one or more microbial cells or virus virions. The initial speed of the droplet movement is about 100 m/s (200 miles/h) in a violent sneeze and ranges from 15 to 50 m/s during coughing or shouting. The number of bacteria in a single sneeze varies from 10^4 to 10^6, and viral numbers can be much higher than this. Because of their small size, the droplets evaporate quickly in the air, leaving behind dried mucus in which the airborne pathogens remain embedded.

The human respiratory tract is divided into upper and lower regions, and specific airborne pathogens tend to exploit one region or the other, or sometimes both (**Figure 31.2**). The speed at which air moves through the human respiratory tract varies, and in the lower respiratory tract the rate is quite slow. As air slows down, particles in it stop moving and settle. Large particles settle first and the smaller ones later; only particles smaller than about 3 μm travel as far as the bronchioles in the lower respiratory tract (Figure 31.2).

Mastering Microbiology
Art Activity: Figure 31.2 The human respiratory system

Upper respiratory infections such as the common cold are typically acute and non-life-threatening. By contrast, *lower* respiratory infections, such as bacterial or viral pneumonia, are often chronic and can be quite serious, especially in elderly or immunocompromised individuals. Also, although most common respiratory infections are not serious in an otherwise healthy host, they can set the stage for *secondary infections* that can be life-threatening. For example, death of an elderly person from pneumonia following a severe case of influenza is not an uncommon event.

Most human respiratory pathogens are transmitted from person to person because humans are the only reservoir for the pathogens. However, many airborne pathogens, such as *Streptococcus* spp., cold viruses, and influenza, can also be transmitted by direct contact (for example, by a handshake) or on fomites. Accurate and rapid diagnosis and treatment of respiratory infections are well developed in the clinical setting and, if practiced effectively, can limit

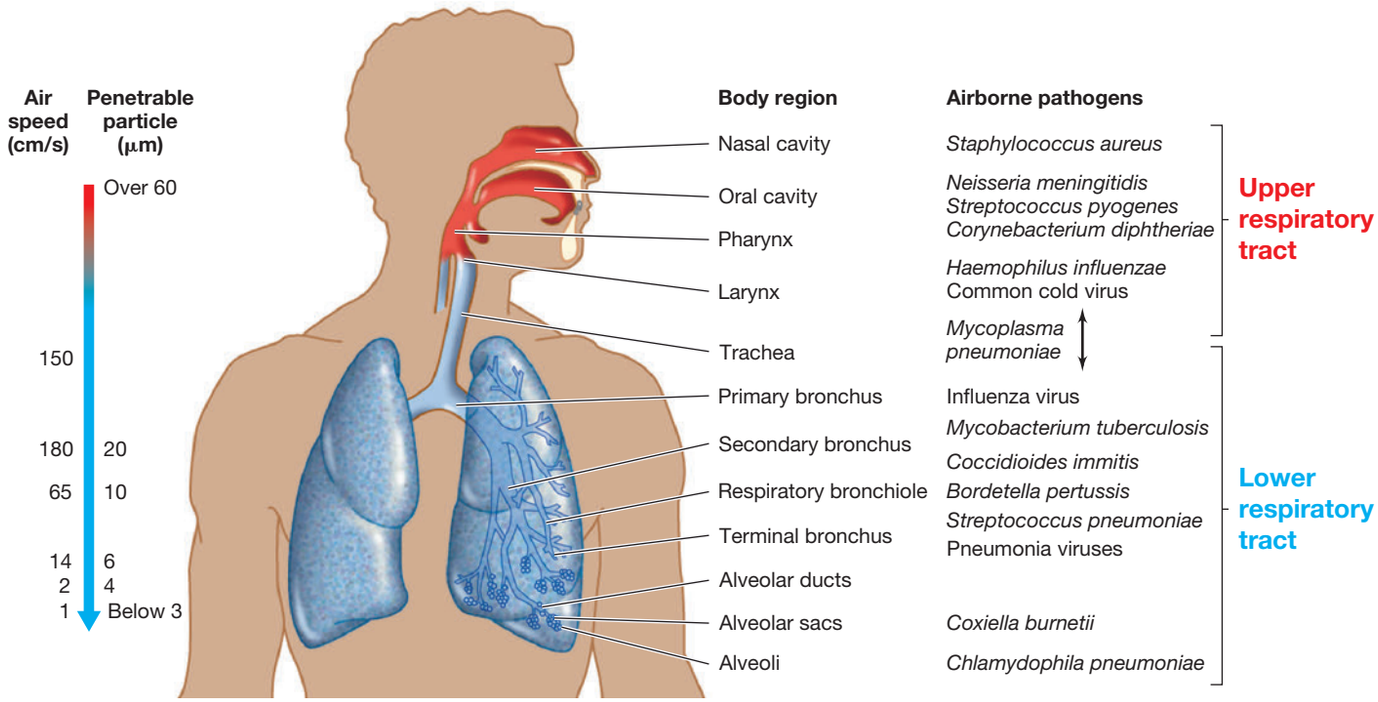


Figure 31.2 The human respiratory system. The microorganisms listed typically initiate infections at the locations indicated. The scale on the left combined with the respiratory tract diagram shows approximately where particles come to rest, determined by their size and air speed.

host damage. Many bacterial and viral pathogens transmitted by an airborne route can be controlled by immunization, and most respiratory bacterial pathogens respond readily to antibiotic therapy. Antiviral therapies, on the other hand, are rather limited, and recovery from viral infections is often due solely to the immune response.

Check Your Understanding

- Why can it be said that respiratory pathogens have exploited an effective means of transmission?
- Identify pathogens more commonly found in the upper respiratory tract. Identify pathogens more commonly found in the lower respiratory tract.

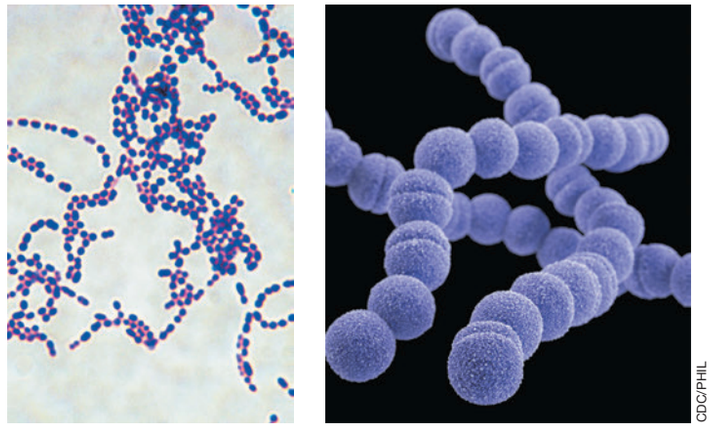
31.2 Streptococcal Syndromes

Streptococcal diseases are transmitted by airborne droplets or by direct contact, and the species *Streptococcus pyogenes* (Figure 31.3) and *Streptococcus pneumoniae* are the most important human respiratory pathogens. Streptococci are nonsporulating, homofermentative but aerotolerant gram-positive cocci (◀ Section 16.6). Cells of *S. pyogenes* typically grow in elongated chains (Figure 31.3), as do many other species of the genus. Pathogenic strains of *S. pneumoniae* grow in pairs or short chains, and virulent strains produce an extensive polysaccharide capsule (see Figure 31.11). Virulent strains of *Streptococcus* can form vicious purulent (pus-forming) wounds in humans and other warm-blooded (endothermic) animals (Figure 31.4 and see Figure 31.10). Many other serious conditions whose symptoms are less dramatic than these are also associated with streptococcal infections.

Streptococcus pyogenes

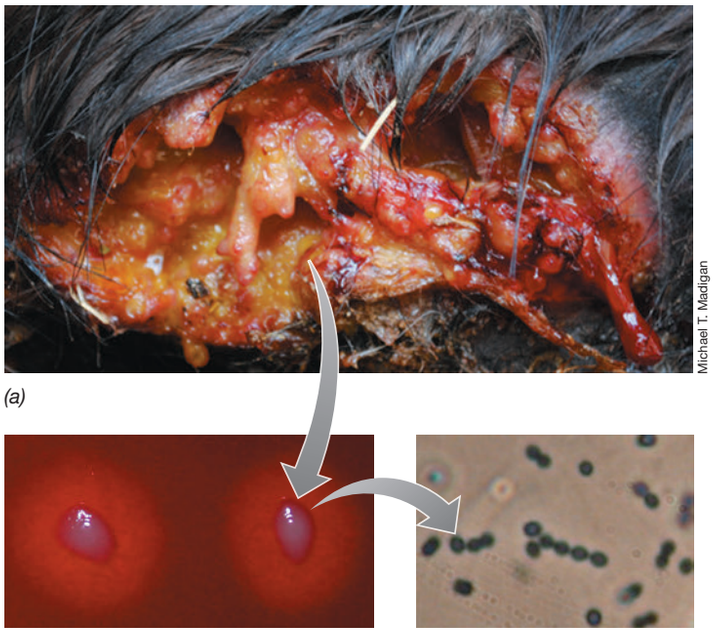
Streptococcus pyogenes (Figure 31.3), the major species in the *group A streptococci*, is frequently isolated from the upper respiratory tract of healthy adults. Although numbers are typically low, serious infections are possible if host defenses are weakened or a new, highly virulent strain is encountered.

S. pyogenes is the cause of *streptococcal pharyngitis*, better known as *strep throat* (Figure 31.5). Most clinical isolates of *S. pyogenes* produce an exotoxin (◀ Sections 25.6 and 25.7) that lyses red blood



(a) (b)

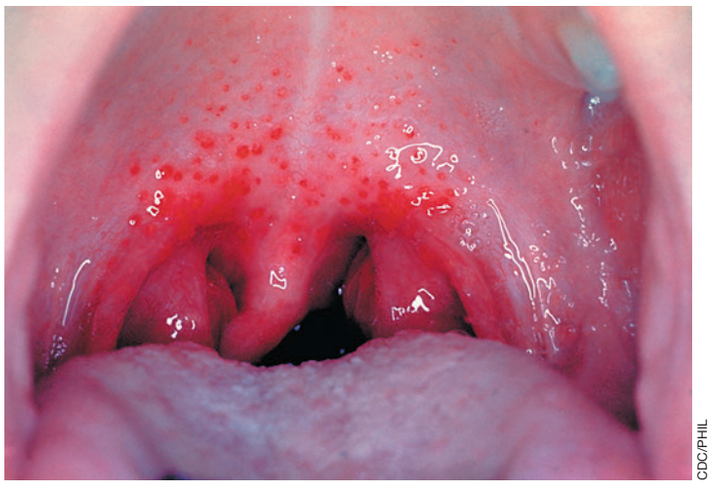
Figure 31.3 *Streptococcus pyogenes*. *(a)* Gram stain of cells of *Streptococcus pyogenes*. Cells grow in chains and range in size from 0.6 to 1 μm in diameter. *(b)* Computer-generated image of a scanning electron micrograph of cells of *S. pyogenes*.



Michael T. Madigan

Figure 31.4 Pus-forming wound from β-hemolytic streptococci. *(a)* Pus and coagulated blood from a *Streptococcus equi* infection of a horse's salivary glands (the salivary glands have burst open from the infection). *(b)* Colonies of *S. equi* showing β-hemolysis on blood agar (compare with Figure 31.8). *(c)* Phase-contrast photomicrograph of cells of *S. equi*. Cells are 1 μm in diameter.

cells in culture media, a condition called *β-hemolysis* (Figure 31.4*b* and see Figure 31.8). Streptococcal pharyngitis is characterized by a severe sore throat, enlarged tonsils with red spots on the soft palate (Figure 31.5), tender cervical lymph nodes, and a mild fever and feeling of general malaise. *S. pyogenes* can also cause infections of the middle ear (*otitis media*) and of the mammary glands (*mastitis*); infections of the superficial layers of the skin called *impetigo* (**Figure 31.6**); *erysipelas*, an acute streptococcal skin infection (**Figure 31.7**); and other conditions linked to the aftereffects of streptococcal infections.



CDC/PHIL

Figure 31.5 A case of strep throat caused by *Streptococcus pyogenes*. The back of the throat is inflamed and shows small red spots, typical of streptococcal pharyngitis.

Franklin H. Top

Figure 31.6 Typical lesions of impetigo. Impetigo is commonly caused by *Streptococcus pyogenes* or *Staphylococcus aureus*.

About half of the clinical cases of severe sore throat are due to *S. pyogenes*; most others are due to viral infections. Because of this, an accurate and rapid diagnosis of a severe sore throat is important. If the sore throat is due to *S. pyogenes*, immediate treatment is important because untreated group A streptococcal infections can lead to serious secondary diseases such as scarlet fever (see Figure 31.9), rheumatic fever, acute glomerulonephritis, and streptococcal toxic shock syndrome. On the other hand, if the sore throat is due to a virus, treatment with antibiotics will be useless and will only promote drug resistance on the part of the normal microbiota.

Clinical tools for quickly diagnosing strep throat are widely available and in routine use in primary care clinics. These tools include, in particular, rapid antigen detection systems that contain antibodies specific for cell surface proteins of *S. pyogenes* (◀ Section 29.7 and Figure 29.21). A more sensitive and accurate confirmation is possible by obtaining an actual culture of *S. pyogenes* from the throat or other suspected lesion on a blood agar plate (**Figure 31.8**). In contrast to rapid tests, however, results of a throat culture may take up to 48 h to process, and such a delay in treatment can have adverse effects, as we consider now.

Scarlet and Rheumatic Fevers and Other Group A Streptococcal Syndromes

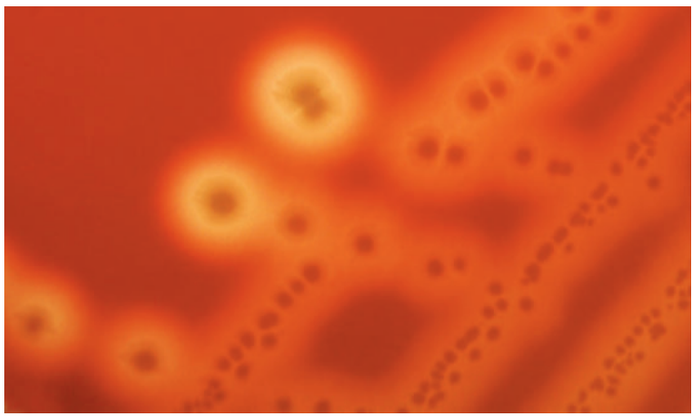
Certain strains of group A streptococci carry a lysogenic bacteriophage that encodes streptococcal pyrogenic exotoxin A (SpeA), SpeB, SpeC, and SpeF. These exotoxins are responsible for most of the symptoms of *streptococcal toxic shock syndrome* and *scarlet fever* (**Figure 31.9**). Streptococcal pyrogenic exotoxins are superantigens that activate and recruit large numbers of T cells to the infected tissues (◀ Sections 25.7 and 28.2). Toxic shock results when the activated T cells secrete cytokines, which in turn activate large numbers of macrophages and neutrophils, causing severe inflammation and tissue destruction.

CDC/Dr. Thomas F. Sellers

Figure 31.7 Erysipelas. Erysipelas is a *Streptococcus pyogenes* infection of the dermis, shown here on the nose and cheeks, characterized by redness and distinct margins of infection. Other commonly infected body sites include the ears and the legs.

Scarlet fever, signaled by a severe sore throat, fever, and characteristic rash (Figure 31.9), is readily treatable with antibiotics or may be self-limiting. But treatment is always advisable because several undesirable conditions can emerge from a case of scarlet fever. Occasionally group A streptococcal infections cause fulminant (sudden and severe) invasive systemic infections, such as cellulitis, a skin infection in subcutaneous layers; or *necrotizing fasciitis*, a rapid and progressive disease resulting in extensive destruction of subcutaneous tissue, muscle, and fat (**Figure 31.10**). Necrotizing fasciitis is a clinical term for the condition caused by "flesh-eating bacteria." In these cases, SpeA, SpeB, SpeC, and SpeF exotoxins and the bacterial cell surface M protein function as superantigens; the associated host inflammation results in extensive tissue destruction and can be fatal (Figure 31.10).

Untreated or insufficiently treated *S. pyogenes* infections may lead to other severe conditions 1 to 4 weeks after the onset of infection.



Michael T. Madigan

Figure 31.8 β-Hemolysis. The ability of a bacterium to lyse red blood cells and form a clear zone around a colony on a blood agar plate indicates secretion of the protein β-hemolysin. See also Figures 25.17*a* and 31.4*b*.

Franklin H. Top

Figure 31.9 Scarlet fever. The typical rash of scarlet fever results from the activity of the pyrogenic exotoxins produced by *Streptococcus pyogenes*.

For example, the immune response to the invading pathogen can produce antibodies that cross-react with host tissue antigens of the heart, joints, and kidneys, resulting in damage to these tissues. The most serious of these syndromes is **rheumatic fever** caused by rheumatogenic strains of *S. pyogenes*. These strains contain cell surface antigens that are similar in structure to heart valve and joint proteins. Thus, rheumatic fever is, in effect, an *autoimmune* complication (◀ Section 28.1) because antibodies directed against streptococcal antigens cross-react with heart valve and joint antigens, causing inflammation and tissue destruction. Damage to host tissues may be permanent and is often exacerbated by subsequent streptococcal infections that lead to recurring bouts of rheumatic fever. Another streptococcal syndrome is *acute poststreptococcal glomerulonephritis*, a painful kidney disease. This "immune complex" disease develops transiently when streptococcal antigen–antibody complexes in the blood lodge in the glomeruli (filtration membranes of the kidney) and cause inflammation, a serious condition called *nephritis*.

Streptococcus pneumoniae

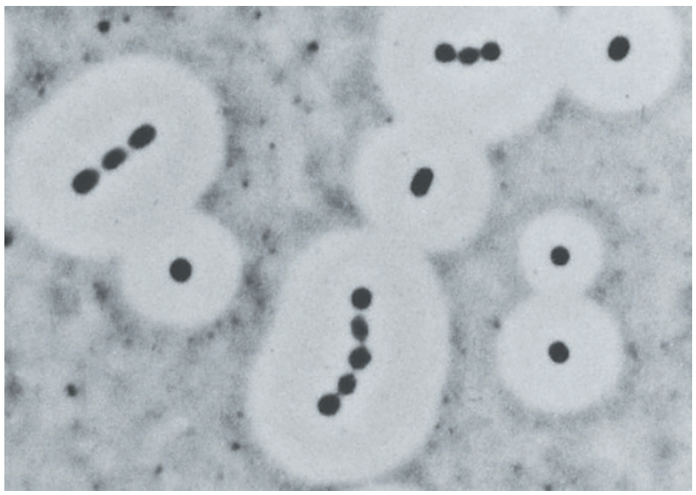
A second major human streptococcal pathogen is *Streptococcus pneumoniae* (**Figure 31.11**), a species that can cause invasive lung infections, typically as secondary infections to other respiratory disorders. Encapsulated strains of *S. pneumoniae* (Figure 31.11; ◀ Figure 25.4) are particularly virulent because they are especially invasive. Cells invade the lower respiratory tract where the capsule enables the cells to resist phagocytosis yet generate a strong host inflammatory response. Pneumonia results from the accumulation of recruited phagocytic cells and fluid. Cells of *S. pneumoniae* can then spread from the focus of infection as a bacteremia, sometimes infecting the bones, middle ear, and heart valves (endocarditis). *S. pneumoniae* infection is often the cause of death in elderly persons whose death is reported to be from "respiratory failure."

Unlike the case with *S. pyogenes*, effective vaccines are available for preventing infection by the most common strains of

Figure 31.10 Necrotizing fasciitis (flesh-eating bacteria). Soft tissue infection of human hip and thigh by group A *Streptococcus pyogenes*. The flesh has split open to reveal muscle tissues.

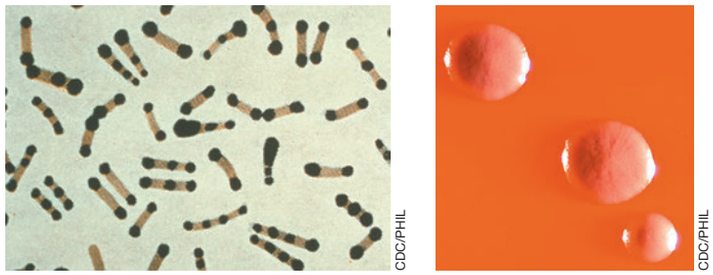
S. pneumoniae. An older vaccine widely used in adults consisted of a mixture of 23 capsular polysaccharides from the most prevalent pathogenic strains. The vaccine is recommended for those over age 60, healthcare providers, individuals with compromised immunity, and any other high-risk population. A newer conjugate vaccine (◀ Figure 28.9) called PREVNAR 13® is an update of the traditional vaccine. It is effective against the 13 *S. pneumoniae* strains most commonly seen today and is recommended for adults age 50 or older.

S. pneumoniae infections typically respond quickly to penicillin therapy, but up to 30% of pathogenic isolates now exhibit resistance to this drug. Resistance to the antibiotics erythromycin and cefotaxime is also found in some strains, but thus far, all strains have been found sensitive to vancomycin, an antibiotic held in reserve for treating pneumonia and several other bacterial diseases where antibiotic resistance is widespread (◀ Section 28.7).



Isaac Shechmeister

Figure 31.11 *Streptococcus pneumoniae*. India ink negative stain of cells of *Streptococcus pneumoniae*. An extensive capsule surrounds the cells, which are 1.0–1.2 μm in diameter.



CDC/PHIL

CDC/PHIL

(a) (b)

Figure 31.12 *Corynebacterium diphtheriae*, the causative agent of diphtheria. *(a)* Cells of *C. diphtheriae* showing typical club-shaped appearance. The gram-positive cells are 0.5–1 μm in diameter and may be several micrometers in length. *(b)* Colonies of *C. diphtheriae* grown on a selective medium of blood agar plus tellurite.

Check Your Understanding

- How does *Streptococcus pyogenes* infection cause rheumatic fever, and why is rheumatic fever considered to be an autoimmune complication?
- What is the primary virulence factor for *Streptococcus pneumoniae*?

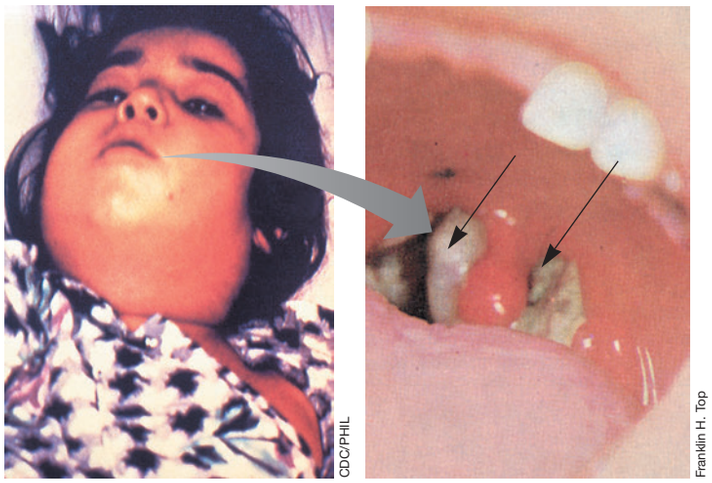
31.3 Diphtheria and Pertussis

Diphtheria is a severe respiratory disease that typically infects young children. Diphtheria is caused by *Corynebacterium diphtheriae*, a gram-positive, nonmotile, and aerobic club-shaped bacterium that forms small, smooth colonies on blood agar plates (**Figure 31.12**). **Pertussis**, also known as **whooping cough**, is a serious respiratory disease caused by infection with *Bordetella pertussis*, a small, gram-negative, aerobic coccobacillus (see Figure 31.14). As with diphtheria, pertussis mostly affects children but can cause serious respiratory disease in adults as well. Both diphtheria and pertussis can be prevented by vaccination and cured with antibiotics.

Diphtheria

Cells of *C. diphtheriae* (Figure 31.12*a*) enter the host from airborne droplets, infecting the tissues of the throat and tonsils and typically causing swelling of the neck (**Figure 31.13*a***). Throat tissues respond to *C. diphtheriae* infection by forming a characteristic lesion called a *pseudomembrane* consisting of damaged host cells and cells of *C. diphtheriae* (Figure 31.13*b*). Not all strains of *C. diphtheriae* cause diphtheria. *Pathogenic* strains of *C. diphtheriae* carry a lysogenic bacteriophage whose genome encodes a powerful exotoxin called *diphtheria toxin*. This toxin inhibits protein synthesis in the host, leading to cell death (◀ Figure 25.13). Death from diphtheria is due to a combination of partial suffocation by the pseudomembrane and tissue destruction by diphtheria exotoxin. *C. diphtheriae* isolated from the throat is diagnostic for diphtheria. Nasal or throat swabs are used to inoculate blood agar containing tellurite (Figure 31.12*b*) or Loeffler's medium, a selective medium that inhibits the growth of most other respiratory pathogens.

Prevention of diphtheria is accomplished with a highly effective toxoid vaccine, part of the *DTP3* or *DTaP* (*d*iphtheria toxoid, *t*etanus toxoid, and *a*cellular *p*ertussis) *vaccine* (◀ Section 28.3). Diphtheria is all but absent from developed countries where this vaccine is



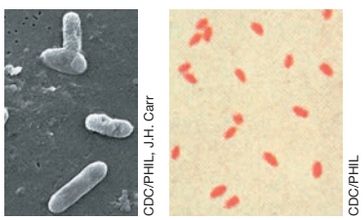
(a) (b)

Figure 31.13 Diphtheria. *(a)* A swollen neck is a common symptom of diphtheria. *(b)* The pseudomembrane (arrows) in an active case of diphtheria restricts airflow and swallowing and is associated with a severe sore throat.

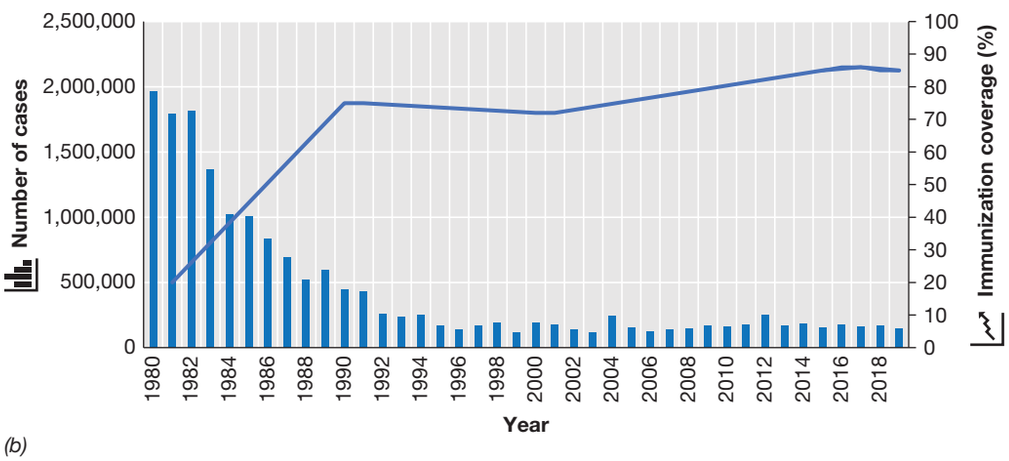
widely used. Penicillin, erythromycin, and gentamicin are generally effective treatments for diphtheria, but in life-threatening cases, diphtheria antitoxin (an antiserum to diphtheria toxoid produced in horses) may be administered in addition to antibiotic therapy.

Pertussis

Pertussis is an acute, highly infectious respiratory disease. Infants less than 6 months old, who are too young to be effectively vaccinated, have the highest incidence of disease and also have the most severe symptoms. Cells of *B. pertussis* (Figure 31.14) attach to ciliated host cells of the respiratory tract and excrete *pertussis exotoxin*. This potent toxin induces synthesis of cyclic adenosine monophosphate (cyclic AMP, ◀ Figure 7.22), which is at least partially responsible for the events that lead to host tissue damage. *B. pertussis* also produces an endotoxin (◀ Section 25.8), which may induce some of the symptoms of whooping cough. Clinically, whooping cough is characterized by a recurrent, violent cough that can last up to 6 weeks. The spasmodic coughing gives pertussis its common name; a whooping sound results from the patient inhaling deep breaths to obtain sufficient air.



(a)



(b)

Figure 31.14 *Bordetella* and pertussis. (*a*) Left, scanning electron micrograph of cells of *Bordetella*; right, Gram-stained cells of *B. pertussis*. Cells of *B. pertussis* are typically coccobacilli 0.4–0.5 µm in diameter and about 1 µm long. (*b*) Pertussis incidence globally has shown a downward trend for the past two decades, associated with an increased uptake of the DTP3 vaccine. Data are from the WHO.

Worldwide, up to 25 million cases and over 150,000 deaths occur each year from pertussis, mostly in developing countries. *B. pertussis* is endemic worldwide, and pertussis remains a problem even in developed countries, usually as a result of inadequate immunization. Globally, there has been a dramatic decrease in the incidence of pertussis since 1980 (Figure 31.14). In 2019, only 145,000 cases of pertussis were reported worldwide as opposed to almost 2 million in 1980. The reduced incidence directly correlates to an increased uptake of the DTP3 vaccine, with WHO data showing that approximately 85% of one-year-olds globally have been immunized against pertussis in 2019. Pertussis is a classic endemic disease (◀ Section 30.1); incidence rises cyclically as populations become susceptible and are exposed to the pathogen. A combination of lax vaccination protocols and the fact that pertussis is a much more common disease than diphtheria have probably fueled the overall higher incidence of pertussis in recent years.

Whooping cough can be treated with ampicillin, tetracycline, or erythromycin, although antibiotics alone do not seem to effect a complete cure, as patients continue to show symptoms and remain infectious for up to 2 weeks after beginning antibiotic therapy. This indicates that the immune response may be as important as antibiotics in ridding the pathogen from the body.

Check Your Understanding

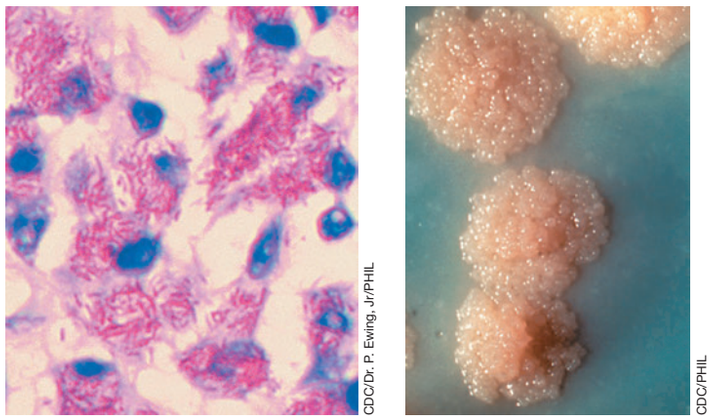
- Contrast the disease symptoms of diphtheria and pertussis.
- What precautions can be taken by parents to reduce the risk of pertussis for infants less than 2 months old?

31.4 Tuberculosis and Leprosy

The famous pioneering microbiologist Robert Koch, the founder of the field of medical microbiology, isolated and described the causative agent of tuberculosis, *Mycobacterium tuberculosis*, in 1882 (◀ Section 1.12). A related species, *Mycobacterium leprae*, causes leprosy (Hansen's disease). Mycobacteria are gram-positive bacteria and share the property of being *acid-fast* because of the waxy mycolic acid constituent of their cell walls (◀ Section 16.11). Mycolic acid allows these organisms to retain the red dye carbol-fuchsin after a mycobacterial smear on a slide is washed in 3% hydrochloric acid in alcohol (Figure 31.15). Colonies of *M. tuberculosis* grow slowly on plates and have a characteristically wrinkled morphology.

Tuberculosis

Tuberculosis (TB) is easily transmitted by the respiratory route, and at one time it was the most important infectious disease of humans. TB kills nearly 1.5 million people



(a) (b)

Figure 31.15 Mycobacteria. *(a)* Acid-fast stained lymph node biopsy from a patient with HIV/AIDS shows cells of *Mycobacterium avium*, a relative of *M. tuberculosis*. Multiple bacilli, stained red with carbol-fuchsin and treated with 3% hydrochloric acid, are evident inside each human cell. The individual rods are about 0.4 μm in diameter and up to 4 μm in length. *(b)* Colonies of *M. tuberculosis*. The rough, wrinkled surface is typical of mycobacterial colonies.

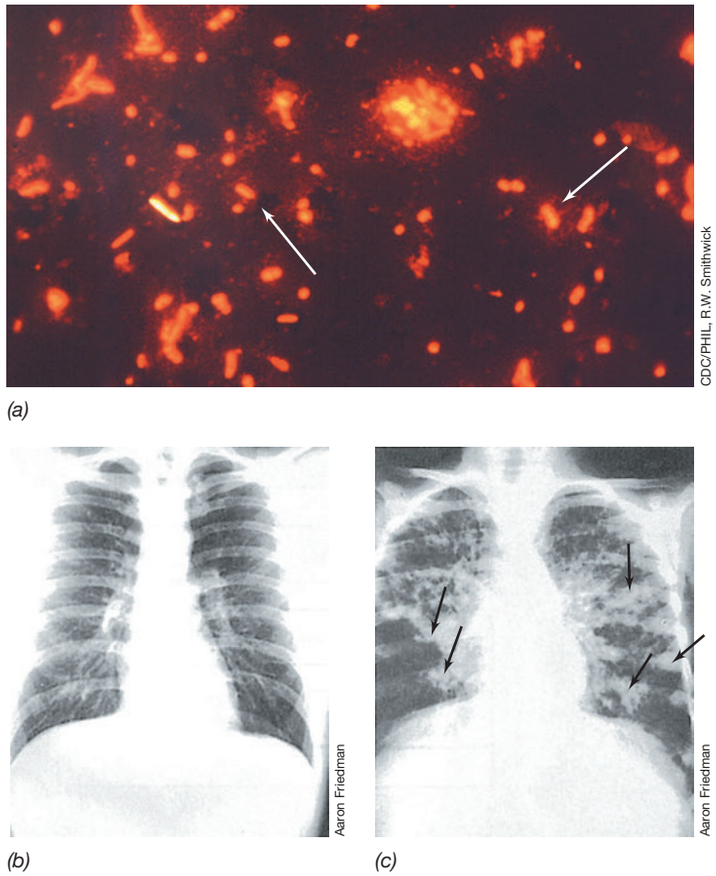
per year, making it the top infectious disease killer worldwide. About one-third of the world's population has been infected with *M. tuberculosis*, though most do not show active disease because cell-mediated immunity (◀ Section 27.8) plays a critical role in the suppression of active disease after infection.

Tuberculosis can take several forms. TB can be a *primary* infection (initial infection) or *postprimary* infection (reinfection). Primary infection typically results from inhalation of droplets containing *M. tuberculosis*, after which the bacteria settle in the lungs and grow. The host mounts an immune response to *M. tuberculosis*, resulting in the formation of aggregates of activated macrophages, called *tubercles*. Bacteria are found in the sputum of individuals with active disease, and areas of destroyed tissue can be seen in chest X-rays (**Figure 31.16**). Mycobacteria survive and grow within macrophages in the tubercles, forming granulomas, and if the disease is not controlled, extensive destruction of lung tissue can occur. If the disease reaches this stage, the pulmonary infection is often fatal.

In most individuals infected with *M. tuberculosis*, however, acute disease does not occur; instead, the infection is asymptomatic. Nevertheless, the infection hypersensitizes the individual to *M. tuberculosis* or its products and typically protects the individual against postprimary infections. A diagnostic skin test, called the **tuberculin test**, can detect this hypersensitivity (◀ Figure 28.4), and many healthy adults are *tuberculin-positive* as a result of previous or current inapparent infections. In most cases, the cell-mediated immune response to *M. tuberculosis* is protective and lifelong. However, some tuberculin-positive patients develop postprimary tuberculosis through reinfection from bacteria that have remained dormant in lung macrophages for years. Because of this, individuals who have a positive tuberculin test are typically treated with anti-tuberculosis drugs for extended periods to ensure that all mycobacteria have been killed.

Antimicrobial therapy of TB has been a major means of controlling the disease. Streptomycin was the first effective anti-tuberculosis antibiotic, but the real revolution in tuberculosis treatment came with the discovery of isonicotinic acid hydrazide, called *isoniazid* (INH). This drug is highly effective and readily absorbed when given orally. Isoniazid is a growth factor analog (◀ Section 28.5 and Table 28.4) of the structurally related molecule nicotinamide; in mycobacteria the drug inhibits mycolic acid synthesis, and this compromises cell wall integrity. Following treatment with isoniazid, mycobacteria lose their acid-fast properties, in keeping with the role of mycolic acid in this staining property.

Treatment of tuberculin-positive individuals is typically achieved with daily doses of isoniazid and the antibiotic rifampin for 2 months, followed by biweekly doses for a total of 9 months. This treatment eradicates pockets of *M. tuberculosis* cells and prevents emergence of antibiotic-resistant derivatives. Multiple drug therapy reduces the possibility that strains having resistance to more than one drug will emerge. Resistance of *M. tuberculosis* to isoniazid and other drugs, however, is increasing, especially in HIV/AIDS patients, in whom TB is a common infection (see Figure 31.46*g*; see also MicrobiologyNow on the opening page of this chapter). Treatment of these strains, called *multidrug-resistant tuberculosis strains*, requires the use of second-line tuberculosis drugs that are generally more toxic, less effective, and more costly than rifampin and isoniazid. Multiple drug resistance is a major reason the incidence of TB is slowly increasing in developed countries worldwide.



(a) (b) (c)

Figure 31.16 Tuberculosis symptoms. *(a)* Sputum sample from a patient with tuberculosis stained by the Smithwick acridine orange method. Cells of *Mycobacterium tuberculosis* are the yellow-orange rod-shaped structures (arrows). *(b)* Normal chest X-ray. The faint white lines are arteries and other blood vessels. *(c)* Chest X-ray showing an advanced case of pulmonary tuberculosis; white patches (arrows) indicate tubercles that are densely populated by viable cells of *M. tuberculosis*.

(a) (b)

Figure 31.17 Lepromatous leprosy lesions on the skin. *(a)* Lepromatous leprosy is caused by infection with *Mycobacterium leprae*. The lesions can contain up to 10^9 bacterial cells per gram of tissue, indicating an active uncontrolled infection with a poor prognosis. *(b)* The palm of the right hand of a leprosy patient showing the claw-like form and digit deformation characteristic of late-stage leprosy.

Leprosy

Mycobacterium leprae, a relative of *M. tuberculosis*, causes the disease *leprosy*, more formally known as *Hansen's disease*. The most serious form of Hansen's disease is *lepromatous* leprosy, characterized by folded, bulblike lesions on the body, especially on cooler parts of the body such as the face and extremities (**Figure 31.17*a***). The lesions are due to the growth of *M. leprae* cells in skin Schwann cells that insulate the nerves, and the lesions contain large numbers of bacterial cells. Like cells of other mycobacteria (Figure 31.15*a*), cells of *M. leprae* from the lesions stain deep red with carbol-fuchsin in the acid-fast staining procedure, providing a definitive demonstration of active infection.

In severe untreated cases of leprosy, the disfiguring lesions lead to destruction of peripheral nerves; muscles then atrophy, and motor function is impaired. The loss of sensation in the extremities leads to inadvertent injuries, such as burns and cuts. Loss of bone calcium leads to a slow shrinking of the digits and their transition to claw-like forms in late-stage leprosy (Figure 31.17*b*). Pathogenicity in the disease is due to a combination of delayed-type hypersensitivity (◀ Section 28.1) and the highly invasive activities of *M. leprae*, which can grow within macrophages and lead to the characteristic lesions (Figure 31.17*a*). Leprosy is transmitted by direct contact as well as by an airborne route, but it is not as highly contagious as TB. Historically, leprosy has been associated with poverty, malnutrition, and poor sanitation and hygiene. Among other things, these factors undoubtedly affect an individual's ability to resist infection.

Many Hansen's disease patients exhibit less-pronounced lesions from which *M. leprae* cells cannot be obtained; these individuals have the *tuberculoid* form of the disease. Tuberculoid leprosy is characterized by a vigorous immune response and a good prognosis for spontaneous recovery. Hansen's disease of either form, and the continuum of intermediate forms, is treated using a multiple drug therapy protocol, which includes some combination of extended therapy of up to 1 year with *dapsone* (4,4′-sulfonylbisbenzeneamine, an inhibitor of folic acid synthesis); *rifampin*, a bacterial RNA polymerase inhibitor; and *clofazimine*, a drug that targets bacterial respiration and ion transport.

Leprosy is considered a neglected tropical disease by the WHO. In 2019, the WHO stated that 202,000 cases of leprosy were reported globally, most of which were confined to countries such as India, Brazil, and Indonesia, with more than 10,000 people infected in each. Until recently, a leprosy diagnosis relied on the identification of *M. leprae* cells from lesions. However, a quick, inexpensive, and specific blood test is now available that greatly assists in diagnosing early-stage leprosy, the most treatable form.

In addition to *M. tuberculosis* and *M. leprae*, several other mycobacteria are human pathogens. These include in particular *M. bovis*, a close relative of *M. tuberculosis* and a common pathogen of dairy cattle. *M. bovis* can initiate classic symptoms of TB in humans; however, a combination of the pasteurization of milk and the culling of infected cattle has greatly reduced the incidence of bovine-to-human transmission of this form of TB.

Check Your Understanding

- Why is *Mycobacterium tuberculosis* a widespread respiratory pathogen?
- Describe three common characteristics of pathogenic mycobacteria.

31.5 Meningitis and Meningococcemia

Meningitis is an inflammation of the meninges, the membranes that compose the protective covering of the central nervous system, that is, the spinal cord and brain. Several different microorganisms, including certain viruses, bacteria, fungi, and protists, can cause meningitis. Here we focus on the severe bacterial form of the disease called *infectious meningitis*, caused by the bacterium *Neisseria meningitidis*.

Pathogen and Disease Syndromes

Neisseria meningitidis, often called the *meningococcus*, is a gram-negative and obligately aerobic coccus about 0.6–1 μm in diameter (**Figure 31.18*a***); it is a relative of the bacterium that causes gonorrhea, *Neisseria gonorrhoeae*. The bacterium is transmitted to a new host, usually via the airborne route from an infected individual, and attaches to the cells of the nasopharynx. Once there, the organism quickly gains access to the bloodstream (bacteremia, ◀ Section 25.2), where it disseminates throughout the body and causes upper respiratory tract symptoms. Meningitis is characterized by the sudden onset of a headache accompanied by vomiting and a stiff neck, and its seriousness is underscored by the fact that meningitis can progress to

(a) (b)

Figure 31.18 *Neisseria meningitidis*. The organism causes meningitis and meningococcemia. *(a)* Gram stain of cells of *N. meningitidis*; cocci are about 0.6–1 μm in diameter. *(b)* Four-month-old infant with gangrene on legs from meningococcemia.

coma and death in less than a day. Instead of, or in addition to, full-blown meningitis, *N. meningitidis* bacteremia sometimes leads to fulminant **meningococcemia**, a condition characterized by intravascular coagulation and tissue destruction (gangrene, Figure 31.18*b*), shock, and death in over 10% of cases.

Meningococcal meningitis often occurs in epidemics, usually in populations living in close proximity, such as in military barracks or college dormitories. Anyone can get meningococcal disease, but the incidence is much higher in infants, school-age children, and young adults. Up to 30% of people carry *N. meningitidis* in their nasopharynx with no apparent harmful effects, and the trigger for conversion from the asymptomatic carrier state to the disease state is unknown.

Diagnosis, Treatment, and Vaccines

Meningococcal meningitis is definitively diagnosed from cultures of *N. meningitidis* isolated from nasopharyngeal swabs, blood, or cerebrospinal fluid. Thayer–Martin medium, a selective medium for the growth of pathogenic *Neisseria*, including both *N. meningitidis* and *N. gonorrhoeae* (◀ Figure 29.6), is used to isolate *N. meningitidis*, and colonies containing gram-negative diplococci (Figure 31.18*a*) are further tested. However, due to the rapid onset of life-threatening symptoms in infectious meningitis, preliminary diagnosis is often based on clinical symptoms and treatment is started before culture tests confirm infection with *N. meningitidis*. Treatment is typically with penicillin, and intravenous application is often needed to speed antibiotic infusion.

Naturally occurring antibodies acquired by subclinical infections with *N. meningitidis* are effective for preventing infectious meningitis in most adults. Vaccines consisting of purified polysaccharides or polysaccharides from the most prevalent pathogenic strains are available to immunize certain susceptible populations, including military recruits and students living in dormitories, especially if an outbreak is in progress. In addition, the antibiotic rifampin is often used to eradicate the carrier state and prevent meningococcal disease in close contacts of infected individuals.

Check Your Understanding

- Identify the symptoms and causes of meningitis.
- Describe the infection by *Neisseria meningitidis* and the resulting development of meningococcemia.

II • Airborne Viral Diseases

Because viruses are so small, they can linger in the tiniest of vapors emitted from an infected individual. Viral diseases transmitted through the air are some of the most contagious infectious diseases known, ranging from the common cold to serious respiratory infections, such as measles and influenza.

31.6 MMR and Varicella-Zoster Infections

The most prevalent and difficult to treat of all human infectious diseases are those caused by viruses. This is because viruses can often remain infectious for long periods in dried mucus (Figure 31.1) or on fomites, and because viruses require host cells for replication. Hence, infectious material can be virtually anywhere, and destroying the virus often means killing the cell as well.

Most viral diseases are acute, self-limiting infections, but some can be problematic in healthy adults. We begin with measles, rubella, mumps, and chicken pox, all common, endemic viral diseases transmitted in infectious droplets by an airborne route.

Measles and Rubella

Measles (*rubeola* or *7-day measles*) affects susceptible children as an acute, highly infectious (◀ Table 30.3), often epidemic disease. The measles virus (**Figure 31.19*a***) is a *paramyxovirus*, a single-stranded, minus-sense RNA virus (◀ Section 11.9) that enters the nose and throat by airborne transmission, quickly leading to a systemic viremia. Symptoms start with nasal discharge and redness of the eyes. As the disease progresses, fever and cough appear and rapidly intensify, followed by a characteristic rash (Figure 31.19*b, c*).

Symptoms of measles generally persist for 7–10 days, and no drugs are available that will eliminate symptoms. However, the measles virus generates a strong immune response. Circulating antibodies to measles virus are measurable within 5 days of infection; these serum antibodies along with cytotoxic T lymphocytes combine to eliminate the virus from the host. Possible measles postinfection complications include middle ear infection, pneumonia, and, in rare cases, measles encephalomyelitis.

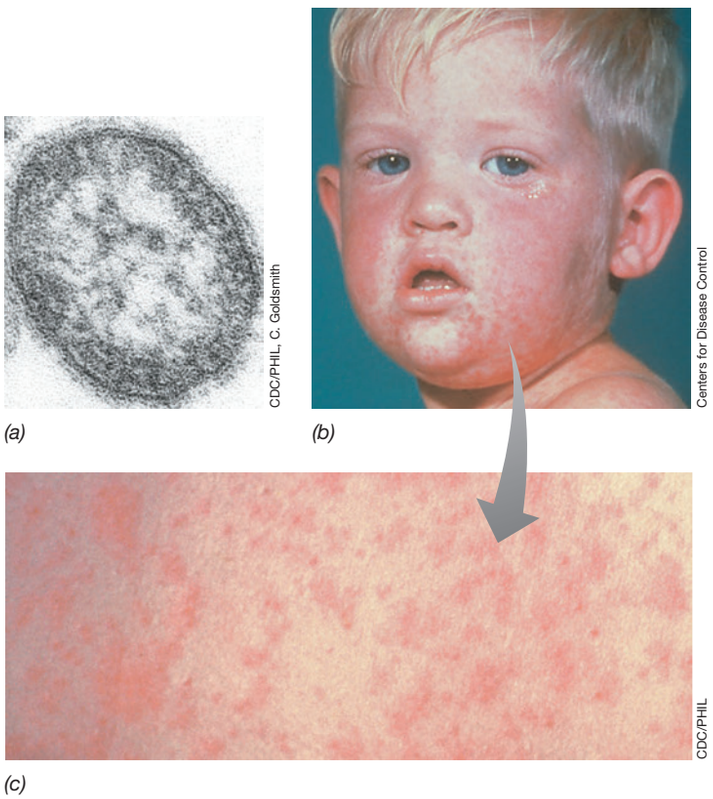


Figure 31.19 Measles in children. *(a)* Transmission electron micrograph of a measles virus virion; a virion is about 150 nm in diameter. *(b, c)* Measles rash. The light pink rash starts on the head and neck, and can spread to the chest, trunk, and limbs. Discrete papules coalesce into blotches as the rash progresses for several days.

Although measles was once a common childhood illness, there has been a dramatic reduction in the incidence of measles globally since the introduction of its vaccine in 1963, with case numbers dropping from above 4 million in 1980 to only 170,000 in 2017. The WHO reports that between 2000 and 2018, the measles vaccine saved 23.2 million lives. However, despite the disease being vaccine-preventable, in the past two years there has been an increase in the global number of cases and deaths from measles. Of the over 360,000 cases of measles reported in 2018, there were 140,000 deaths, most of which were children aged under 5. This number rose again in 2019, with over 870,000 cases reported worldwide. There is also the potential for measles cases to rise due to the SARS-CoV-2 pandemic. As healthcare workers have been dealing with SARS-CoV-2 infections, there has been a decrease in childhood vaccinations globally. For example, over 20 countries suspended measles vaccination campaigns during the pandemic.

Worldwide, measles remains endemic, and despite the existence of a highly effective vaccine, the disease still causes about 100,000 annual deaths, mostly in children. Active immunity to measles is conferred with an attenuated virus preparation as part of the *MMR* (*m*easles, *m*umps, *r*ubella) vaccine (◀ Section 28.3). Because the disease is highly infectious, all public school systems in the United States require proof of measles immunization before a child can enroll. A childhood case of measles generally confers lifelong immunity.

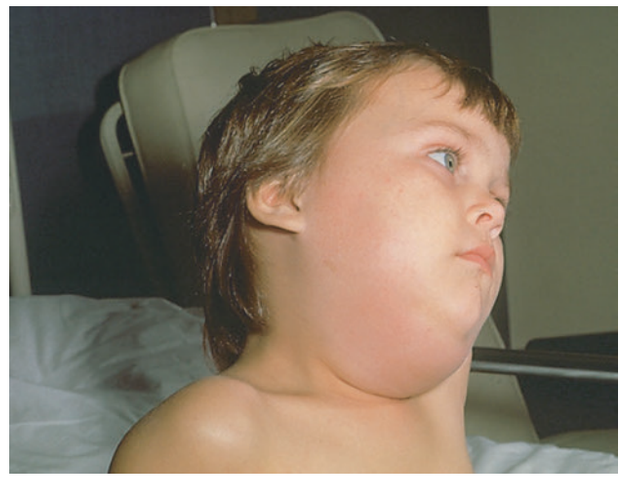
Rubella (sometimes called *German measles* or *3-day measles*) is caused by a single-stranded, positive-sense RNA virus distinct from that of measles (◀ Section 11.8). Symptoms of rubella resemble those of measles (**Figure 31.20**) but are often restricted to just the upper torso. Rubella is less contagious than measles, and thus a significant proportion of the population has never been infected. However, during the first three months of pregnancy, rubella virus can infect the fetus by placental transmission and cause serious fetal abnormalities including stillbirth, deafness, heart and eye defects, and brain damage, events called *congenital rubella syndrome*. Thus, women should not be immunized with the rubella vaccine or contract rubella during pregnancy. Also for this reason, routine childhood immunization against rubella should be practiced. A highly effective attenuated rubella virus is administered as part of the MMR vaccine. As a result of a 15-year-long campaign to eliminate rubella in the Western Hemisphere using this vaccine, the Americas have officially become the first region to be declared rubella-free. Public health officials are in the process of extending the rubella vaccination program worldwide, with the ultimate goal being global eradication of the virus.

Mumps

Mumps, like measles, is caused by a paramyxovirus and is also highly infectious by the airborne route. Mumps is spread by airborne droplets, and the disease is characterized by inflammation of the salivary glands, typically the parotid gland, the largest of the salivary glands, leading to swelling of the jaws and neck (**Figure 31.21**). The virus spreads through the bloodstream and may infect other organs, including the testes and pancreas, and may cause encephalitis in rare cases. As for measles, the immune response rather than drug treatment is what cures a case of mumps. The host immune response produces antibodies to mumps virus surface proteins, and this generally leads to a quick recovery and lifelong immunity to reinfection.

CDC/PHIL

Figure 31.20 Rubella. The rash of rubella (German measles) on the face of a young child.



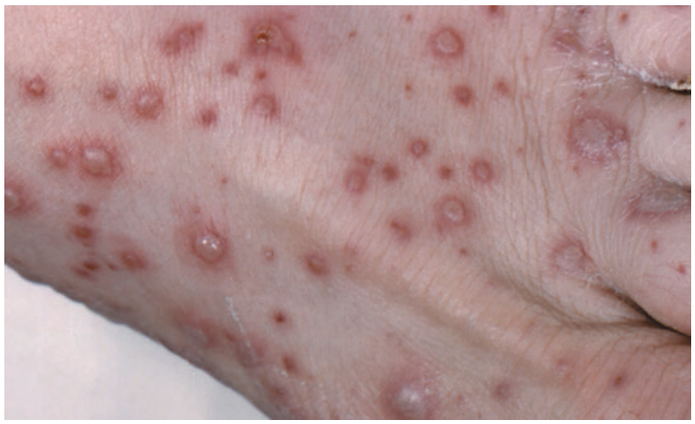
Centers for Disease Control

Figure 31.21 Mumps. Glandular swelling characterizes infection with the mumps virus. Mumps symptoms typically last about one week, and a person is infectious both before and during the symptomatic stages.

An attenuated mumps vaccine (part of the MMR) is highly effective in preventing disease. Hence, the prevalence of mumps in developed countries is low, with disease generally restricted to individuals who did not receive the vaccine. However, outbreaks of mumps in the United States in 2006, 2016, and 2017 each resulted in more than 6000 annual cases. These outbreaks affected mainly young adults (18–34), and as a result, recommendations for immunizations were revised to target school-age children, healthcare workers, and adults who had not previously had mumps.

Chicken Pox and Shingles

Chicken pox (*varicella*) is a common childhood disease caused by the varicella-zoster virus (VZV), a double-stranded DNA herpesvirus (◀ Section 11.7). VZV is a mild but highly contagious disease and is transmitted by infectious droplets, especially when susceptible individuals are in close contact. In school children, for example, close confinement during the winter months leads to the spread of VZV through airborne droplets from infected classmates and through direct contact with chicken pox blisters of other children or contaminated fomites. The virus enters the respiratory tract, multiplies, and is quickly disseminated via the bloodstream, resulting in a systemic papular rash (**Figure 31.22**) that heals quickly without scarring. A live-attenuated varicella vaccine was first developed in Japan in the early 1970s, and there are currently several live-attenuated varicella vaccines available, including both a monovalent vaccine and a combined vaccine that protects against measles, mumps, rubella, and varicella. The United States was the first country to introduce the varicella vaccine into its national vaccination program. Currently, over 36 countries worldwide have



CDC/PHIL, A.D. Langmuir

Figure 31.22 Chicken pox. Papular rash on the foot of an adult. The papules are due to infection by varicella-zoster virus, the herpesvirus that causes chicken pox.

TABLE 31.1 Colds and influenza

Symptoms	Cold	Influenza
Fever	Rare	Common (39–40°C); sudden onset
Headache	Rare	Common
General malaise	Slight	Common; often quite severe; can last several weeks
Nasal discharge	Common and abundant	Less common; usually not abundant
Sore throat	Common	Less common
Vomiting and/or diarrhea	Rare	Common in children
Incidence[a]	340	50

[a]Cases/100 people per year in the United States for recent years. Incidence of all other infectious diseases totals about 30 cases/100 people per year.

Mastering Microbiology
Art Activity: Table 31.1 Colds and influenza

introduced a varicella vaccine into their national vaccination schedule.

Globally, approximately 95% of individuals aged 50 and over have been infected with VZV at some point in their life. VZV establishes a lifelong latent (permanent) infection in nerve cells. The virus can remain dormant there indefinitely, but in some individuals the virus migrates from this reservoir to the skin surface, often years or decades later, causing a painful skin eruption called *shingles* (*zoster*). Shingles most commonly strikes immunosuppressed individuals or the elderly, causing severe blisters and a rash on the head, neck, or upper torso. A number of shingles vaccines are available worldwide. A moderately effective shingles vaccine containing concentrated attenuated virus (marketed as Zostavax®) is available for individuals over 50 years of age, and it is part of the national immunization schedule in the United Kingdom and Australia. The vaccine stimulates antibody- and cell-mediated immunity to VZV, which keeps VZV from migrating out of nerve ganglia to skin cells and triggering shingles symptoms. A recombinant shingles vaccine marketed as Shingrix® is reported to be nearly 90% effective in preventing a case of shingles (if administered in both primary and booster injections) and is the shingles vaccine recommended by the Advisory Committee on Immunization Practices of the United States Centers for Disease Control and Prevention.

Check Your Understanding

- How do the genomes of the measles virus (rubeola) and the German measles virus (rubella) differ?
- Describe the potential serious outcomes of infection by measles, mumps, rubella, and VZV viruses.
- Identify the effects of immunization on the incidence of measles, mumps, rubella, and chicken pox.

31.7 The Common Cold

Colds are the most common of infectious diseases. Colds are typically upper respiratory tract viral infections that are transmitted via droplets spread from coughs, sneezes, and respiratory secretions. Colds are usually of short duration, lasting a week or so, and the symptoms are milder than other respiratory diseases such as influenza. Table 31.1 contrasts the usually distinct symptoms and incidence of colds and influenza.

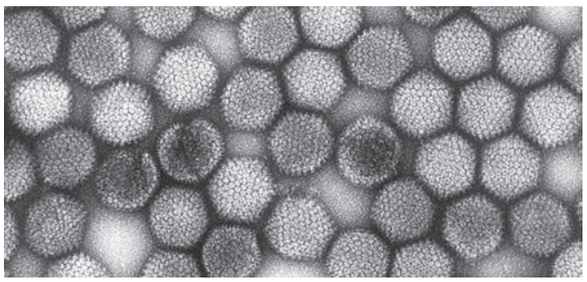
Symptoms and Transmission of the Common Cold

Cold symptoms include *rhinitis* (inflammation of the nasal region, especially the mucous membranes), nasal obstruction, watery nasal discharges, muscle aches, and a general feeling of malaise, usually without fever. *Rhinoviruses* are single-stranded plus-sense RNA viruses of the picornavirus group (◀ Section 11.8) and are the most common causes of colds. Over 150 different rhinoviruses have been identified. About one-quarter of all colds are due to infections with other viruses. These include in particular the *adenoviruses* (Figure 31.23*a*) and *coronaviruses* (Figure 31.23*b*). Coxsackie viruses, respiratory syncytial viruses (RSV), and orthomyxoviruses are collectively responsible for only a small percentage of common colds.

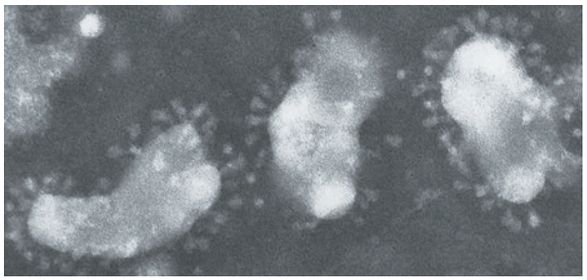
Aerosol transmission is a major means of spreading colds, although experiments with volunteers suggest that direct contact and indirect contact involving fomites are also important means of transmission, perhaps even more important than aerosols. Incidence of the common cold rises when people are indoors in the winter months, although it is possible to "catch a cold" at any time of year. Most antiviral drugs are ineffective against common cold viruses, although some have shown promise for preventing the onset of symptoms following rhinovirus exposure. Moreover, new antiviral drugs are being designed based on knowledge of the three-dimensional structure of cold viruses. For example, antirhinovirus drugs that bind to the virus and change its surface properties in such a way as to prevent it from attaching to host cells have been developed. But thus far, most "cold drugs" on the market simply help to reduce the severity of symptoms—primarily the cough, nasal discharges, congestion, and headache.

Treatment

Because colds are generally self-limiting and not serious diseases, treatment is aimed at controlling symptoms, especially nasal discharges, with antihistamine and decongestant drugs. A plethora of such drugs are available without prescription, each touting its superior features. Many of these help control cold symptoms fairly well, but some have unwanted side effects, especially drowsiness. The severity of symptoms of any cold event is a function of the virulence



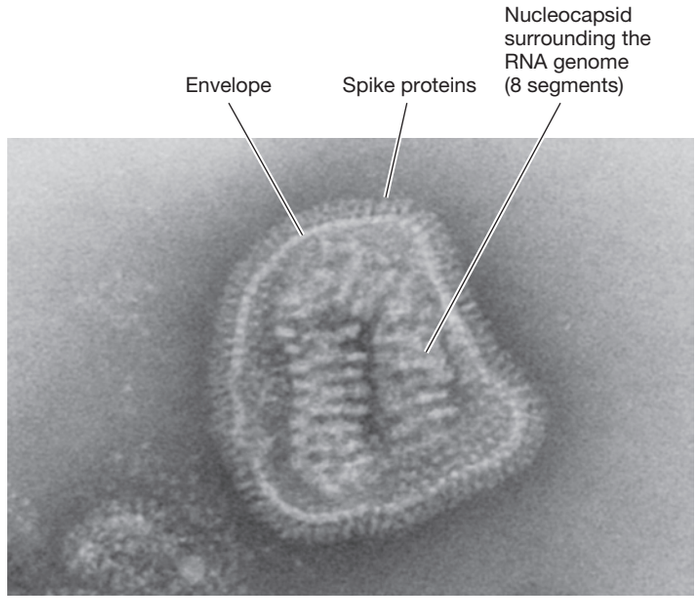
(a)



(b)

Heather Davies and D. Tyrell

Figure 31.23 Transmission electron micrographs of common cold viruses. *(a)* Human adenovirus; a virion is about 90 nm in diameter. *(b)* Human coronavirus; a virion is about 60 nm in diameter.



CDC/PHIL, E.L. Palmer, M.L. Martin, and F. Murphy

Figure 31.24 Influenza A virus. The virus contains a single-stranded, negative-sense RNA genome in eight segments; a virion is about 100 nm in diameter. Major factors in the success of influenza virus as a pathogen are antigenic drift and antigenic shift (see Figure 31.26).

of the cold virus, the overall health and well-being of the person at the time of infection, and the nature of supportive factors during infection. In the long run, immunity is more important in ridding the body of a cold virus than anything drugs can achieve. Cold viruses induce an antibody-mediated immune response that targets the current cold virus. However, the number of immunologically unique strains of each type of cold virus makes long-term immunity to the common cold unobtainable.

On average, a person in the United States contracts about three colds per year compared with less than one case of influenza per person per year (Table 31.1). This is an indication of the ease of transmission of the common cold and the large number of different viruses that cause the same general symptoms. Thus, the common cold is a recurrent event that humans must simply live with. Preventive measures such as avoiding contact with major common cold fomites (door handles and other surfaces touched by others) and with one's inner nostrils, coupled with frequent hand washing, are best practices for avoiding the common cold.

Check Your Understanding

- Define the cause and symptoms of common colds.
- Discuss the possibilities for effective treatment and prevention of colds.

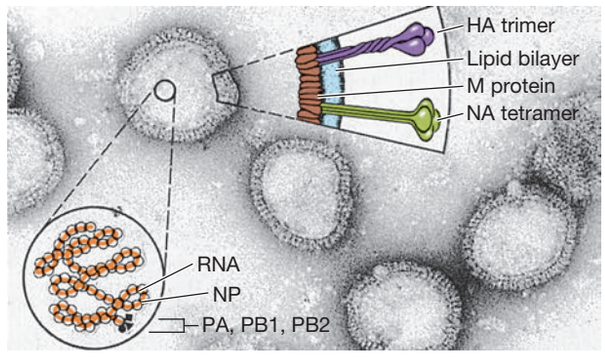
31.8 Influenza

Influenza is a highly infectious airborne disease of viral origin. Influenza viruses contain a single-stranded, negative-sense, segmented RNA genome surrounded by an envelope composed of protein, a lipid bilayer, and external glycoproteins (**Figure 31.24**; ◀ Section 11.9). There are three classes of influenza viruses: influenza A, B, and C. Here we consider only influenza A because it is the most important human pathogen.

Antigenic Drift and Antigenic Shift

Each strain of influenza A virus can be identified by a unique set of surface glycoproteins. These glycoproteins are *hemagglutinin* (HA or the "H antigen") and *neuraminidase* (NA or the "N antigen"). Each virus has one type of HA and one type of NA on its viral capsid and is named for the antigens it contains; for example, "H1N1." The HA antigen is important in *attaching* the influenza virus to host cells, while the NA antigen is instrumental in *releasing* the virus from host cells; each antigen is composed of several individual proteins (**Figure 31.25**).

Infection or immunization with influenza virus results in the production of antibodies that react with the HA and NA glycoproteins.



Irene T. Schulze

Figure 31.25 Influenza virus structure. Major viral coat proteins are: HA, hemagglutinin (three copies make up the HA coat spike); NA, neuraminidase (four copies make up the NA coat spike); M, coat protein; NP, nucleoprotein; PA, PB1, PB2, and other internal proteins, some of which have enzymatic functions.

When these antibodies bind to HA or NA, the virus is blocked from either attaching or releasing and is effectively neutralized, stopping the infection process. However, over time, the viral genes encoding the HA and NA glycoprotein antigens mutate, rendering minor changes to their amino acid sequence and hence antigenic structure. Mutations that alter as few as one amino acid in the glycoprotein can affect how an antibody binds to these antigens. This slight variation in the structure of influenza viral surface antigens is at the heart of a phenomenon in influenza biology called **antigenic drift**. As a result of these subtle yet important changes, host immunity to a given virus strain diminishes as the strain mutates, and reinfection with the mutated strain can occur. This phenomenon is why last year's influenza vaccine may work only poorly against this year's crop of influenza viruses (**Figure 31.26*a***).

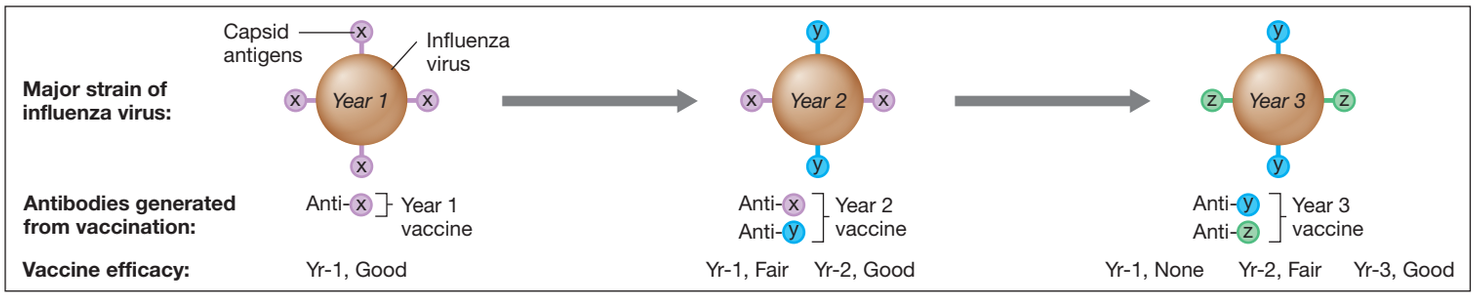
In addition to antigenic drift, there is a second feature of influenza virus biology that aids virulence. The single-stranded RNA genome of influenza viruses is *segmented*, with genes found on each of eight distinct segments (◀ Figure 11.24*b*). During virus maturation in the host cell, the viral RNA segments are packaged randomly. To be infective, a virus must be packaged so it contains one copy of each of the eight gene segments. Occasionally, however, more than one strain of influenza virus infects a single animal at one time. In such cases, if the two strains infect the same cell, both viral genomes are replicated; when genome packaging occurs, the segments from the two strains may intermix. The result is a genetically unique virus that is now a *new virus strain*. This mixing of gene fragments between different strains of influenza virus is called *reassortment*.

Unique *reassortant viruses* trigger the phenomenon known as **antigenic shift** (Figure 31.26*b*), a major change in a surface antigen resulting from the total replacement of the RNA that encoded it. Antigenic shift can immediately and completely change one or both of the major HA and NA viral glycoproteins in a major way. As a result, reassortant viruses are essentially unrecognized by immune responses to previous influenza infections. Reassortant viruses also frequently display one or more unique virulence properties that help to trigger unusually strong clinical symptoms and are the usual catalysts of influenza *pandemics*, which we consider shortly.

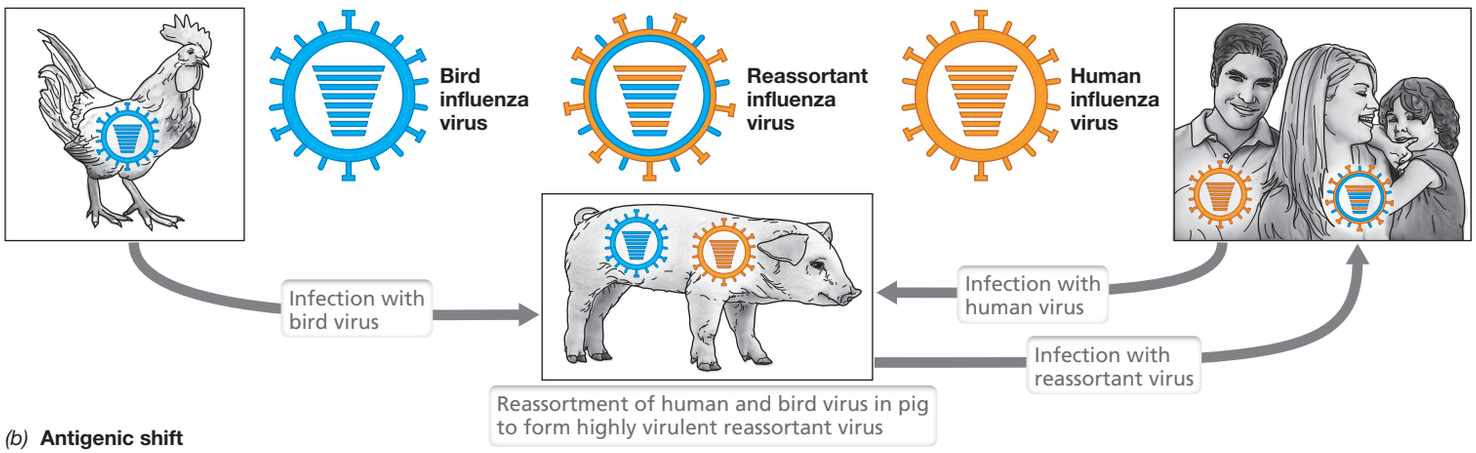
Mastering Microbiology
Art Activity: Figure 31.26 Antigenic drift and antigenic shift in influenza virus biology

Symptoms and Treatment of Influenza

Human influenza virus is transmitted from person to person through the air, primarily in droplets expelled during coughing and sneezing (Figure 31.1). The virus infects the mucous membranes of the upper respiratory tract and occasionally invades the lungs. Symptoms include a low-grade fever lasting up to a week, chills, fatigue, head and muscle aches, a cough and/or a sore throat, and general malaise (Table 31.1). Most of the serious consequences of seasonal influenza occur not from



(a) **Antigenic drift**



(b) **Antigenic shift**

Figure 31.26 Antigenic drift and antigenic shift in influenza virus biology. *(a)* Antigenic drift. A new vaccine is prepared each year against the major strain of influenza circulating among the population. However, vaccine efficacy wanes with time as immunologically new surface antigens appear from mutations in genes encoding viral surface proteins. *(b)* Antigenic shift. Influenza strains that originate in birds and humans can also infect certain other animals, such as swine, and may cause a reassortment of viral genetic material. For example, if a pig becomes infected with both bird and human viruses simultaneously, the viral genomes can be mixed, forming reassortant viruses. If such viruses, which now contain several unique antigens, infect humans, influenza pandemics can be triggered because of an ineffective immune response (◀ Section 30.8).

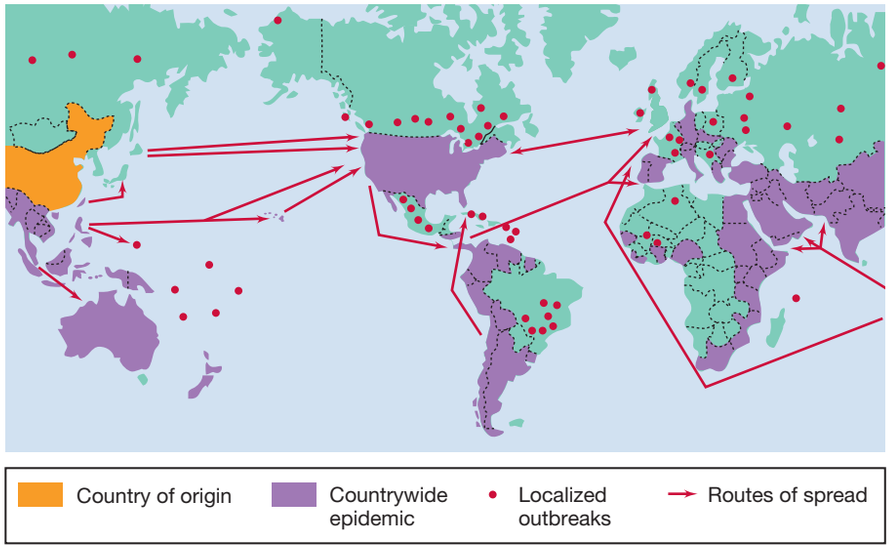


Figure 31.27 An influenza pandemic. Map of the Asian influenza pandemic of 1957. Lax agricultural practices with poultry and swine coupled with human interactions with these animals allowed the reassortment of influenza viral genomes from the three host species, producing a new strain for which there was no immune memory in humans. See Section 30.8 for more coverage of influenza pandemics.

the disease itself but from bacterial secondary infections, especially in persons whose resistance has been lowered by the influenza infection. For example, in infants and the elderly, influenza can be followed by bacterial pneumonia (Section 31.2), sometimes in fatal form.

Most individuals develop protective immunity to the infecting strain of influenza virus, making it impossible for that strain or a very closely related strain to cause widespread infection (an epidemic) until the virus encounters another susceptible population. Immunity occurs from both antibody- and cell-mediated immune responses directed at HA and NA glycoproteins. Influenza epidemics can be controlled by immunization. Developing an effective vaccine, however, is complicated by the large number of existing influenza viral strains resulting from antigenic drift and antigenic shift (Figure 31.26). Through careful worldwide surveillance, samples of the major emerging strains of influenza virus are obtained each year before the onset of seasonal epidemics and used to prepare that year's vaccine. In most years, this approach confers adequate protective immunity, but sometimes the vaccine is only marginally effective. For example, those who received the vaccine during the 2017–18 flu season were only 36% less likely to contract influenza than those who received no vaccine. There is evidence, however, that the annual vaccine does lessen the severity of symptoms and shorten the duration of disease in those who do become ill.

Most human influenza viruses respond to antiviral drugs. The adamantanes—*amantadine* and *rimantadine*—are synthetic amines that inhibit viral replication, and the neuraminidase inhibitors *oseltamivir* (Tamiflu®) and *zanamivir* (Relenza®) (◀ Table 28.5) block release of newly replicated human influenza virions. These drugs are often used early on to shorten the course and severity of infection, especially in the elderly and immunocompromised.

Influenza Pandemics

Influenza pandemics—worldwide epidemics—are much less frequent than outbreaks and epidemics, occurring from 10 to 40 years apart (◀ Section 30.8). Flu pandemics result from antigenic shift, and virtually all have been due to avian and human influenza viruses reassorting in swine (Figure 31.26*b*) because swine can propagate both avian and human influenza viruses. This results in a highly virulent influenza strain for which there is no preexisting immunity in humans.

The "Spanish flu" pandemic of 1918 was the most catastrophic in recorded history, and the extreme virulence of the 1918 H1N1 virus is thought to have triggered host production and release of unusually large amounts of inflammatory substances, resulting in systemic inflammation and more severe symptoms than those typical of yearly flu epidemics. The 1957 Asian flu was also a significant pandemic (**Figure 31.27**), beginning in China and spreading to the United States and shortly thereafter to Europe and South America. In this case, the pandemic influenza strain was a highly virulent H2N2 virus, differing antigenically from all previous strains. Pandemic influenza A (H1N1) 2009 virus, nicknamed the "swine flu," spread much more rapidly in 2009 than even the 1957 Asian flu, starting in Mexico and spreading quickly to the United States, Europe, and Central and South America. H1N1 was a classic case of influenza virus genome reassortment in swine (Figure 31.26*b*), and from the swine reservoir, a highly virulent virus emerged to infect humans. Influenza A H5N1, nicknamed the "bird flu," emerged in Hong Kong in 1997, and is the major virus health officials are monitoring closely today. H5N1 has been detected in birds in many countries, and if this virus were to infect swine and form an easily transmissible reassortant virus that subsequently jumps to humans (Figure 31.26*b*), such a virus could initiate a very deadly influenza pandemic.

Check Your Understanding

- Distinguish between antigenic drift and antigenic shift in influenza.
- Discuss the possibilities for effective immunization programs for influenza and compare them to the possibilities for immunization for colds.

III • Direct-Contact Bacterial and Viral Diseases

Several infectious diseases are spread from person to person or from fomites or animals to people through direct nonsexual contact. These diseases range from local infections, such as those caused by *Staphylococcus aureus*, to serious systemic infections associated with high mortality, such as that caused by Ebola virus.

Some pathogens are spread primarily by direct contact with an infected person or by direct contact with blood or excretions from an infected person. Many of the respiratory diseases we have just discussed can also be spread by direct contact, but here we consider diseases spread primarily through direct contact with infected

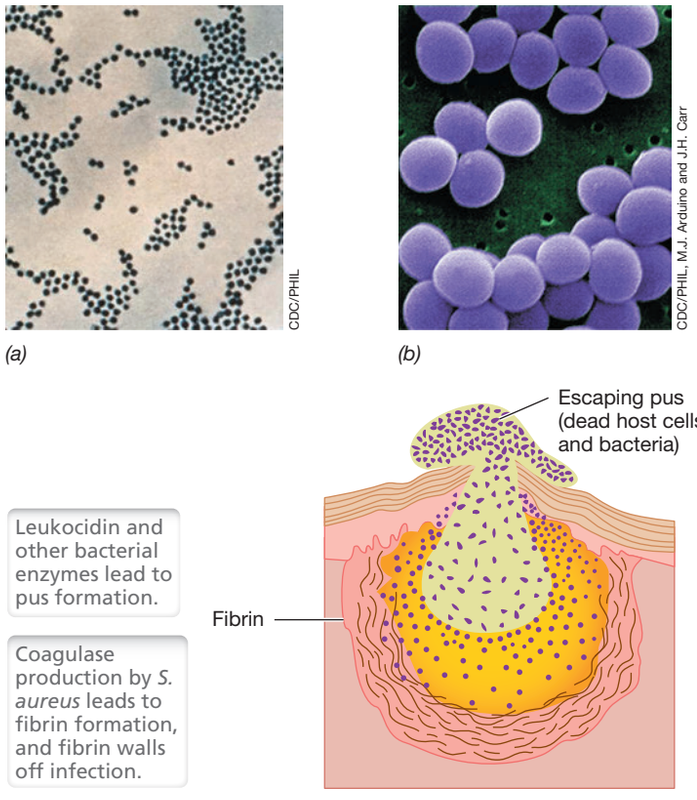


Figure 31.28 ***Staphylococcus aureus* and *S. aureus* infections.** Cells divide in several planes, giving the appearance of a cluster of grapes. *(a)* Gram stain; an individual coccus is about 1 μm in diameter. *(b)* Scanning electron micrograph of cells. *(c)* Structure of a boil. Staphylococci initiate a localized skin infection and become walled off by coagulated blood and fibrin through the activity of the enzyme coagulase, a major virulence factor. The ruptured boil releases pus, consisting of dead host cells and bacteria. See also Figure 31.29.

individuals rather than by an airborne route. These include staphylococcal infections, gastric ulcers, certain types of hepatitis, and the highly dangerous Ebola hemorrhagic fever.

31.9 *Staphylococcus aureus* Infections

The genus *Staphylococcus* contains pathogens of humans and other animals. Staphylococci commonly infect skin and wounds and may also cause pneumonia. Most staphylococcal infections result from the transfer of staphylococci in the normal microbiota of an infected, asymptomatic individual to a susceptible individual. Others result from toxemia following the ingestion of contaminated food ("staph food poisoning," ▶ Section 33.8).

Staphylococci are nonsporulating, gram-positive cocci about 0.5–1.5 μm in diameter that divide in multiple planes to form irregular clusters of cells (**Figure 31.28*a, b***). They are resistant to drying and tolerate high concentrations of salt (up to 10% NaCl) when grown on artificial media. Staphylococci are readily dispersed in dust particles through the air and on surfaces. In humans, two species are important: *Staphylococcus epidermidis*, a nonpigmented species usually found on the skin or mucous membranes, and *Staphylococcus aureus*, a yellow-pigmented species. Both species are potential pathogens, but *S. aureus* is more commonly associated with human disease. Both species are frequently present in the normal microbiota of the upper respiratory tract and the skin (Figure 31.2 and see Figure 31.30*b*), making many people potential carriers (◀ Sections 30.1 and 30.3).

Epidemiology and Pathogenesis

Staphylococcal diseases include acne, boils, pimples, impetigo, pneumonia, osteomyelitis, carditis, meningitis, and arthritis. Many of these diseases are *pyogenic* (pus-forming; Figure 31.28*c* and **Figure 31.29**). Those strains of *S. aureus* that cause human disease produce a variety of potent virulence factors (◀ Section 25.3). At least four different *hemolysins* (proteins that lyse red blood cells, see Figure 31.8) have been recognized, and a single strain often produces several. A key virulence factor produced by *S. aureus* is *coagulase*, an enzyme that converts the soluble blood glycoprotein fibrinogen to an insoluble protein, fibrin, forming a localized clot (◀ Figure 25.12*b*). Clotting induced by coagulase results in the accumulation of fibrin around the bacterial cells, making it difficult for host immune cells to contact the bacteria and initiate phagocytosis (◀ Section 26.7). Most *S. aureus* strains also produce *leukocidin*, a protein that destroys white blood cells. Production of leukocidin

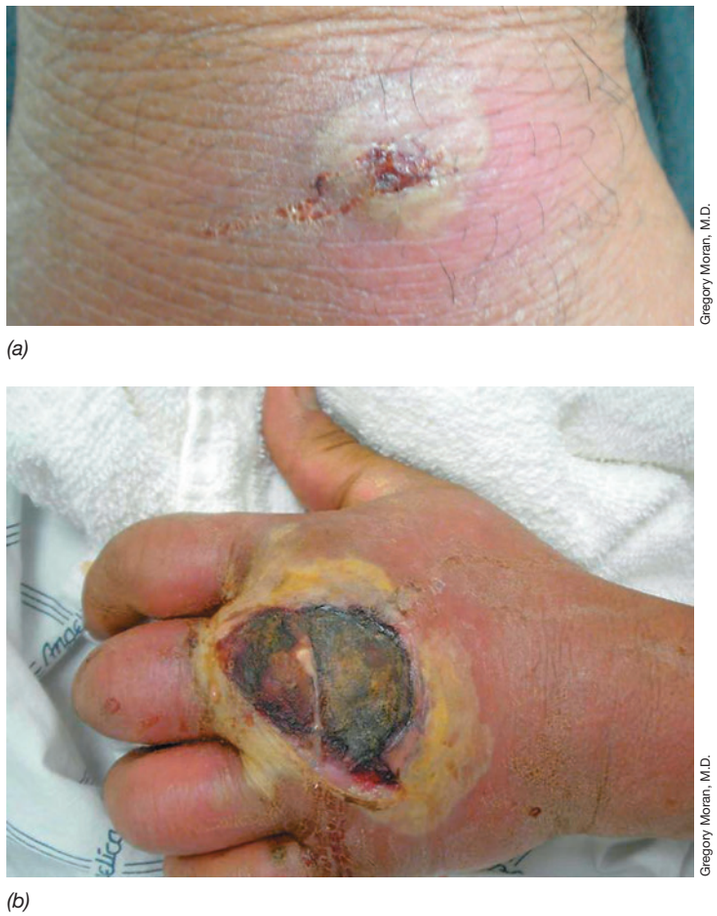


Figure 31.29 **Pus-forming staphylococcal wounds.** *(a)* A typical pus-forming wound on the hand. Pus lies just under the epidermal layer. *(b)* Abscess on the hand caused by a methicillin-resistant strain of *Staphylococcus aureus* (MRSA strain). If no treatment for a pus-forming wound is sought or if penicillin is administered first, MRSA infections can cause extensive tissue destruction, as shown here.

in skin lesions such as boils and pimples results in host cell destruction and is one of the factors responsible for pus accumulation (Figures 31.28 and 31.29). Some strains of *S. aureus* also produce other virulence proteins, including *hyaluronidase, fibrinolysin, lipase, ribonuclease*, and *deoxyribonuclease*.

Staphylococcus aureus Toxic Shock

S. aureus can also cause **toxic shock syndrome (TSS)**, a serious outcome of certain staphylococcal infections characterized by high fever, rash, vomiting, diarrhea, and in some cases, death. TSS was first recognized in women and was associated with the use of highly absorbent tampons, where conditions in the vagina promoted growth of toxic shock strains of *S. aureus*. However, today, TSS is seen in both men and women and is typically triggered by staphylococcal infections following surgery.

The symptoms of TSS result from an exotoxin (◀ Section 25.6) called *toxic shock syndrome toxin-1*. This very potent exotoxin is a superantigen (◀ Sections 25.7 and 28.2) that is released during cell growth and recruits large numbers of T cells to the site of infection. These then cause a major inflammatory response that is fatal in about 70% of cases. TSS can also result from superantigen exotoxins secreted by other pathogens, including *Streptococcus pyogenes*, the causative agent of several streptococcal syndromes (Section 31.2).

Diagnosis and Treatment and the MRSA Epidemic

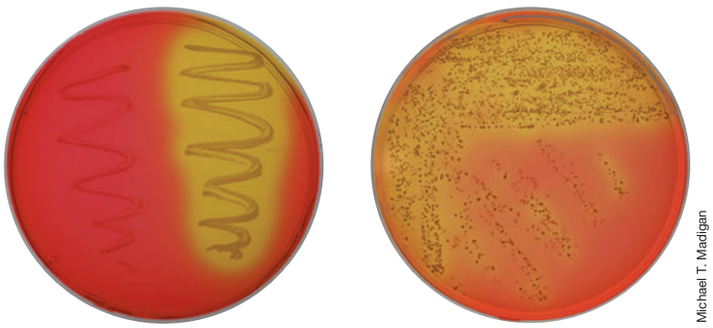
To diagnose an *S. aureus* infection through laboratory culture, a specimen, typically from a pus-forming wound (Figure 31.29*a*), is cultured on a selective and differential medium containing 7.5% NaCl, mannitol, and phenol red, a pH indicator (mannitol–salt agar, **Figure 31.30**). The salt inhibits the growth of nonhalotolerant bacteria while allowing the halotolerant (◀ Section 4.15) staphylococci to grow. In addition, because *S. aureus* ferments mannitol, it generates acidity that changes the medium from red to yellow; other staphylococci, such as *S. epidermidis*, do not (Figure 31.30).

In clinical laboratories, the polymerase chain reaction (PCR, ◀ Section 12.1) is used to amplify genes unique to *S. aureus* from DNA isolated directly from a clinical sample (◀ Section 29.8), and this allows for a quicker diagnosis; results from a laboratory culture take 24 h. For specific identification of methicillin-resistant strains of *S. aureus* (MRSA), a special selective and differential medium is available (see Explore the Microbial World, "MRSA—A Formidable Clinical Challenge" in Chapter 29), as well as a PCR protocol that identifies *mecA* (the gene that encodes methicillin resistance in MRSA strains) and a rapid immunological test where cells of *S. aureus* in suspension are agglutinated by antibodies to specific cell surface proteins (◀ Figure 29.16).

Historically, *S. aureus* infections have been treated with various penicillin and cephalosporin antibiotics. However, extensive use of these antibiotics for many years has selected for resistant strains that now predominate, especially in the clinical environment. Surgical patients, for example, may acquire staphylococci from healthcare personnel who are asymptomatic carriers of drug-resistant strains. As a result, appropriate antimicrobial drug therapy for *S. aureus* infections is a major problem in healthcare environments. The antibiotics clindamycin and various tetracycline drugs are currently used to treat MRSA infections.

MRSA infections (Figure 31.29*b*) are becoming more common. Over 80,000 cases of MRSA are reported each year in the United States, but infections are actually probably closer to ten times this number. Many of these cases are hospital-acquired (nosocomial) MRSA (◀ Section 29.2), but many others are not. Because of the potential severity of MRSA infections, it is important to rapidly identify these strains in clinical specimens so that an effective treatment is begun as soon as possible. Delayed treatment of a MRSA infection, whether due to hesitation to seek treatment or treatment with an ineffective antibiotic, can lead to extensive tissue damage (Figure 31.29*b*).

Prevention of staphylococcal infections is virtually impossible because many people are asymptomatic carriers of *S. aureus*, either on their skin or in their upper respiratory tract. However, identification and treatment of MRSA-carrying healthcare providers who serve in surgical or nursery units has helped limit transmission of these very aggressive strains. As is true of many direct-contact diseases, MRSA transmission can also be greatly diminished by practicing good basic hygiene, avoiding contact with the personal items (including clothing and towels) of others, keeping wounds covered, and for those in the healthcare profession, following the established practices of clinical microbiology safety (◀ Section 29.1).



(a) (b)

Figure 31.30 Mannitol–salt agar in the isolation of staphylococci. *(a)* Mannitol–salt agar (MSA) is both selective and differential for staphylococci. The presence of 7.5% NaCl makes MSA selective and phenol red makes it differential. Left, *Staphylococcus epidermidis*; right, *Staphylococcus aureus*. *(b)* A nasal swab of the senior author of this text supports the observation that most humans are carriers of *S. aureus*.

Check Your Understanding

- What is the normal habitat of *Staphylococcus aureus*? How does *S. aureus* spread from person to person?
- What is MRSA, and why is it a health problem?

31.10 *Helicobacter pylori* and Gastric Diseases

Helicobacter pylori is a gram-negative, highly motile and spiral-shaped bacterium (**Figure 31.31**) associated with gastritis, ulcers, and gastric cancers. This bacterium colonizes the non-acid-secreting mucosa of the stomach and the upper intestinal tract. It is estimated that half the world's population is chronically infected with *H. pylori*. Up to 80% of gastric ulcer patients have concomitant *H. pylori* infections, and up to 50% of asymptomatic adults in developing countries are

Figure 31.31 ***Helicobacter pylori.*** Colorized scanning electron micrograph of cells attached to the mucous lining of the stomach. Cells range in length from 3 to 5 μm and are about 0.5 μm in diameter. Note the flagella.

chronically infected. Although there is no known nonhuman reservoir of *H. pylori*, infection occurs at high incidence within families, suggesting person-to-person transmission. *H. pylori* infections also occasionally occur in clusters, suggesting that transmission from common sources such as food or water is also possible.

The *Helicobacter* Infection Process

H. pylori is only slightly invasive and colonizes the surfaces of the gastric mucosa, where it is protected from the effects of stomach acids by the gastric mucus layer. Cells of *H. pylori* reach these relatively protected regions by employing a chemoreceptor (◀ Sections 2.11 and 7.6) that tracks the gradient of urea produced by gut epithelia to direct flagellar rotation up the gradient. The organism is strongly ureolytic and cleaves the urea into ammonia and bicarbonate, which help buffer the region of cell colonization from stomach acids (ammonia is a strong base).

After *H. pylori* cells colonize the mucosa, a combination of virulence factors and host responses cause inflammation, tissue destruction, and ulceration. Pathogen products such as the cytotoxin VacA (an exotoxin), urease, and an autoimmune response triggered by *H. pylori* lipopolysaccharide all contribute to localized tissue destruction and ulceration. Individuals who acquire *H. pylori* tend to have chronic infections unless they are treated with antibiotics. Treatment is both straightforward and important, as chronic inflammation of the gastroduodenum (gastritis) due to untreated *H. pylori* infection may lead to the development of gastric cancers.

H. pylori and Clinical Disease

Clinical signs of *H. pylori* infection include belching and stomach (epigastric) pain. Definitive diagnosis requires the isolation or observation of *H. pylori* from a gastric ulcer biopsy. However, a simple diagnostic test for the *H. pylori* enzyme urease is used for a noninvasive diagnosis. In this test, a small amount of ^{13}C- or ^{14}C-labeled urea ($H_2N{-}CO{-}NH_2$) is ingested; if *H. pylori* is present, the bacterium will hydrolyze the urea, forming labeled CO_2 and ammonia. Hence, the presence of labeled CO_2 in the patient's breath is highly suggestive of *H. pylori* infection.

The best evidence for a causal association between *H. pylori* and gastric ulcers comes from antibiotic treatments for the disease. Long-term treatment with antacids helps alleviate gastric ulcer symptoms temporarily, but most patients relapse within 1 year. However, by treating the *cause* rather than the *effect* of the disease, actual cures can be obtained. *H. pylori* infection is typically treated with a combination of drugs, including the antibacterial compound metronidazole, an antibiotic such as tetracycline or amoxicillin, and a bismuth-containing antacid preparation. The combination treatment, administered for 14 days, abolishes the *H. pylori* infection and provides a true cure.

Like the link with gastric ulcers, the link between *H. pylori* infection and certain forms of gastric cancers, in particular, gastric adenocarcinoma (the most prevalent form of gastric cancer), is also strong. Gastric cancers are the second leading cause of cancer deaths worldwide. Although how *H. pylori* infection actually triggers adenocarcinomas is unclear, it is believed that long-term inflammation caused by infection with this bacterium coupled with host and possibly environmental factors combine to predispose an individual to stomach malignancies. For their contributions to unraveling the connection between *H. pylori* and peptic and duodenal ulcers, the Australian scientists Robin Warren and Barry Marshall were awarded the 2005 Nobel Prize in Physiology or Medicine.

Check Your Understanding

- Describe infection by *Helicobacter pylori* and the resulting development of a gastric ulcer.
- How can gastric ulcers be diagnosed? How can they be cured?

31.11 Hepatitis

Hepatitis is a liver inflammation, commonly caused by an infectious agent. Hepatitis sometimes results in acute illness followed by destruction of functional liver anatomy and cells, a condition known as **cirrhosis**. Hepatitis due to infection can cause chronic or acute disease, and some forms lead to liver cancer.

Although many viruses and a few bacteria can cause hepatitis, a restricted group of viruses is often associated with liver disease. Hepatitis viruses A, B, C, D, and E are phylogenetically diverse viruses (**Table 31.2**) but share in common their ability to infect the liver, with hepatitis viruses A, B, and C accounting for the majority of cases worldwide. The incidence of hepatitis A and B has decreased significantly over the past 25 years, primarily because of the availability of effective vaccines and increases in surveillance (**Figure 31.32**). By comparison, with no vaccine available, hepatitis C infections have gradually risen in recent years, although those infected can be cured of infection with HCV-specific drugs.

Mastering Microbiology
Art Activity: Figure 31.32 Hepatitis A, B, and C in the United States

Hepatitis A and E viruses, although occasionally transmitted person to person, are more commonly transmitted enterically by contaminated food or water. We cover hepatitis A viral disease in Chapter 33. Here our focus is on hepatitis viruses transmitted by direct contact, with the major focus on hepatitis B, the causative agent of "bloodborne hepatitis."

TABLE 31.2 Hepatitis viruses

Disease	Virus and genome[a]	Vaccine	Clinical illness	Transmission route
Hepatitis A	*Hepatovirus* (HAV) ssRNA	Yes	Acute	Enteric (food)
Hepatitis B	*Orthohepadnavirus* (HBV) dsDNA	Yes	Acute, chronic, oncogenic	Parenteral, sexual
Hepatitis C	*Hepacivirus* (HCV) ssRNA	No	Chronic, oncogenic	Parenteral
Hepatitis D	*Deltavirus* (HDV) ssRNA	No	Fulminant, only with HBV	Parenteral
Hepatitis E	*Caliciviridae* family (HEV) ssRNA	No	Fulminant disease in pregnant women	Enteric (water)

[a]Examples and discussion of each of these genomes can be found in Chapter 11 (◀ Figures 11.2 and 11.3).

Epidemiology

Infection with *hepatitis B virus* (HBV) is called bloodborne hepatitis (or serum hepatitis) because it is transmitted in blood or in body fluids in contact with blood. HBV is a hepadnavirus, a partially double-stranded DNA virus (◀ Section 11.11). The mature virus particle containing the viral genome is called a *Dane particle* (**Figure 31.33**). HBV causes acute, often severe disease that can lead to liver failure and death. Chronic HBV infection can lead to cirrhosis and liver cancer.

HBV is transmitted by a *parenteral route*, which means "outside the gut." The main means of HBV transmission is from blood transfusions, contact with infected blood in a hypodermic needle, and from mother to child during childbirth. HBV may also be transmitted through exchanges of body fluids during sex. The number of new HBV infections has remained low and more or less constant since the year 2000 (Figure 31.32). Nevertheless, over 100,000 people worldwide and nearly 5000 people in the United States die yearly from liver failure or liver cancer caused by chronic HBV infection.

Hepatitis D virus (HDV) is a *defective virus* (◀ Section 9.7) that lacks genes encoding its own capsid. HDV is also transmitted by parenteral routes, but because it is a defective virus, it cannot replicate and form an intact virion unless the cell is also infected with HBV. The HDV genome replicates independently but relies on HBV to produce capsid proteins (which are the same as those used by HBV) to form infectious virions. Thus, HDV infections are always coinfections with HBV, and therefore the HBV vaccine also indirectly protects against HDV.

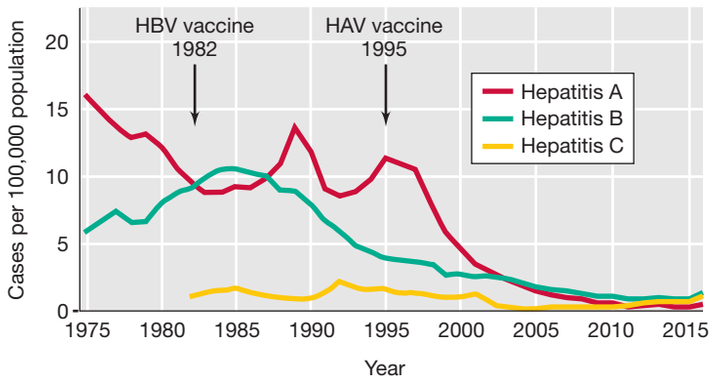


Figure 31.32 Hepatitis A, B, and C in the United States. In 2016 there were 2007 reported cases of hepatitis A, 3218 reported cases of hepatitis B, and 2967 reported cases of hepatitis C. The number of actual new cases of hepatitis A, B, or C infection is likely to be much higher than the reported new cases. Data obtained from the CDC, Atlanta, Georgia, USA.

Hepatitis C virus (HCV) is also transmitted parenterally, most commonly by sharing of needles and syringes for injection of illicit drugs. HCV generally produces a mild or even asymptomatic disease at first, but later up to 85% of those infected develop chronic hepatitis, with up to 20% proceeding to chronic liver disease and cirrhosis. Chronic infection with HCV leads to hepatocarcinoma (liver cancer) in 3–5% of infected individuals. The latency period for development of cancer can be several decades after the primary infection. Only a fraction of the estimated 25,000 annual new infections with HCV in the United States are recognized and formally reported (Figure 31.32). Large numbers of HCV-related deaths occur annually as a result of chronic HCV infections that develop into liver cancer. HCV-induced liver disease accounts for up to 10,000 of the 25,000 annual deaths due to liver cancer, other chronic liver diseases, and cirrhosis.

Other Aspects of Hepatitis Syndromes

Hepatitis is an acute disease of the liver, a vital organ that plays a role in several key metabolic processes, including carbohydrate, lipid, and protein syntheses, as well as detoxification and many other functions. Symptoms of hepatitis include fever, jaundice (yellowing of the skin and the whites of the eyes, Figure 31.33*b*), and liver enlargement and cirrhosis. All hepatitis viruses cause similar acute symptoms and cannot be readily distinguished based on clinical findings alone. Chronic hepatitis infections, usually caused by HBV or HCV, are often asymptomatic or produce very mild symptoms, but nonetheless cause serious liver disease, even in the absence of liver cancer.

Diagnosis of hepatitis is based on a combination of clinical symptoms and laboratory tests that assess liver function, especially key liver enzymes. Cirrhosis is diagnosed by visual examination of biopsied liver tissue. Virus-specific molecular assays are typically used to confirm a diagnosis, positively identify the type of hepatitis virus, and determine a course of treatment. Many of the immunological and molecular diagnostic tools discussed in Chapter 29 are used in hepatitis diagnoses. These include enzyme immunoassays that target viral-specific proteins or antiviral antibodies in a blood sample, immunoblots (Western blots), and immunofluorescence (microscopic) methods. Polymerase chain reaction (PCR) tests are also used to detect hepatitis viral genomes in blood or in liver tissue obtained by biopsy.

Infection with HAV or HBV can be prevented with effective vaccines. HBV vaccination is recommended and in most cases is

(a) (b)

Figure 31.33 Hepatitis. *(a)* Hepatitis B virus. The arrow indicates a complete HBV virion, which is called a Dane particle. A Dane particle is about 40 nm in diameter. *(b)* Jaundice, a yellowing of the facial skin and eye conjunctiva, is a common symptom of hepatitis infections and results from the accumulation of bilirubin (a by-product of degraded red blood cells) that results from reduced liver function.

required for school-age children in the United States. No effective vaccines are available for the other hepatitis viruses. For those unvaccinated, the practice of *universal precautions* will prevent infection. The precautions prescribe a high level of vigilance and aseptic handling and containment procedures to deal with patients, body fluids, and infected waste materials (◀ Section 29.1). Most treatment of hepatitis is supportive, providing rest and time for the immune system to attack the infection and allow liver damage to be repaired. In some cases, in particular for HBV infections, some antiviral drugs are available that offer effective treatment. Those infected with any of the known strains of hepatitis C virus can be treated with a specific antiviral drug that in most cases effects a complete cure.

Check Your Understanding

- What host organ do hepatitis viruses attack? How are hepatitis A, B, and C viruses transmitted?
- Describe potential prevention and treatment methods for hepatitis A, B, and C viruses.

31.12 Ebola: A Deadly Threat

A recent example of a highly infectious and deadly serious emerging pathogen that spreads by direct contact is Ebola virus, which ravaged parts of West and central Africa in 2014, 2015, and 2019 (◀ Section 30.7 and Figure 30.10). Ebola has infected over 31,000 people and killed over 12,000 of them.

Ebola emerged in 1976 in Zaire, and since then several small outbreaks have occurred in West African countries. But not until the 2014 outbreak in Guinea, Liberia, Nigeria, Senegal, and Sierra Leone did the disease kill such large numbers of people. However, nearly as fast as the disease reemerged, strong efforts to contain the spread of infection were put in place and these, along with unknown natural events that control cycles of this disease, combined to significantly reduce Ebola incidence. By the end of 2015, only a handful of cases were reported. Ebola reemerged in 2019 in the Democratic Republic of Congo with over 2000 deaths reported. Today, significant epidemiological surveillance for Ebola remains in place because of the ease of transmission of the virus and thus the rapidity by which a single infection can trigger an outbreak.

Ebola: The Virus and Its Transmission

Ebola hemorrhagic fever is caused by a *filovirus*, a filamentous virus that can take on many shapes (**Figure 31.34*a***). The Ebola virus genome contains single-stranded and linear RNA of the negative sense, similar in this respect to influenza and rabies virus genomes. The genome contains only 19 kilobases of RNA, enough to encode just seven proteins; about a third of the genome encodes the RNA-dependent RNA polymerase (RNA replicase) needed to replicate the genome of negative-sense RNA viruses (◀ Section 11.9).

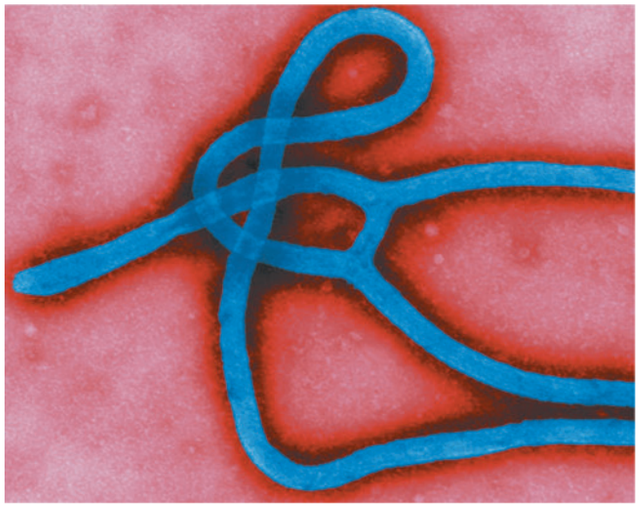
Ebola virus is transmitted from person to person by direct contact through breaks in the skin or mucous membranes as well as by body fluids (including semen) and fomites (bedding, clothing, utensils) contaminated with the virus. The ease with which Ebola can be transmitted seems remarkable compared with other pathogens that rely on direct-contact transmission. For example, in a few documented instances of Ebola transmission to healthcare workers, significant precautions had been taken to ensure that full-body personal protective equipment (PPE, ◀ Section 29.1) was in place to specifically prevent such transmission. Thus, the disease is not only deadly for those infected but can be an extremely dangerous risk for healthy medical providers as well. If PPE is not worn, healthcare workers who either treat patients or dispose of dead Ebola victims run a high risk of becoming infected. Thus, PPE is routinely worn by international health organization workers who might come in contact with an Ebola patient (Figure 31.34*b*).

The natural reservoir of Ebola virus that triggered the West African outbreak is unknown, although related filoviruses, such as hantavirus (▶ Section 32.2), are known to be spread from arthropods and rodents. Among the suspected reservoirs of Ebola are a variety of animals and possibly insects that inhabit tropical forests. In addition to person-to-person transmission, likely responsible for virtually all of the cases in the recent West Africa epidemic, natural Ebola infection in humans probably originates from an animal bite. In this regard, bats, and in particular fruit bats in which the virus has been documented, may be a major disease reservoir.

Ebola: The Disease and Its Treatment

Ebola virus migrates from the initial site of infection to lymph nodes, from which it travels systemically to infect the liver and spleen. Once the virus has entered the body, several different types of cells can become infected. One to two weeks postinfection, an Ebola patient experiences an abrupt fever and general malaise, conditions that make Ebola difficult to distinguish from many other tropical diseases, including malaria. But then more severe symptoms appear. These typically include severe fever and fatigue, diarrhea, nausea, vomiting and abdominal pain, and major loss of appetite. Bleeding through the skin and blood in vomit and feces can occur, but such bleeding is not a common symptom.

Ebola virus causes major problems in the liver, killing liver cells and disrupting normal blood clotting events. It is thought that the virus triggers host cells to release various cytokines that cause widespread inflammation (◀ Section 26.8) and internal bleeding; these lead to multiple organ failures, shock, and renal failure. The mortality



(a)

(b)

Figure 31.34 Ebola. *(a)* Colorized transmission electron micrograph of a negatively stained preparation of Ebola virus virions. A virion is about 80 nm in diameter. *(b)* Ugandan Red Cross workers donning their personal protective equipment before collecting the body of an Ebola victim.

rate in the West African Ebola outbreak averaged 35–70% depending on access to treatment, the initial state of health of those infected, viral load (abundance of the virus in the blood), and age; mortality was as high as 85% among infected people over the age of 45.

There is currently no drug treatment for Ebola, but survival rates among those that receive supportive care to help alleviate symptoms are significantly higher than in those that do not. Therapy includes the maintenance of fluids and electrolytes, oxygen supplements, and transfusions of blood to replace that lost from internal bleeding. Ebola survivors develop an antibody-mediated immune response to the virus, and some treatment success has been achieved by transfusing blood or serum from Ebola survivors into those infected. Vaccines for Ebola are in development, and several promising candidates have emerged. Experimental vaccines have been administered with considerable success to healthcare workers in the Democratic Republic of Congo outbreak, and thus it is likely that putting a person's immune system to work against Ebola—through vaccination—will be the best preventive measure against the disease. However, it is unlikely that an Ebola vaccine would help an already infected person, considering the rapidity with which the disease progresses and the major organ damage that viral infection triggers.

Much has been learned from recent Ebola outbreaks concerning the logistics of handling large-scale outbreaks of such a deadly disease. The ease by which Ebola is transmitted made a public education campaign about the dangers of Ebola just as important as dealing with the morbidity and mortality of the outbreak. When epidemiologists develop a better understanding of the natural reservoirs of Ebola virus, outbreaks like that in West Africa—which likely began by animal-to-person transmission—may well be preventable by reducing or eliminating the major reservoirs and educating the populace about the dangers of encounters with known reservoirs. Also, rigorous campaigns to educate people such as family members who put themselves at risk by handling an Ebola patient or the corpse of an Ebola victim without PPE in place are also helping to reduce spread of Ebola when a case or case cluster does appear.

Check Your Understanding

- What do influenza virus and Ebola virus have in common? In what ways do their modes of transmission differ?
- Contrast mortality rates for influenza and Ebola hemorrhagic fever. Which is the more serious disease?

IV • Sexually Transmitted Infections

Diseases spread through sexual contact may be caused by bacteria, viruses, protists, or fungi. Sexually transmitted infections are characterized by latency, persistence, and recurrence, and in some cases are inapparent. Although most sexually transmitted diseases are curable, currently only one type of sexually transmitted infection is preventable by immunization.

Sexually transmitted infections (STIs), also called *sexually transmitted diseases* (*STDs*), are caused by a wide variety of bacteria, viruses, protists, and even fungi (Table 31.3). Unlike respiratory pathogens that can be shed constantly in large numbers by an infected individual, sexually transmitted pathogens are typically found only in body fluids from the genitourinary tract (and blood, in the case of HIV). Because they require a protected and moist environment, sexually transmitted pathogens preferentially and sometimes exclusively colonize the genitourinary tract.

Because the transmission of STIs is limited to sexual activity, their spread can be controlled by sexual abstinence and minimized by the effective use of condoms that stop the exchange of body fluids during sex. With the exception of HIV/AIDS and genital herpes, most STIs are curable, and many can have only minor symptoms. These realities, combined with the fact that those infected are sometimes reluctant to seek treatment, make treatment of STIs an ongoing

TABLE 31.3 Sexually transmitted infections and treatment guidelines

Disease	Causative organism(s)[a]	Recommended treatment
Gonorrhea	*Neisseria gonorrhoeae* (B)	Cefixime or ceftriaxone, *and* azithromycin or doxycycline
Syphilis	*Treponema pallidum* (B)	Benzathine penicillin G
Chlamydia trachomatis infections	*Chlamydia trachomatis* (B)	Doxycycline or azithromycin
Nongonococcal urethritis	*C. trachomatis* (B) or *Ureaplasma urealyticum* (B) or *Mycoplasma genitalium* (B) or *Trichomonas vaginalis* (P)	Azithromycin or doxycycline Metronidazole
Lymphogranuloma venereum	*C. trachomatis* (B)	Doxycycline
Chancroid	*Haemophilus ducreyi* (B)	Azithromycin
Genital herpes	Herpes simplex 2 (V)	No known cure; symptoms can be controlled by several antiviral drugs
Genital warts	Human papillomavirus (HPV) (certain strains)	No known cure but preventable by vaccine; symptomatic warts can be removed surgically, chemically, or by cryotherapy
Trichomoniasis	*Trichomonas vaginalis* (P)	Metronidazole
Acquired immunodeficiency syndrome (AIDS)	Human immunodeficiency virus (HIV)	No known cure; several drugs can stop viral replication and slow disease progression; prophylactic drug available
Pelvic inflammatory disease	*N. gonorrhoeae* (B) or *C. trachomatis* (B)	Cefotetan and doxycycline
Vulvovaginal candidiasis	*Candida albicans* (F)	Butoconazole

[a]B, bacterium; V, virus; P, protist; F, fungus.

public health challenge. However, delaying or forgoing treatment of STIs only serves to maintain lines of transmission and can lead to long-term health problems, such as infertility, cancer, heart disease, degenerative nerve disease, birth defects, stillbirth, or destruction of the immune system, any of which can result in death.

31.13 Gonorrhea, Syphilis, and Chlamydia

Gonorrhea, *syphilis*, and sexually transmitted *Chlamydia* infections are ancient STIs. Whereas the pathogenesis and symptoms of gonorrhea and chlamydial infections are similar in several ways, these STIs are quite different from syphilis. In the United States, cases of gonorrhea and sexually transmitted *Chlamydia* peaked following the introduction of birth control pills in the mid-1960s, and these STIs are still quite prevalent today; cases of syphilis, on the other hand, are less prevalent (**Figure 31.35**). However, the incidence of all of these STIs has risen significantly in recent years. Between 2013 and 2017 in the United States, reported cases of gonorrhea increased 67%, *Chlamydia* incidence rose by 22%, and diagnoses of syphilis jumped 76% (Figure 31.35).

Gonorrhea

Neisseria gonorrhoeae, often called the *gonococcus*, causes gonorrhea, a highly prevalent bacterial STI of more than half a million reported cases in the United States in 2017. *N. gonorrhoeae* is a gram-negative and obligately aerobic diplococcus related biochemically and phylogenetically to *Neisseria meningitidis* (Section 31.5). Cells of *N. gonorrhoeae* are killed rapidly by drying, sunlight, and ultraviolet radiation and thus normally do not survive away from the mucous membranes of the pharynx, conjunctiva, rectum, or genitourinary tract (**Figure 31.36**). Because of this, gonorrhea can be transmitted only by intimate person-to-person contact. We discussed the clinical microbiology and diagnosis of gonorrhea in Section 29.3.

Mastering Microbiology
Art Activity: Figure 31.35 Reported cases of gonorrhea and syphilis in the United States

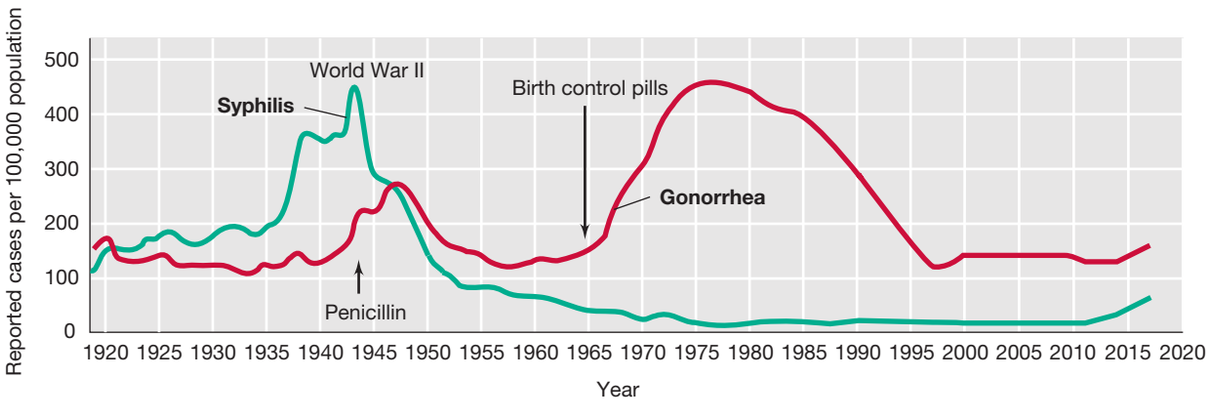
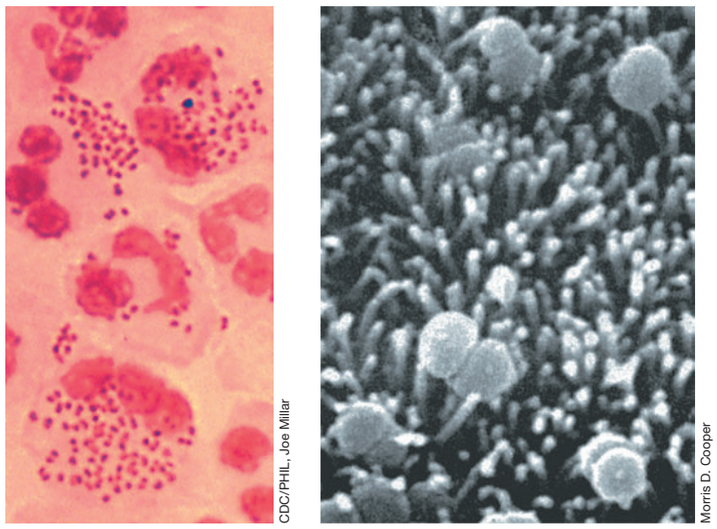


Figure 31.35 Reported cases of gonorrhea and syphilis in the United States. Note the downward trend in disease incidence after the introduction of antibiotics and the upward trend in the incidence of gonorrhea after the introduction of birth control pills. In 2017 there were 555,608 new cases of gonorrhea and 30,644 new cases of primary and secondary syphilis in the United States.



(a) (b)

Figure 31.36 The causative agent of gonorrhea, *Neisseria gonorrhoeae*. *(a)* Gram stain of a urethral discharge. *(b)* Scanning electron micrograph of the microvilli of human fallopian tube mucosa with cells of *N. gonorrhoeae* attached to the surface of epithelial cells. Cells of *N. gonorrhoeae* are about 0.8 μm in diameter. *Neisseria* species are *Betaproteobacteria* (◀ Section 16.2).

The symptoms of gonorrhea are quite different in the male and female. In females, gonorrhea may be asymptomatic or cause a mild vaginitis that is difficult to distinguish from vaginal infections caused by other organisms; hence, the infection may easily go unnoticed. Complications from untreated gonorrhea in females, however, can lead to a chronic condition called *pelvic inflammatory disease* (*PID*), which can cause severe abdominal pain and sterility. In men, *N. gonorrhoeae* causes an infection of the urethral canal characterized by painful urination (dysuria) and urethral discharge of pus. Complications from untreated gonorrhea affecting both males and females include damage to heart valves and joint tissues due to inflammatory reactions from immune complexes that deposit in these areas. In addition to disease in adults, *N. gonorrhoeae* can also cause eye infections in newborns. Infants born of infected mothers may acquire an eye infection called *ophthalmia neonatorum* from passage through the birth canal. Therefore, prophylactic treatment of the eyes of all newborns with an ointment containing erythromycin is mandatory in much of the United States to prevent gonococcal and other bacterial eye infections in infants.

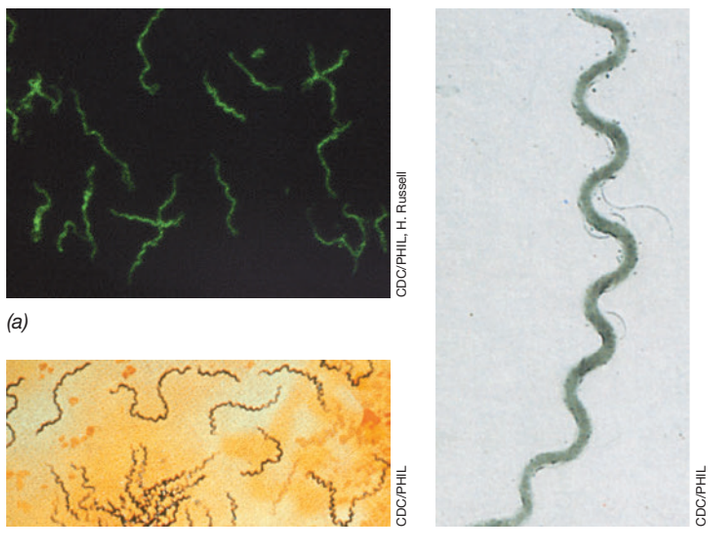
Treatment of gonorrhea with penicillin was the method of choice until the 1980s when strains of *N. gonorrhoeae* resistant to penicillin emerged. The quinolones ciprofloxacin, ofloxacin, or levofloxacin were also used, but by 2006, a significant fraction of *N. gonorrhoeae* strains isolated in the United States had developed resistance to these drugs as well. Strains resistant to penicillin and quinolones respond to alternative antibiotic therapy with a single dose of the β-lactam antibiotics cefixime or ceftriaxone, especially when given in combination with azithromycin (◀ Figure 28.18).

Despite the fact that drugs are still effective in treating gonorrhea, incidence of gonorrhea remains high (Figure 31.35) for at least three reasons. First, although anti-gonococcal antibodies are generated by an infection, they are strain-specific and provide no protection from infection by other strains of *N. gonorrhoeae*. As a consequence, gonorrhea reinfection is possible and quite common in high-risk populations (primarily sex workers and those with multiple sex partners). In addition, within a single *N. gonorrhoeae* strain, antigenic switches can thwart the immune response. For example, by mutation *N. gonorrhoeae* can alter the structure of its pilus proteins, thus creating new serotypes to challenge the immune response. Second, oral contraceptives cause a rise in vaginal pH; when this occurs, lactic acid bacteria normally found in the adult vagina fail to develop, and this reduces competition for colonization by *N. gonorrhoeae*. Finally, and most importantly, symptoms of gonorrhea in the female are often so mild that the disease may go unrecognized; a promiscuous infected female can then infect many males.

Syphilis and Yaws

Syphilis is caused by the bacterium *Treponema pallidum* subspecies *pallidum*, a spirochete having a long, thin, and tightly coiled morphology (**Figure 31.37**). Like *N. gonorrhoeae*, this pathogen is sensitive to environmental stress and drying, and thus syphilis is transmitted only through intimate sexual contact or from mother to fetus during pregnancy (the biology of the spirochetes and the genus *Treponema* is discussed in Section 15.17). A closely related spirochete, *Treponema pallidum* subspecies *pertenue*, causes the tropical disease *yaws* and is more resistant to environmental stressors. Therefore, unlike syphilis, yaws is easily spread by casual (nonsexual) contact through breaks in the skin. Although rarely fatal, about 100,000 new cases of yaws appear every year, mostly in children, producing ulcerous lesions that can be disabling and disfiguring (see Figure 31.39).

Syphilis is often transmitted simultaneously with gonorrhea as a coinfection. However, syphilis is potentially the more serious disease. Despite its lower incidence, syphilis kills nearly 100,000 people per year worldwide, whereas gonorrhea kills fewer than 1000 people



(a) (b) (c)

Figure 31.37 The syphilis spirochete, *Treponema pallidum*. *(a)* Cells from a chancre stained with a fluorescent antibody measure 0.15 μm wide and 10–15 μm long. *(b)* Silver-stained (Fontana method) preparation of a specimen from a syphilitic chancre. *(c)* Shadow-cast electron micrograph of a cell of *T. pallidum*. The endoflagella are typical of spirochetes (◀ Section 15.17).

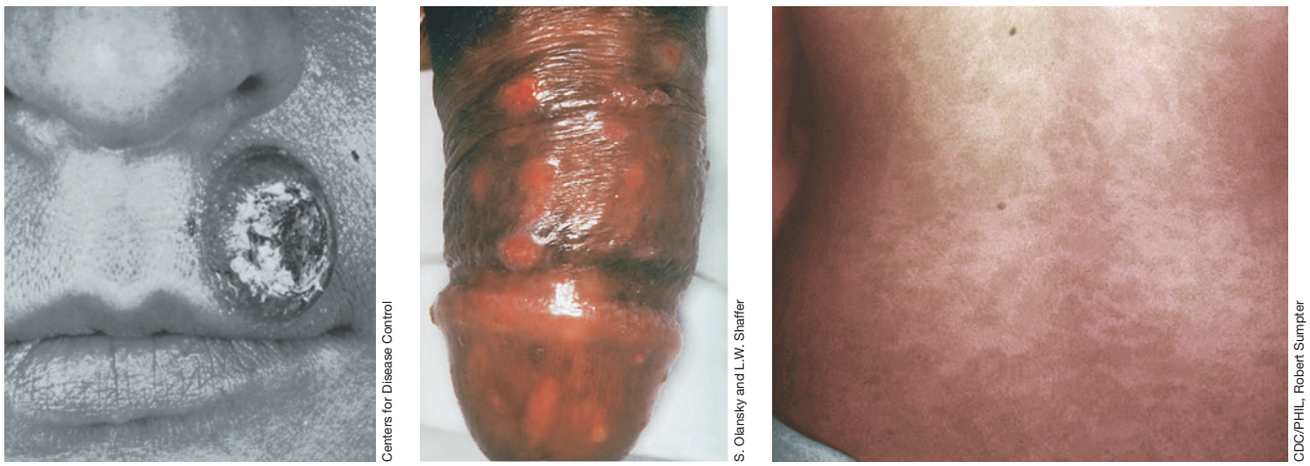


Figure 31.38 Primary and secondary syphilis. *(a)* Chancres on the lip and *(b)* the penis in cases of primary syphilis. The chancre is the characteristic lesion of primary syphilis at the site of infection by *Treponema pallidum*. *(c)* Syphilitic rash on the lower back of a patient showing secondary syphilis.

per year (in some of these cases, the individuals are in poor health to begin with or have coinfections with other pathogens, and so the cause of death may not be due to a single pathogenic agent). Cases of syphilis in the United States have increased dramatically in recent decades, however, with over 30,644 new infections reported in 2017 from a low of around 6000 just ten years earlier. The increase in syphilis incidence may partly be linked to the success of new HIV/AIDS treatments (Section 31.15). Those infected with HIV are often coinfected with *T. pallidum*, and new anti-retroviral drugs that extend the life span of HIV/AIDS patients allow time for the slowly progressing symptoms of advanced syphilis to appear, thus resulting in diagnosis and case reporting.

Neither subspecies of *T. pallidum* (Figure 31.37) is able to penetrate intact skin, and therefore initial infection takes place through tiny breaks in the epidermis. Initial syphilitic infection is evident with the appearance of a hard lesion called a *chancre*, usually on the penis of males or the vagina, cervix, or perineal region of females. In about 10% of cases, infection is extragenital, usually in the oral region (**Figure 31.38*a***). During pregnancy, the pathogen can be transmitted from an infected woman to the fetus; the disease acquired by the infant is called **congenital syphilis**. For yaws, lesions generally appear on the legs, face, or back where the skin has been broken and direct contact with the spirochete has occurred. The "weeping" lesions of yaws are characterized by a fluid exudate and white, splotchy patches around the perimeter of the ulcer (**Figure 31.39**).

Stages of Syphilis

Syphilis is a more complex disease than yaws because it progresses through a series of increasingly serious stages. Syphilis always begins with a localized infection called *primary syphilis*, and it is at this stage that *T. pallidum* multiplies at the site of entry and the characteristic hard chancre appears, usually within 2 weeks to 2 months (Figure 31.38*a, b*). Microscopy of a syphilitic chancre exudate reveals the actively motile spirochetes (Figure 31.37*a, b*). In most cases, the chancre heals spontaneously, and *T. pallidum* disappears from the site. In untreated cases, however, the highly invasive cells spread from the initial site to various parts of the body, such as the mucous membranes, eyes, joints, bones, or central nervous system, where extensive multiplication occurs. A hypersensitivity reaction to the invading bacteria often produces the key symptom of *secondary syphilis* at this time—the development of a generalized, often coppery looking skin rash over the whole body (Figure 31.38*c*).

In the absence of treatment, the subsequent course of the disease varies from case to case. About 25% of infected individuals spontaneously recover and are free of any further disease symptoms. Another one-fourth exhibit no further symptoms but maintain a persistent, chronic syphilitic infection. Roughly half of untreated patients develop *tertiary syphilis*, with symptoms ranging from relatively mild infections of the skin and bone to serious and even fatal infections of the cardiovascular system or central nervous system. This may occur many years after the primary infection. Involvement of the nervous system can cause paralysis or other severe neurological damage (insanity is common in fatal infections). Relatively low

Figure 31.39 Yaws lesion on the face of a young girl caused by *Treponema pallidum* subspecies *pertenue*. The tropical disease yaws, caused by a different subspecies of the same bacterium that causes syphilis, produces splotchy, "weeping" lesions, often with white perimeters. Like syphilis, the debilitating disease can be treated successfully by administering penicillin-based antibiotics.

numbers of *T. pallidum* are present in individuals with tertiary syphilis; most of the symptoms probably result from inflammation due to delayed-type hypersensitivity reactions (◀ Section 28.1) to the pathogen. Tertiary syphilis can still be treated, usually with long-term intravenous antibiotic administration, but prior neurological damage from the syphilitic infection is typically irreversible.

Although several laboratory tests can be used to diagnose *T. pallidum* infections (Chapter 29), the most important physical sign is an ulcerous lesion—either a splotchy, weeping ulcer with yaws or a hard chancre with syphilis. Unlike the case with gonorrhea, penicillin remains highly effective in treating both syphilis and yaws, and both diseases can typically be cured by a single injection of benzathine penicillin G. Oral azithromycin has also been highly effective in treating yaws, often in just a single dose, but some resistance to this antibiotic has emerged among strains of *T. pallidum* that cause syphilis.

Chlamydial Syndromes

A number of sexually transmitted diseases can be ascribed to infection by the obligately intracellular bacterium *Chlamydia trachomatis* (**Figure 31.40**). This organism is one of a small group of parasitic bacteria that form their own phylum (the *Chlamydiae*) of *Bacteria* (◀ Section 16.15). Because *C. trachomatis* must be grown in host cells (tissue culture), its rapid isolation and identification is not as straightforward as for *Neisseria gonorrhoeae*.

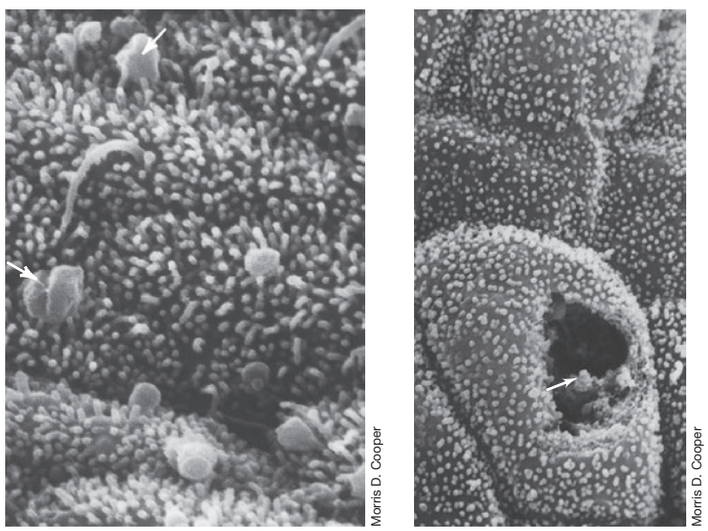
The total incidence of sexually transmitted *C. trachomatis* infections significantly outnumbers the incidence of gonorrhea, making chlamydial infections the most prevalent bacterial STIs in the world. In the United States alone, over 1.7 million chlamydial cases were reported in 2017, nearly half of which were among 15- to 24-year-old females. Because of their often inapparent nature, the actual incidence of sexually transmitted chlamydial infections may be closer to 4 million cases every year. *C. trachomatis* also causes a serious eye infection called *trachoma*, which is the number one cause of infectious blindness in the world, but the strains of *C. trachomatis* responsible for STIs are usually distinct from those that cause trachoma. Chlamydial infections may also be transmitted congenitally to the newborn in the birth canal, causing newborn conjunctivitis and pneumonia.

Nongonococcal urethritis (*NGU*) due to *C. trachomatis* is one of the most frequently observed sexually transmitted diseases in males and females, but the infections are often inapparent. In a small percentage of cases, chlamydial NGU leads to serious acute complications, including testicular swelling and prostate inflammation in men and cervicitis, pelvic inflammatory disease, and fallopian tube damage in women. These are due to the ability of *C. trachomatis* to trigger an overblown immune response and inflammation in the host. During NGU, cells of *C. trachomatis* can attach to microvilli of fallopian tube cells, enter, multiply, and eventually lyse the cells (Figure 31.40*b*). Untreated NGU in a female can thus lead to infertility, ectopic pregnancy, and chronic pelvic pain. Infections with the protist *Trichomonas vaginalis* can cause symptoms similar to those of chlamydial NGU, and we consider trichomoniasis along with other parasitic infections in Chapter 34.

Chlamydial NGU is frequently observed as a secondary infection following gonorrhea. Both *N. gonorrhoeae* and *C. trachomatis* are often transmitted to a new host simultaneously. However, treatment of gonorrhea does not eliminate chlamydial infection. Although cured of gonorrhea, these patients are still infected with *Chlamydia* and eventually experience an apparent recurrence of gonorrhea that is instead a case of chlamydial NGU. Thus, patients treated for gonorrhea with drugs such as cefixime or ceftriaxone are also given azithromycin or doxycycline to treat a potential coinfection with *C. trachomatis*. A variety of clinical techniques including nucleic acid and immunological analyses are available for making a positive diagnosis of *C. trachomatis* infection, but drug therapy in the absence of a positive diagnosis is often prescribed.

Lymphogranuloma venereum (*LGV*) is a sexually transmitted disease caused by distinct strains of *C. trachomatis* (referred to as LGV 1, 2, and 3). The disease occurs most frequently in males and is characterized by infection and swelling of the lymph nodes in and about the groin. From the infected lymph nodes, chlamydial cells may travel to the rectum and cause a painful inflammation of rectal tissues called *proctitis*. LGV has the potential to cause regional lymph node damage and the complications of proctitis. It is the only chlamydial infection that invades beyond the epithelial cell layer.

We move on to the final two sections of this chapter to look at sexually transmitted diseases of viral origin.



(a) (b)

Figure 31.40 Cells of *Chlamydia trachomatis* (arrows) attached to human fallopian tube tissues. *(a)* Cells attached to the microvilli of a fallopian tube. *(b)* A damaged fallopian tube containing a cell of *C. trachomatis* (arrow) in the lesion.

Check Your Understanding

- How are gonorrhea, syphilis, and chlamydial infections diagnosed?
- Explain at least one potential reason for the higher incidence of gonorrhea as compared with syphilis.
- Compare and contrast the symptoms of yaws and syphilis. How are these diseases treated?

31.14 Herpes Simplex Viruses (HSV) and Human Papillomavirus (HPV)

STIs caused by herpesviruses and human papillomaviruses are extremely prevalent among sexually active adults and are often more difficult to diagnose and treat than are bacterial STIs.

Herpes

Herpesviruses are a large group of double-stranded DNA viruses (◀ Section 11.7), many of which are human pathogens. The herpes simplex viruses are responsible for both cold sores and genital infections.

Herpes simplex 1 virus (*HSV-1*) infects the epithelial cells around the mouth and lips, causing cold sores (also called fever blisters) that occasionally spread to other parts of the face in severe infections (**Figure 31.41**). HSV-1 is spread via direct contact with infectious lesions or through saliva. The incubation period of HSV-1 infections is short (3–5 days), and the lesions heal without treatment in 2–3 weeks. However, latent herpes infections are common, because the virus typically persists in low numbers in nerve ganglia. Recurrent acute herpes infections can then occur when the virus is triggered by coinfections with other pathogens or by bodily stress. Oral herpes caused by HSV-1 is quite common and apparently has no long-term harmful effects on the host, beyond the discomfort of the oral blisters.

Herpes simplex 2 virus (*HSV-2*) infections are associated primarily with the anogenital region, where the virus causes painful blisters on the penis of males or on the cervix, vulva, or vagina of females (**Figure 31.42**). HSV-2 infections are generally transmitted by direct sexual contact, and the disease is most easily transmitted when active blisters are present but may also be transmitted during asymptomatic periods, even when the infection is presumably latent. HSV-2 occasionally infects other sites such as the mucous membranes of the mouth and can also be transmitted to a newborn by contact with herpetic lesions in the birth canal at birth. The disease in the newborn varies from latent infections with no apparent damage to systemic disease resulting in brain damage or even death. To avoid herpes infections in newborns, delivery by cesarean section is advised for pregnant women with genital herpes infections.

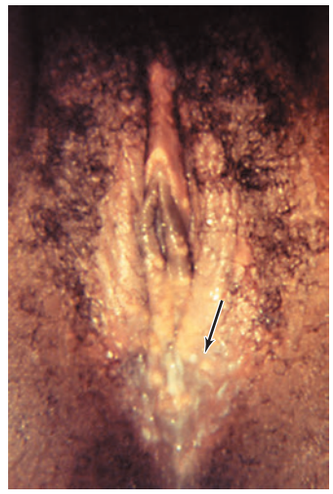
The long-term effects of genital herpes infections are not fully understood. However, studies have indicated a significant correlation between genital herpes infections and cervical cancer in females. Genital herpes infections are presently incurable, although a limited number of drugs have been successful in controlling the infectious blister stages. The guanine analog acyclovir (**Figure 31.43**), given orally and also applied topically, is particularly effective in limiting the shed of active virus (both HSV-1 and HSV-2) from blisters and promoting the healing of blistering lesions (Figures 31.41 and 31.42). Acyclovir, and the related drugs valacyclovir and vidarabine, are nucleoside analogs that interfere with herpesvirus DNA replication, thus inhibiting viral multiplication.

(a)

(b)

Figure 31.41 Herpes simplex 1 virus infections. *(a)* A severe case of herpes blisters on the face due to infection with herpes simplex 1 virus. *(b)* Close-up view of herpes blisters by the eye.

(a)



(b)

Figure 31.42 Herpes simplex 2 virus infections. Herpes simplex 2 virus blisters on the *(a)* penis and *(b)* vulva. As for herpes type 1, acute type 2 herpes infections can seemingly be cured only to reappear later from a persistent virus infection (◀ Section 5.7).

Human Papillomavirus

As for herpesviruses, **human papillomaviruses (HPV)** comprise a family of double-stranded DNA viruses. Of more than 100 different strains, about 30 are transmitted sexually, and several of these cause genital warts and cervical cancer. Up to 80% of women aged over 50 have had at least one HPV infection. There are 14 strains of HPV that cause cervical cancer. HPV types 16 and 18 cause 70% of cervical cancers and pre-cancerous cervical lesions. Cervical cancer is the fourth most common cancer among women globally, with an estimated 570,000 new cases in 2018, resulting in 311,000 fatalities.

Most HPV infections are asymptomatic, with some progressing to cause genital warts. Others cause cervical neoplasia (abnormalities in cells of the cervix), and a few progress to cervical cancers. Most HPV infections resolve spontaneously but, as with many viral infections, there is no adequate treatment or cure for active infections. Because human papillomaviruses are potentially oncogenic (cancer-causing), available HPV vaccines, such as the widely used HPV

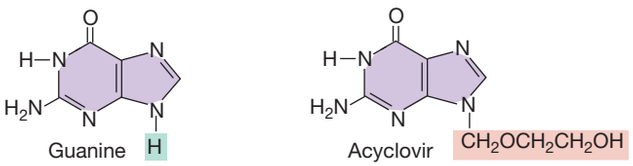


Figure 31.43 Guanine and the guanine analog acyclovir. Acyclovir has been used therapeutically to control genital herpes (HSV-2) blisters (Figure 31.42).

9-valent vaccine (marketed as Gardasil®), are currently recommended for use in females 11–26 years of age. The HPV vaccine is also recommended for males to prevent anal and penile cancers and also because immunized males do not carry HPV and thus cannot infect females. In addition, the HPV vaccine should reduce incidence of certain neck and throat cancers in both males and females caused by the same sexually transmitted strains of HPV.

Check Your Understanding

- Describe pertinent clinical features and treatment protocols for herpes simplex viruses and human papillomaviruses.
- Why are these diseases more difficult to diagnose than gonorrhea or syphilis?

31.15 Human Immunodeficiency Virus (HIV) and AIDS

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV). Worldwide, nearly 80 million people have been infected with HIV, with an estimated 6000 more added each day, and over 35 million have died. In the United States, from a total of 5 cases diagnosed in 1981, over 1.1 million people are infected with HIV today. We covered some aspects of the epidemiology of HIV/AIDS in Section 30.8 and will pick up on that theme here.

HIV and a Definition of AIDS

HIV is of two types, *HIV-1* and *HIV-2*, but because more than 99% of global AIDS cases are due to HIV-1, we focus on HIV-1 here. HIV-1 is a retrovirus (◀ Sections 5.7 and 11.11) that replicates in macrophages and T cells of the human immune system (Chapters 26 and 27). HIV infection eventually leads to the destruction of key immune system cells, virtually eliminating the host immune response. Death from AIDS is usually the result of a secondary infection, typically one caused by an **opportunistic pathogen**, pathogens that in a healthy individual would be controlled by the immune system.

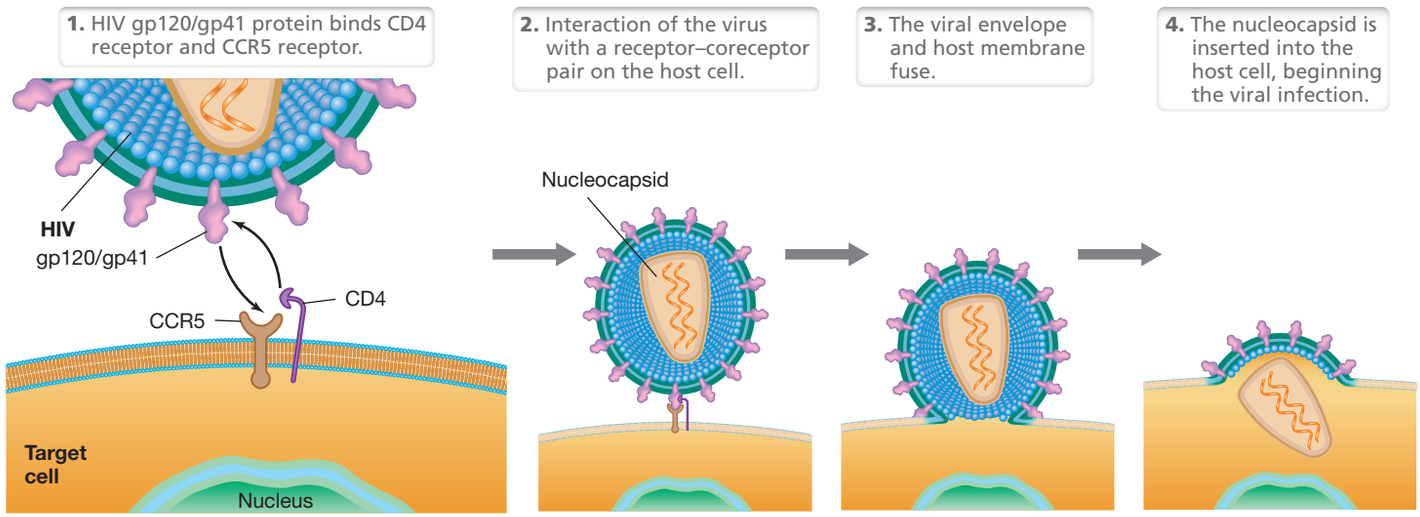
Mastering Microbiology
Art Activity: Figure 31.44 Infection of a CD4 target cell with HIV

The current definition of a case of HIV/AIDS is a patient who tests positive for HIV in immunological and/or nucleic acid–based tests and meets at least one of the following two criteria:

1. A CD4 T cell number of less than 200/μl of whole blood (the normal count is 600–1000/μl) or a CD4 T cell/total lymphocytes percentage of less than 14%.
2. A CD4 T cell number of more than 200/μl *and* any of the following diseases: candidiasis, coccidioidomycosis, cryptococcosis, histoplasmosis, cystoisosporiasis, *Pneumocystis jirovecii* pneumonia, cryptosporidiosis, or toxoplasmosis of the brain (all fungal or protozoal diseases, Chapter 34); pulmonary tuberculosis or other mycobacterial infections, or recurrent *Salmonella* septicemia (bacterial diseases); cytomegalovirus infection, HIV-related encephalopathy, HIV wasting syndrome, chronic ulcers, or bronchitis due to herpes simplex (viral infections); or certain malignant diseases such as invasive cervical cancer, Kaposi's sarcoma, Burkitt's lymphoma, primary lymphoma of the brain, or immunoblastic lymphoma; or recurrent pneumonia due to any agent.

Pathogenesis of HIV/AIDS

HIV infects cells that have the CD4 cell surface protein. The two cell types most commonly infected are macrophages and T-helper (Th) lymphocytes, both of which are important components of the immune system. Infection normally occurs first in macrophages. At the macrophage cell surface, the CD4 molecule binds to the gp120/gp41 capsid protein of HIV as the virus interacts with the macrophage receptor CCR5 (**Figure 31.44**). CCR5 is a coreceptor for HIV and, together with CD4, forms the docking site where the HIV envelope fuses with the host cytoplasmic membrane; this is required for the viral nucleocapsid to be inserted into the cell (Figure 31.44*b*). Within the macrophage, HIV replicates and makes an



(a) **Interaction of HIV with a host cell** (b) **Fusion of the HIV envelope with the host cell facilitates nucleocapsid entry**

Figure 31.44 Infection of a CD4 target cell with HIV. *(a)* Recognition and binding of HIV by CCR5 and CD4 receptors. *(b)* The viral nucleocapsid eventually enters the cell. Details of the replication of the HIV genome were shown in Figure 11.27.

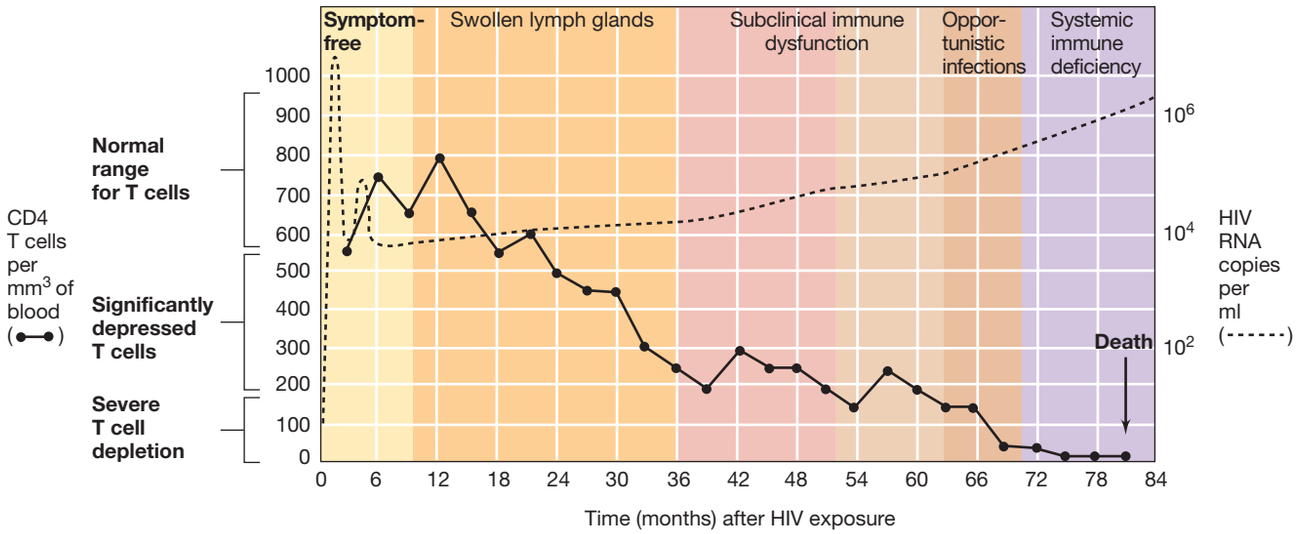


Figure 31.45 Decline of CD4 T lymphocytes and progress of HIV infection. During the typical progression of untreated AIDS, there is a gradual loss in the number and functional ability of the CD4 T cells, while the viral load, measured as HIV-specific RNA copies per milliliter of blood, gradually increases after an initial decline.

altered form of gp120 that recognizes a different coreceptor, CXCR4, on Th cells. HIV virions are released from macrophages and proceed to infect and replicate in Th lymphocytes. Infected Th cells that produce HIV no longer divide and are eventually diminished by attrition.

In some HIV/AIDS patients, HIV infection does not progress immediately to killing host immune cells. HIV can exist in a dormant state as a provirus; under these conditions, the reverse-transcribed HIV genome, now in the form of DNA, is integrated into host chromosomal DNA (◀ Figure 11.27). At this point the cell may show no outward sign of infection. Indeed, HIV DNA can remain latent for long periods, replicating only as the host cell DNA replicates. However, sooner or later, HIV begins to replicate, and progeny viruses are produced and released from the cell.

Symptoms of HIV/AIDS

Ongoing HIV infection results in a progressive decline in CD4 cell numbers. In a healthy human, CD4 cells constitute about 70% of the total T cell pool. In those with HIV/AIDS, CD4 numbers steadily decrease, and by the time opportunistic infections begin to appear, CD4 cells are all but absent (**Figure 31.45**). The progression of untreated HIV infection to AIDS follows a typical pattern. First, there is an intense immune response to HIV, and HIV numbers drop. But eventually, the immune response is overwhelmed, and HIV levels slowly increase while CD4 T cells slowly decrease. When T cell numbers have dropped below about 200/mm^3 of blood, infections by opportunistic pathogens can occur (Figure 31.45).

Opportunistic infections caused by normally controllable protists, fungi, bacteria, and viruses occur with high prevalence in those with HIV/AIDS and are typically the actual cause of death (**Figure 31.46**). The most common opportunistic disease in HIV/AIDS patients is pneumonia caused by the fungus *Pneumocystis jirovecii* (Figure 31.46*d*), but infections by various molds, yeasts, protists, and bacteria are also seen (Figure 31.46). Bacterial infections are less common than those of eukaryotic pathogens, but when they occur, they are frequently of strongly antibiotic-resistant bacteria, such as multiple-drug-resistant *Mycobacterium tuberculosis*.

Eukaryotic opportunistic pathogens are difficult to treat in general because many of the drugs used to treat infections from fungi and protists have significant negative side effects on the host, which of course is also a eukaryote. A cancer frequently seen in HIV/AIDS patients is *Kaposi's sarcoma*, a cancer of the cells lining the blood vessels and characterized by purple splotches on the skin, especially in the extremities (**Figure 31.47**). Kaposi's sarcoma is caused by coinfection of HIV and human herpesvirus 8 (HHV-8) and is rarely seen outside of HIV/AIDS patients.

Diagnosing HIV/AIDS

HIV infection is typically diagnosed by identifying antibodies to the pathogen in a patient blood sample. An enzyme immunoassay (EIA, ◀ Figure 29.20) is used for HIV screening purposes, typically for screening done on a large scale, such as with donated blood. A positive HIV EIA must be confirmed by an HIV immunoblot (Western blot, ◀ Figure 29.22) or by immunofluorescence (◀ Section 29.6) to rule out the possibility of a false-positive screening test. Rapid and inexpensive HIV tests are also available for preliminary screening of blood in clinics. One test requires only a single drop of patient blood and detects the gp41 HIV surface antigen (Figure 31.44) by producing a visible agglutination reaction. A second uses saliva as a source of anti-HIV antibodies and yields a colored product. In general, however, these rapid tests are not as sensitive or specific as the standard HIV EIA and thus positive tests should be confirmed by more sensitive and specific tests. Unfortunately, no matter how sensitive or specific, none of the antibody tests will detect those who have recently acquired the virus and are infectious but have not yet made a detectable antibody response to HIV, which may require a period of 6 weeks or more following infection.

Mastering Microbiology
Art Activity: Figure 31.45 Decline of CD4 T lymphocytes and progress of HIV infection

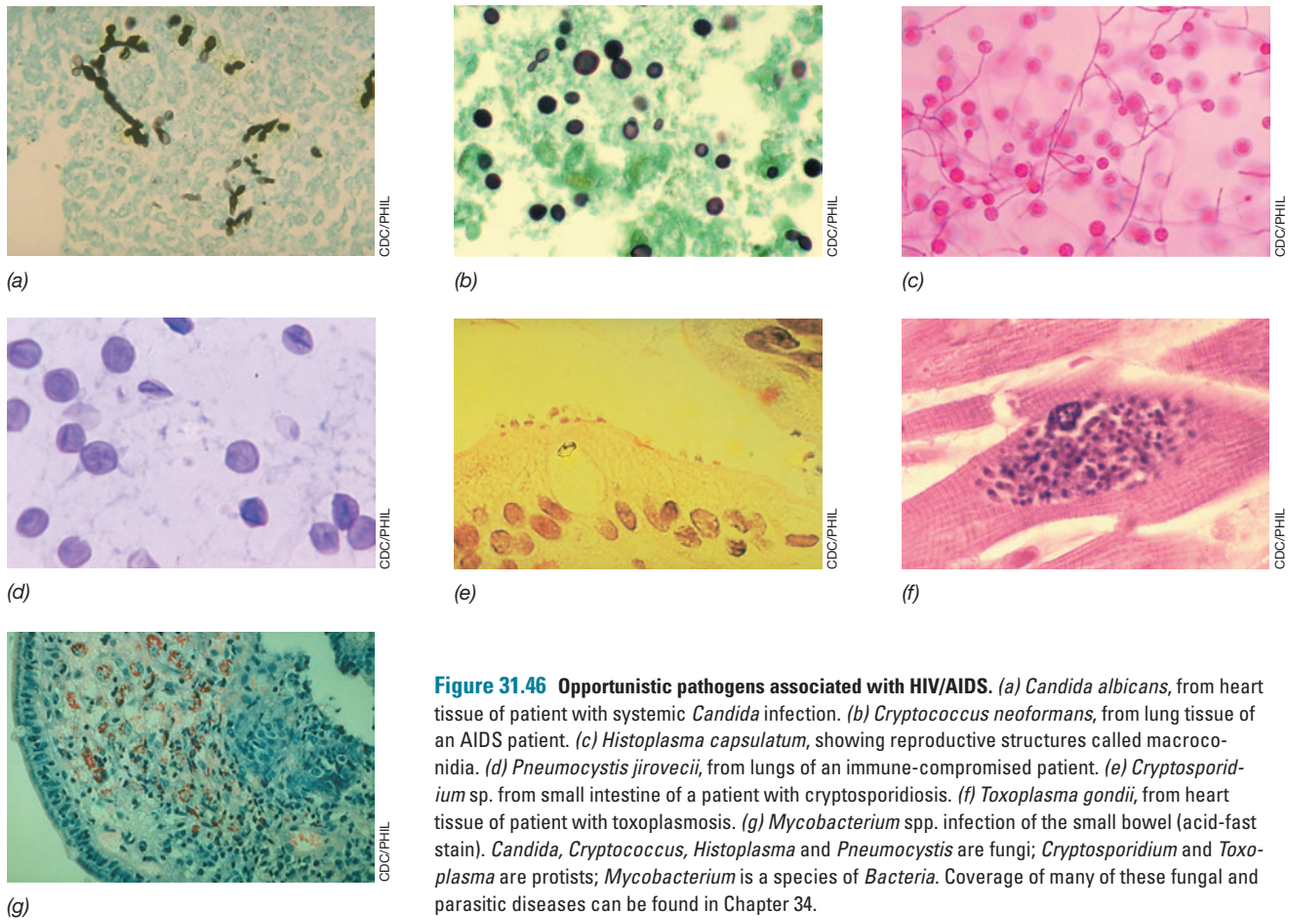


Figure 31.46 Opportunistic pathogens associated with HIV/AIDS. *(a) Candida albicans*, from heart tissue of patient with systemic *Candida* infection. *(b) Cryptococcus neoformans*, from lung tissue of an AIDS patient. *(c) Histoplasma capsulatum*, showing reproductive structures called macroconidia. *(d) Pneumocystis jirovecii*, from lungs of an immune-compromised patient. *(e) Cryptosporidium* sp. from small intestine of a patient with cryptosporidiosis. *(f) Toxoplasma gondii*, from heart tissue of patient with toxoplasmosis. *(g) Mycobacterium* spp. infection of the small bowel (acid-fast stain). *Candida, Cryptococcus, Histoplasma* and *Pneumocystis* are fungi; *Cryptosporidium* and *Toxoplasma* are protists; *Mycobacterium* is a species of *Bacteria*. Coverage of many of these fungal and parasitic diseases can be found in Chapter 34.

Diagnostic procedures also are available that directly measure the number of HIV virions in a blood sample. These tests use a virus-specific reverse transcription–polymerase chain reaction assay (RT-PCR, ◀ Sections 12.1 and 29.8). RT-PCR estimates the number of HIV virions present in the blood, called the **viral load** (Figure 31.48). The RT-PCR test for HIV load is not routinely used to screen for HIV because it is costly and technically demanding. However, after an initial diagnosis, the test is often used to monitor progression of an HIV infection (Figure 31.48) and the effectiveness of chemotherapy.

Treatment of HIV/AIDS

The prognosis for an *untreated* HIV-infected individual is poor, as opportunistic pathogens or malignancies (Figures 31.46 and 31.47) eventually kill virtually all infected persons. Long-term studies indicate that the average person infected with HIV progresses through several stages of decreasing immune function, with CD4 cells dropping from a normal range of 600−1000/mm^3 of blood to near zero over a period of 5–7 years (Figure 31.45). Although the rate of decline varies from one HIV-infected individual to another, it is rare for an HIV-positive individual to live for more than 10 years without anti-HIV drug therapy (see MicrobiologyNow in the Chapter 27 opener for exceptions).

Several drugs have been developed that delay the progression of HIV/AIDS and significantly prolong the life of those infected with HIV. Therapy is aimed at reducing the viral load of HIV-infected individuals to below detectable levels. The strategy to accomplish this is called *highly active anti-retroviral therapy* (HAART) and is carried out by administering at least three anti-retroviral drugs at once to inhibit the replication of HIV and prevent the development of drug-resistant strains. Multiple drug therapy, however, is not a cure for HIV infection. In individuals who have no detectable viral load after drug treatment, a significant viral load returns if therapy is interrupted or discontinued, or if multiple drug resistance develops.

Effective anti-HIV drugs fall into four categories, including two classes of *reverse transcriptase inhibitors*, various *protease inhibitors, fusion inhibitors*, and *integrase inhibitors*. Reverse transcriptase is the enzyme that converts the single-stranded RNA genome of HIV into cDNA and then double-stranded DNA and is essential for viral replication (◀ Sections 5.7 and 11.11). Cells lack reverse transcriptase, and thus reverse transcriptase inhibitors are virus-specific. *Azidothymidine* (AZT), also called zidovudine, closely resembles the nucleoside thymidine but lacks the correct attachment site for the next base in a replicating nucleotide chain, resulting in termination of the growing DNA chain. AZT is thus a **nucleoside reverse transcriptase inhibitor** (Figure 31.49*a*). **Nonnucleoside reverse transcriptase inhibitors**, such as *nevirapine* (Figure 31.49*b*), inhibit the activity of reverse transcriptase in a different way by interacting with the protein and altering the conformation of the catalytic site.

(a) (b)

Figure 31.47 Kaposi's sarcoma. Lesions are shown as they appear on *(a)* the heel and lateral foot, and *(b)* the distal leg and ankle.

Other anti-HIV drugs include the **protease inhibitors**, such as *saquinavir* (◀ Figure 28.20*b*). These are peptide analogs that bind to the active site of the processing enzyme, *HIV protease*, thus inhibiting the processing required to activate retroviral polypeptides; this effectively inhibits viral maturation. **Fusion inhibitors** include *enfuvirtide*, a synthetic peptide that functions by binding to the gp41 protein on HIV capsids (Figure 31.44); this stops fusion of the viral envelope and the CD4 cell cytoplasmic membrane. Finally, there are the **integrase inhibitors**, such as *elvitegravir* and *raltegravir*. These drugs target HIV integrase, the protein that integrates the HIV genome into host cell DNA. The interference with integration of viral DNA into the host cell genome interrupts the HIV replication cycle.

All anti-HIV drugs rapidly decrease the viral load when given to HIV-infected individuals, but drug-resistant strains of HIV arise quickly if only a single drug is administered. A typical HAART protocol for treatment of an established HIV infection includes at least one protease or nonnucleoside reverse transcriptase inhibitor plus a combination of two nucleoside reverse transcriptase inhibitors. A resistant virus would, therefore, have to develop resistance to three drugs simultaneously, an unlikely event. A patient receiving this combination therapy is then monitored to track changes in viral load (Figures 31.45 and 31.48). An effective HAART protocol reduces viral load to nondetectable levels within several days. Drug therapy is then continued and the patient monitored for viral load indefinitely. If the viral load again reaches detectable limits, the drug cocktail is changed because an increase in viral load indicates the emergence of drug-resistant HIV.

In addition to drug resistance, some HAART protocols must be modified because of host toxicity. In many cases, nucleoside analogs are not well tolerated by patients, presumably because they interfere with host functions such as cell division. In general, the nonnucleoside reverse transcriptase inhibitors and the protease inhibitors are better tolerated because they target virus-specific functions. However, drug resistance and host toxicity are major problems in all forms of HIV therapy. Thus, new chemotherapeutic agents and drug protocols are constantly being developed and tailored to the needs of individual patients.

HIV/AIDS Prevention

Although a number of experimental vaccines are in development, several of which are currently undergoing clinical trials, no HIV vaccine has been approved for clinical use. Public education about how HIV/AIDS is transmitted, sexual abstinence, and avoidance of high-risk behavior remain the best strategies to prevent HIV/AIDS. HIV spread is linked to promiscuous sexual activities and other activities that involve exchange of body fluids, which include not only men who have sex with men, but also prostitution and intravenous drug use where needles are shared. In some countries, the fastest growing mode of HIV transmission is actually between promiscuous heterosexual partners. Effective prevention of HIV transmission therefore

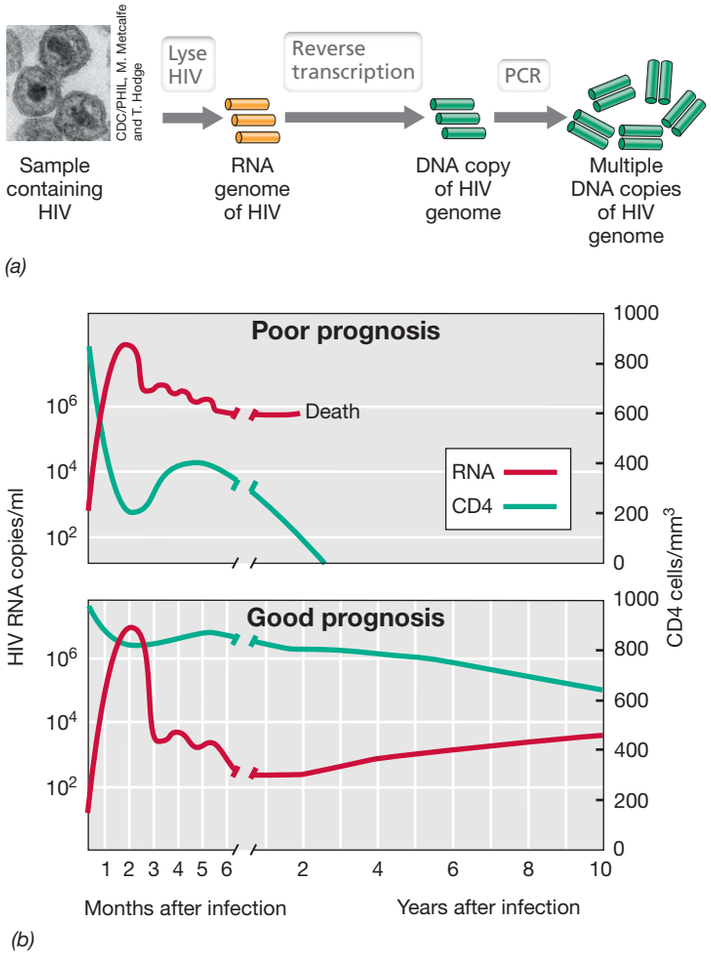
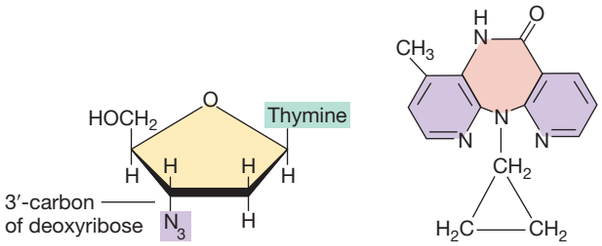


Figure 31.48 Monitoring of HIV load. *(a)* Procedure for detecting HIV by reverse transcription–polymerase chain reaction (RT-PCR) techniques. *(b)* Time course for HIV infection as monitored by HIV load and CD4 T cell counts. In the upper panel, a viral load greater than 10^4 copies/ml correlates with below normal CD4 cell numbers (normal = 600–1000/mm^3), indicating a poor prognosis and early death of the patient. In the lower panel, a viral load less than 10^4 copies/ml correlates with normal CD4 cell numbers, indicating a good prognosis and extended survival of the patient. Data are adapted from the CDC, Atlanta, Georgia, USA.



(a) **Azidothymidine (zidovudine)** *(b)* **Nevirapine**

Figure 31.49 HIV/AIDS chemotherapeutic drugs. *(a)* Azidothymidine (AZT), also called zidovudine, a nucleoside reverse transcriptase inhibitor. This nucleoside analog is missing the –OH group on the 3'-carbon, causing nucleotide chain elongation to terminate when the analog is incorporated, inhibiting virus replication. *(b)* Nevirapine, a nonnucleoside reverse transcriptase inhibitor, binds directly to the catalytic site of HIV reverse transcriptase, also inhibiting elongation of the nucleotide chain.

requires avoiding these high-risk behaviors, using condoms whenever the health of a sex partner is in doubt, and, for anyone who has engaged in high-risk behavior, undergoing an HIV blood test.

Check Your Understanding

- Review the definition of HIV/AIDS. Which symptoms of HIV/AIDS are shared by all HIV/AIDS patients?
- What does the enzyme reverse transcriptase do, and why is it a good target for anti-HIV drugs?
- What is HAART therapy for treating AIDS patients?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Airborne Bacterial Diseases

31.1 Bacterial and viral respiratory pathogens are transmitted in air. Most respiratory pathogens are transferred from person to person via respiratory aerosols generated by coughing, sneezing, talking, or breathing, or by direct or fomite contact. Respiratory pathogens infect either the upper or lower respiratory tracts and sometimes both.

Q What enables *Staphylococcus* and *Mycobacterium* to survive drying and persist in dust for long periods?

31.2 Streptococcal diseases include strep throat and pneumococcal pneumonia. *Streptococcus pyogenes* infections may progress into serious conditions such as scarlet and rheumatic fevers, and pneumococcal pneumonia can have high mortality. Both pathogens can be cultured, and both are treatable with antimicrobial drugs, including penicillin.

Q Describe one method to diagnose strep throat quickly. Why may this method be better than a throat culture?

31.3 Diphtheria is an acute respiratory disease caused by *Corynebacterium diphtheriae*. Early childhood immunization is effective for preventing this very serious respiratory disease. Whooping cough is an endemic disease caused by *Bordetella pertussis*. Immunization of children, adolescents, and adults can control its propagation and spread.

Q What causes a strain of *C. diphtheriae* to be pathogenic? Describe how such a strain may cause death in an infected patient?

31.4 Tuberculosis is one of the most prevalent and dangerous infectious diseases in the world. Its incidence is increasing in developed countries in part because of the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. The pathology of tuberculosis and other mycobacterial syndromes such as Hansen's disease (leprosy) is influenced by the cell-mediated immune response.

Q Describe the process of infection by *Mycobacterium tuberculosis*. Does infection always lead to active tuberculosis? Why or why not? How is exposure to *M. tuberculosis* detected in humans?

31.5 *Neisseria meningitidis* is a common cause of meningococcemia and meningitis in young adults and occasionally occurs in epidemics in enclosed populations. Bacterial meningitis and meningococcemia can have high mortality rates, and treatment and prevention strategies including vaccines are available.

Q How is *Neisseria meningitides* typically transmitted, and what are the symptoms of full-blown meningitis caused by this bacterium?

II • Airborne Viral Diseases

31.6 Viral respiratory diseases are highly infectious and may cause serious health problems, although most are controllable and not life-threatening. The measles/mumps/rubella (MMR) vaccine is highly effective in controlling these diseases.

Q Compare and contrast measles, mumps, and rubella. Include a description of the pathogen, major symptoms encountered, and any potential consequences of these infections. Why is it important that women be vaccinated against rubella before puberty?

31.7 Colds are the most common infectious viral diseases. Usually caused by a rhinovirus, colds are generally mild and self-limiting diseases; over-the-counter "cold drugs" may help to moderate symptoms but are not a cure. Each infection induces specific, protective immunity, but the large

number of cold viruses precludes complete protective immunity or vaccines.

Q Why are colds such common respiratory diseases, and why are vaccines not used to prevent colds?

31.8 Influenza is caused by an RNA virus that contains a segmented genome and is easily transmitted by the airborne route. Influenza outbreaks occur annually as a result of the plasticity of the influenza genome. Antigenic drift varies the nature of the viral envelope of influenza viruses in minor ways, causing influenza seasonal epidemics, while antigenic shift varies the virus in major ways and can trigger periodic influenza pandemics. Surveillance and immunization are used to control influenza.

Q Why is influenza such a common respiratory disease? How are annual influenza vaccines developed?

III • Direct-Contact Bacterial and Viral Diseases

31.9 Staphylococci are usually benign inhabitants of the upper respiratory tract and skin, but several serious diseases can result from pyogenic infection or from the activity of staphylococcal superantigen exotoxins. Antibiotic resistance is common, even in community-acquired infections. MRSA strains of *Staphylococcus aureus* can be very difficult to treat and cause significant tissue damage.

Q Distinguish between pathogenic staphylococci and those that are part of the normal microbiota.

31.10 *Helicobacter pylori* infection is the common cause of gastric ulcers. As an infectious disease, gastric ulcers are best treated with antibiotics to promote a permanent cure.

Q Describe the evidence linking *Helicobacter pylori* to gastric ulcers. How can these ulcers be cured?

31.11 Viral hepatitis can result in acute liver disease, which may be followed by chronic liver disease (cirrhosis). Hepatitis B and C viruses in particular are transmitted by direct contact and can cause chronic infections leading to liver cancer. Vaccines are available for hepatitis viruses A and B. Viral hepatitis is still a major public health problem because of the high infectivity of the viruses and the lack of effective treatments.

Q Describe the major hepatitis viruses. How are they related to one another? How is each spread?

31.12 Ebola hemorrhagic fever is a deadly viral disease spread by direct contact through the skin or from contaminated body fluids. Mortality rates from Ebola are near the highest of all diseases. Treatment is primarily supportive of symptoms, but vaccine trials have shown that effective vaccination protocols are possible.

Q The Ebola virus cannot depend on the host to synthesize its genome; why not?

IV • Sexually Transmitted Infections

31.13 Gonorrhea, syphilis, and chlamydial infections caused by *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Chlamydia trachomatis*, respectively, are sexually transmitted infections (STIs) with potential serious consequences if infections are not treated. In the United States, the incidence of all three of these STIs has increased in recent years, with chlamydial infections being the most prevalent of bacterial STIs. The tropical disease yaws is not an STI but is caused by a subspecies of *Treponema pallidum* closely related to the syphilitic spirochete.

Q Why did the incidence of gonorrhea rise dramatically in the mid-1960s, while the incidence of syphilis actually decreased at the same time?

31.14 Herpes simplex viruses cause incurable infections transmitted by oral or genital contact with herpes simplex 1 or herpes simplex 2, respectively. Human papillomaviruses cause widespread STIs that may lead to cervical and other cancers, but effective HPV vaccines are available.

Q Describe the viruses that cause genital herpes and genital warts. In each case, is a preventive vaccine available? Is treatment of these STIs possible, and if so, is it an effective cure? Why or why not?

31.15 HIV is a retrovirus that destroys the immune system, leading to AIDS, and opportunistic pathogens eventually kill the host. There is no effective cure or vaccine for HIV infection, although antiviral drugs may slow or stop the progress of AIDS. Preventing HIV infection requires education and avoidance of high-risk behaviors involving exchange of body fluids.

Q Describe how human immunodeficiency virus (HIV) effectively shuts down both antibody-mediated and cell-mediated immunity. What is HAART therapy?

APPLICATION QUESTIONS

1. Why is it that you get a cold or two each year but if you have had a case of measles, it was a one-time occurrence?
2. Your college roommate goes home for the weekend, becomes extremely ill, and is diagnosed with bacterial meningitis at a local hospital. Because he was away, university officials are not aware of his illness. What should you do to protect yourself against meningitis? Should you notify university health officials?
3. Contrast an HIV infection with an infection by any other viral pathogen considered in this chapter, regardless of mode of transmission. Why do untreated cases of HIV infection almost always lead to death whereas untreated cases of chicken pox, influenza, or even hepatitis typically do not?
4. Discuss how the quarantines and lockdowns occurring throughout the world due to the spread of SARS-CoV-2 may impact the incidence of influenza globally. How do you think these public health precautions will affect the transmission of other airborne diseases such as measles? (Hint: you may want to look to these resources: (1) https://www.nature.com/articles/d41586-020-01011-6 and (2) https://www2.health.vic.gov.au/about/publications/researchandreports/seasonal-influenza-reports-2020)

CHAPTER GLOSSARY

Antigenic drift a minor change in influenza virus antigens due to gene mutation

Antigenic shift a major change in influenza virus antigen due to gene reassortment between different strains

Cirrhosis breakdown of normal liver architecture, resulting in fibrosis and compromised liver function

Congenital syphilis syphilis contracted by an infant from its mother during pregnancy

Fusion inhibitor a synthetic polypeptide that binds to viral glycoproteins, inhibiting fusion of viral and host cell membranes

Hepatitis liver inflammation, commonly caused by an infectious agent

Human papillomavirus (HPV) a sexually transmitted virus that causes genital warts, cervical neoplasia, and cancer

Integrase inhibitor a drug that interrupts the HIV replication cycle by interfering with integrase, the HIV protein that catalyzes the integration of viral dsDNA into host cell DNA

Meningitis inflammation of the meninges (brain tissue), sometimes caused by *Neisseria meningitidis* and characterized by sudden onset of headache, vomiting, and stiff neck, often progressing to coma within hours

Meningococcemia a rapidly progressing severe disease caused by *Neisseria meningitidis* and characterized by septicemia, intravascular coagulation, and shock

Nonnucleoside reverse transcriptase inhibitor a nonnucleoside compound that inhibits the action of retroviral reverse transcriptase by binding directly to the catalytic site

Nucleoside reverse transcriptase inhibitor a nucleoside analog compound that inhibits the action of retroviral reverse transcriptase by competing with nucleosides

Opportunistic pathogen an organism that causes disease only in the absence of normal host resistance

Pertussis (whooping cough) a disease caused by a respiratory tract infection with *Bordetella pertussis*, characterized by a deep, persistent cough

Protease inhibitor a compound that inhibits the action of viral protease by binding directly to the catalytic site, preventing viral protein processing

Rheumatic fever an inflammatory autoimmune disease triggered by an immune response to infection by *Streptococcus pyogenes*

Scarlet fever the characteristic reddish rash with fever and sore throat resulting from an exotoxin produced by *Streptococcus pyogenes*

Sexually transmitted infection (STI) an infection that is usually transmitted by sexual contact

Toxic shock syndrome (TSS) the acute systemic shock resulting from a host response to an exotoxin produced by *Staphylococcus aureus*

Tuberculin test a skin test for previous infection with *Mycobacterium tuberculosis*

Viral load a quantitative assessment of the amount of virus in a host organism, usually in the blood

Vectorborne and Soilborne Bacterial and Viral Diseases 32

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II Arthropod-Transmitted Bacterial and Viral Diseases 1023

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MICROBIOLOGYNOW

The Historical Emergence of an Ancient and Deadly Pathogen

Although a handful of other diseases throughout history have claimed more human lives—namely smallpox, measles, and perhaps influenza—no other infectious disease has elicited more fear and dread than *plague*.

Causing an estimated 75 million deaths, the "Black Death," as it came to be called during devastating European outbreaks in the 14th century, is indeed a prolific killer. As described in this chapter, plague is accompanied by a particularly grim suite of symptoms caused by the gram-negative bacterium *Yersinia pestis* (photo, cells of *Y. pestis* [yellow] embedded in a stringy matrix on spines [purple] that line the digestive tract of the rat flea, the vector for this pathogen). As the name *Y. pestis* implies, plague is a classic example of a pestilence that can quickly attain epidemic proportions, and often has. But what triggers the emergence of deadly pathogens like *Y. pestis*?

Recent evidence suggests that, under certain conditions, highly virulent pathogens can emerge over a relatively short period of time. In the case of plague, this emergence was closely linked to the appearance of large Eurasian farming settlements of up to 20,000 inhabitants about 6000 years ago. By combining archaeological evidence with molecular analyses of DNA extracted from ancient human remains, researchers investigating the decline of Neolithic European populations discovered that multiple lineages of *Y. pestis*—some now extinct—may have been largely responsible, triggering epidemics more ancient than any previously known. Newly established trade routes (made possible by large-animal domestication, metallurgy, and wheeled transport) connecting densely populated, unsanitary habitations likely created favorable conditions for the transmission of *Y. pestis*, resulting in the sudden collapse of many of the earliest agricultural settlements in Europe.

Studies of this nature reveal just how quickly pathogens can emerge and shape human activities, as observed in recent years with the Ebola and Zika viruses.

Source: Rascovan, N., et al. 2018. Emergence and spread of basal lineages of *Yersinia pestis* during the Neolithic decline. *Cell 176*: 1. doi:10.1016/j.cell.2018.11.005.

In this chapter we focus on pathogenic bacteria and viruses transmitted to humans by animals, arthropods, or soil. Animal-transmitted pathogens have their origins in nonhuman vertebrates, and these infected animal populations can transmit infections to humans. Some arthropods are disease vectors, spreading pathogens to new hosts from a bite. Soilborne pathogens are transmitted to humans through either direct contact with soil or contact with contaminated animal fur or hides. A few of the diseases we will explore in this chapter produce only mild symptoms and are typically self-limiting, but most are highly dangerous with life-threatening symptoms and high mortality rates. These include such dreaded diseases as rabies, hantavirus syndromes, yellow fever, and plague.

Table 32.1 summarizes several of the vectorborne and soilborne diseases we will encounter in this chapter and includes the pathogen responsible for each and its mode of transmission.

TABLE 32.1 Viral and bacterial pathogens of selected vector- and soilborne diseases

Disease	Pathogen	Mode of transmission
Viral diseases		
Rabies	Rabies virus (*Rhabdoviridae*)	Infected mammal bite
Hantavirus pulmonary syndrome	Hantavirus (*Hantaviridae*)	Rodent excreta inhalation/ingestion
Lassa fever	Lassa virus (*Arenaviridae*)	Rodent excreta inhalation/ingestion
Oropouche fever	Oropouche virus (*Peribunyaviridae*)	*Culicoides* biting midge
Yellow fever	Yellow fever virus (*Flaviviridae*)	*Aedes aegypti* mosquito
Dengue fever	Dengue virus (*Flaviviridae*)	*Aedes aegypti* mosquito
Zika virus disease	Zika virus (*Flaviviridae*)	*Aedes aegypti* mosquito
Chikungunya disease	Chikungunya virus (*Togaviridae*)	*Aedes aegypti* mosquito
West Nile fever	West Nile virus (*Flaviviridae*)	*Culex quinquefasciatus* mosquito
Bacterial diseases		
Typhus	*Rickettsia prowazekii*	Body or head louse
Spotted fever rickettsiosis	*Rickettsia rickettsii*	Dog or wood tick
Ehrlichiosis	*Ehrlichia chaffeensis*	Ticks (various)
Q fever	*Coxiella burnetii*	Contact with infected animals
Lyme disease	*Borrelia burgdorferi*	Deer tick
Plague	*Yersinia pestis*	Rat flea
Anthrax	*Bacillus anthracis*[a]	Inhalation/ingestion/broken skin
Tetanus	*Clostridium tetani*[a]	Deep puncture wound
Gas gangrene	*Clostridium perfringens*[a]	Injury causing tissue ischemia

[a]Transmission of these species is typically via endospores found in soil or on soil-contaminated surfaces; in the case of anthrax, inhalation of airborne endospores may occur, leading to pulmonary disease (◀ Section 30.9).

Mastering Microbiology Art Activity: Figure 32.1 Rabies cases in wild and domestic animals in the United States

Mastering Microbiology Art Activity: Figure 32.2 Rabies virus

I • Animal-Transmitted Viral Diseases

Although not as prevalent as arthropod-borne viruses, animal-transmitted viruses, including rabies virus and hantavirus, are some of the most lethal pathogens known. Despite vaccines of high efficacy for use in both animals and humans, rabies alone kills tens of thousands of people every year worldwide.

Some diseases are transmitted from animals to humans. A **zoonosis** is an animal disease transmissible to humans, generally by direct contact, aerosols, or bites (Table 32.1). Immunization and veterinary care can control many infectious diseases in domesticated animals, reducing the transfer of zoonotic pathogens to humans. However, wild animals neither receive veterinary care nor are they immunized, making them a source of potential zoonoses. Diseases in animals may be **enzootic**, present endemically in certain populations, or **epizootic**, with incidence reaching epidemic proportions. In the first part of the chapter we focus on two typically enzootic viral diseases, rabies and hantavirus syndromes, both of which can be transmitted to humans.

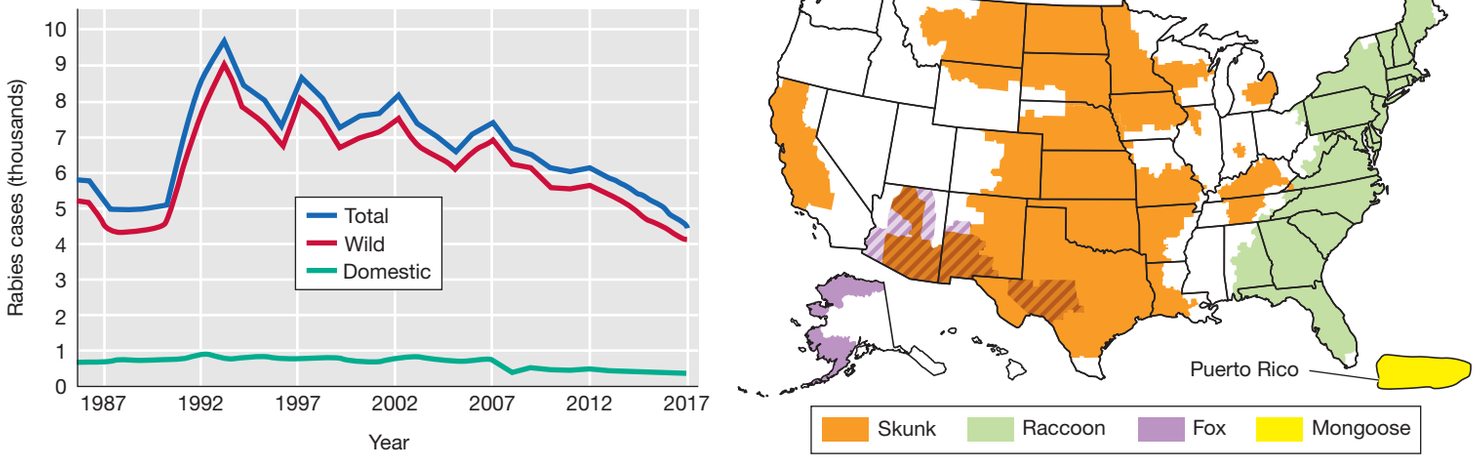
32.1 Rabies Virus and Rabies

Rabies occurs in wild animals, and the major enzootic reservoirs of rabies virus in the United States are raccoons, skunks, coyotes, foxes, and bats. A small number of rabies cases also occur annually in domestic animals (Figure 32.1).

Symptoms and Pathology of Rabies

Rabies is caused by a rhabdovirus, a single-stranded minus-sense RNA virus (◀ Section 11.9) that infects cells of the central nervous system in most endothermic (warm-blooded) animals, almost invariably leading to death once symptoms have developed. The virus (Figure 32.2*a*) enters the body from virus-contaminated saliva through a wound from a bite or through contamination of mucous membranes. Rabies virus multiplies at the site of inoculation and travels to the central nervous system. The incubation period before the onset of symptoms is highly variable and depends on the host; the size, location, and depth of the inoculating wound; and the titer of rabies virions transmitted in the bite. In dogs, the incubation period for rabies is less than 2 weeks. By contrast, in humans, 9 months or more may pass before rabies symptoms become apparent in an infected individual.

Rabies virus proliferates in the brain, especially in the thalamus and hypothalamus. Infection leads to fever, excitation, dilation of the pupils, excessive salivation, and anxiety. In advanced rabies, swallowing triggers uncontrollable spasms of the throat muscles when victims try to drink, ultimately leading to a fear of even the sight of water, a condition called *hydrophobia* (an early name for rabies). Death of rabies victims eventually results from respiratory paralysis. In humans, an *untreated* rabies infection in which symptoms have begun is almost always fatal. Fortunately for both domestic animals and humans, an effective rabies vaccine exists, and this keeps the incidence of rabies low in domestic animals (Figure 32.1*a*) and a rarity in humans in developed countries.



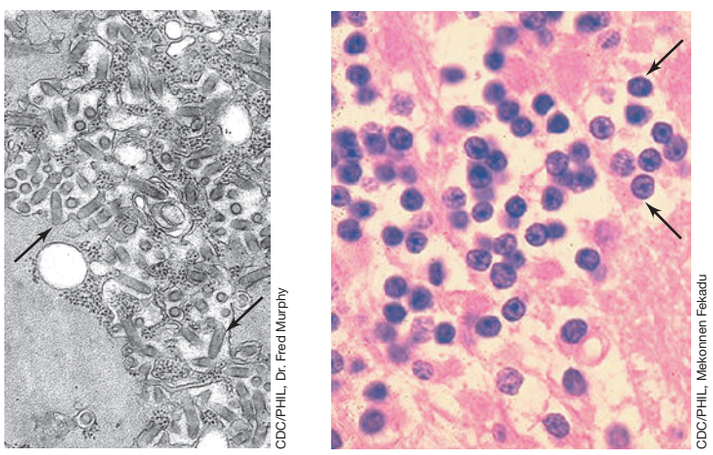
(a) **Incidence of rabies in the United States**

(b) **Major vectors of rabies in the United States and Puerto Rico**

Figure 32.1 Rabies cases in wild and domestic animals in the United States. *(a)* Incidence of rabies by year. Human cases are fewer than five per year. *(b)* Major vectors of rabies virus. In some areas, for example, southwest Texas, both skunks and foxes are the major vectors (shown by hatched lines). Over 90% of all reported rabies cases occur in wild animals. However, actual numbers are probably significantly higher than shown in part *a* because of undiagnosed cases and undiscovered rabid animal carcasses. Bats (not shown) are reservoirs of rabies virus throughout the continental United States and in Alaska. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Diagnosis, Treatment, and Prevention of Rabies

Rabies is diagnosed in the laboratory by examining tissue samples for the virus. Fluorescent antibodies that bind to rabies virus in brain tissues are used to confirm a case of rabies in a postmortem examination. Infected nerve cells stained for light microscopy also show viral inclusions called *Negri bodies* in their cytoplasm, and these characteristic structures confirm rabies virus infection as well (Figure 32.2*b*).



(a) *(b)*

Figure 32.2 Rabies virus. *(a)* The bullet-shaped rabies virions (arrows) shown in this transmission electron micrograph of a tissue section from a rabid animal are about 75 × 180 nm. *(b)* Pathology of rabies in humans. In brain tissue, rabies virus causes characteristic cytoplasmic inclusions called Negri bodies (arrows in photomicrograph), which contain rabies virus antigens. Negri bodies are about 2–10 μm in diameter.

Because rabies is such a serious disease, firm guidelines for treating possible human exposure to rabies have been established, and the details can be found in the rabies section of the World Health Organization website (http://www.WHO.int). In summary, the guidelines state that if a wild or stray animal is suspected of being rabid, it should be immediately examined for evidence of the rabies virus. If a domestic animal, generally a dog, cat, or ferret, bites a human, especially if the bite is unprovoked, the animal should be held in quarantine for 10 days to check for signs of rabies. If the animal exhibits rabies symptoms, or if a definitive diagnosis of its illness cannot be made after 10 days (or if the animal could not be retained for examination), the human should be passively immunized with rabies immune globulin (purified human antibodies to rabies virus) injected at both the site of the bite and intramuscularly. The patient should also be actively immunized with a rabies virus vaccine. Because of the very slow progression of rabies in humans, this combination of passive and active immune therapy (◀ Section 27.2) is nearly 100% effective, stopping the onset of the disease.

Rabies is prevented largely through immunization. An inactivated rabies vaccine is used in the United States for both humans and domestic animals. Prophylactic rabies immunization is practiced for individuals at high risk, such as veterinarians, animal control personnel, animal researchers, and individuals who work in rabies research or rabies vaccine production laboratories. The rabies problem is primarily with wild animals (Figure 32.1), where traditional means of vaccination are impossible. However, experimental trials with an oral rabies vaccine administered in food "baits" have reduced the incidence and spread of rabies in limited geographic areas. If herd immunity (◀ Section 30.2) could be established in some of the key carriers of rabies (Figure 32.1*b*), it might be possible

to reduce incidence of the disease dramatically. Some regions, such as Hawaii and Great Britain, are rabies-free, and any animal imported into these areas is subject to temporary quarantine to ensure the animal is healthy.

Although rabies is vaccine-preventable, nearly 60,000 people per year die from rabies worldwide, primarily in developing countries in Africa and Asia where rabies is enzootic in domestic animals because of inadequate vaccination practices. Worldwide, nearly 14 million people receive prophylactic treatment for rabies after exposure annually, and in the United States, over 20,000 individuals receive such treatment. Fewer than five cases of human rabies are reported in the United States each year, nearly always the result of bites from wild animals, most frequently from bats. Because domestic animals often have exposure to wild animals, dogs and cats are routinely vaccinated against rabies beginning at 3 months of age. Large farm animals, especially horses, are often immunized against rabies as well. One other rare but possible mode of rabies transmission is from organ and tissue transplants. In 2013, a rabies death in the U.S. was linked to a transplanted kidney received from a donor who died from rabies that was misdiagnosed as severe gastroenteritis. Past cases of rabies transmission in a cornea and other transplants have also been documented where the donor had yet to show signs of clinical rabies.

Check Your Understanding

- What is the procedure for treating a human bitten by an animal if the animal cannot be found?
- What major advantage does an oral vaccine have over a parenteral (injected) vaccine for rabies control in wild animals?

32.2 Hantavirus and Hantavirus Syndromes

Hantaviruses cause two severe, emerging diseases, **hantavirus pulmonary syndrome (HPS)**, an acute respiratory and cardiac disease, and **hemorrhagic fever with renal syndrome (HFRS)**, an acute disease characterized by shock and kidney failure. Both diseases are caused by hantaviruses transmitted from infected rodents. Hantavirus is named for Hantaan, Korea, the site of a hemorrhagic fever outbreak where the virus was first recognized as a human pathogen.

Symptoms and Pathology of Hantavirus Syndromes

Similar to influenza viruses, hantaviruses are enveloped viruses (**Figure 32.3*a***) having single-stranded minus-sense RNA genomes arranged in segments (◀ Section 11.9). However, hantaviruses are genetically and functionally more closely related to other hemorrhagic fever viruses, such as Lassa fever virus and Oropouche fever virus (Table 32.1). Hantaviruses asymptomatically infect an estimated 10–15% of rodents, including mice, rats, lemmings, and voles. The virus is transmitted from these reservoirs to humans by inhalation of virus-contaminated rodent excreta. Humans are accidental hosts and are infected only when they come into contact with rodents, their waste, or their saliva.

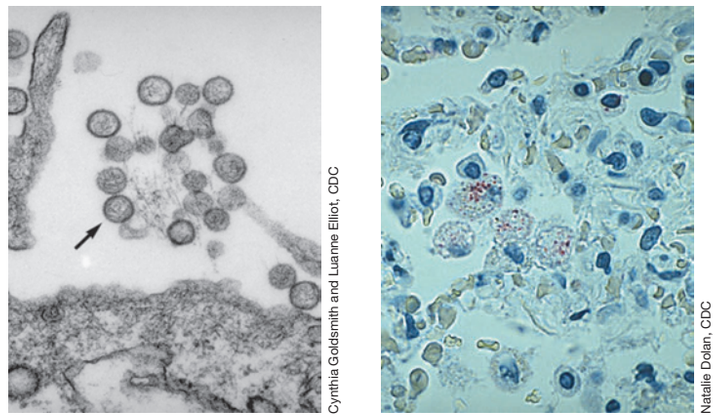
HPS is characterized by a sudden onset of fever, nausea, muscle pain, a reduction in the number of blood platelets along with an increase in the number of circulating leukocytes, and hemorrhaging. Death (if it occurs) takes several days, and it is usually a result of systemic shock and cardiac complications precipitated by leakage of fluid into the lungs, causing suffocation and heart failure. These symptoms are typical of hantaviruses, but other symptoms such as kidney failure are common, depending on the strain of virus causing the disease. HFRS is characterized by intense headache, back and abdominal pain, renal dysfunction, and various hemorrhagic complications. HFRS strains are most prevalent in hantavirus outbreaks in Eurasia, whereas HPS strains are more prevalent in the Americas. At nearly 40%, the mortality rate for HPS strains is significantly higher than for HFRS strains (1–15%).

Hantaviruses can be cultured in the laboratory, but because of the danger involved, they must be handled with precautions for biosafety level 4 (BSL-4; ◀ Section 29.1). In the world of infectious diseases, hantavirus, Ebola, and other BSL-4 viral pathogens are considered "the worst of the worst" and are thus handled in the United States by the Viral Special Pathogens Branch of the Centers for Disease Control and Prevention in Atlanta (Georgia, USA).

Epidemiology, Diagnosis, and Prevention of Hantavirus Syndromes

A significant HPS outbreak in the United States occurred near the Four Corners region of Arizona, Colorado, New Mexico, and Utah in 1993. The outbreak resulted from an enlarged population of deer mice in the spring of 1993. The previous winter was mild and was followed by abundant spring rains, triggering unusually high food levels for the mice. The HPS outbreak caused 27 deaths among 48 infected people (56% mortality), illustrating the potential danger of outbreaks due to pathogens that can be directly transmitted from animal reservoirs. In total from 1993 to January 2017, there were 728 cases of HPS in the United States, with 262 deaths (36% mortality), mostly in western states. On a global basis, it is estimated that 200,000 infections occur annually, chiefly in China, Korea, and Russia, but mortality rates are typically low.

Hantavirus syndromes can be diagnosed using immunological techniques that identify anti-hantavirus antibodies in a blood



(a) (b)

Figure 32.3 Hantavirus. *(a)* A transmission electron micrograph of the Sin Nombre hantavirus. The arrow indicates one of several virions that are about 100 nm in diameter. *(b)* Immunofluorescent staining of Andes hantavirus antigens in alveolar macrophages. Each granular dark blue–stained area indicates cellular infection of an individual macrophage that contains many hantavirus virions (each cell is about 15 μm in diameter).

sample. These include immunoassays (Figure 32.3*b* and ◀ Section 29.5) that detect both exposure to the virus and the strength of the immune response. The presence of the viral RNA genome from circulating virions can also be detected using RT-PCR (◀ Sections 12.1 and 29.8) on patient tissue or blood samples.

There is no virus-specific treatment or vaccine for hantavirus diseases. Treatment amounts to isolation, rest, rehydration, and alleviation of other symptoms. Hantavirus infection can be prevented by avoiding rodent contact and rodent habitat. Destruction of mouse habitat, restricting food supplies (for example, keeping human food in sealed containers), and aggressive rodent extermination measures are the only effective controls; animal surveys have shown that areas that have experienced a hantavirus outbreak have a high proportion of mice that carry the virus.

Check Your Understanding

- Why might hantaviruses be considered a serious public health threat in the United States?
- Describe the spread of hantaviruses to humans. What are some effective measures for preventing infection by hantaviruses?

II • Arthropod-Transmitted Bacterial and Viral Diseases

Arthropods such as mosquitoes and ticks are reservoirs for a diversity of pathogenic bacteria and viruses that infect humans, and these microbes are responsible for considerable human morbidity and mortality anywhere these animal vectors exist.

Pathogens can be spread to new hosts from the bite of an infected arthropod. In the bacterial and viral diseases we consider here—the rickettsial illnesses; yellow and dengue fevers; Lyme, Chikungunya, and Zika virus diseases; and plague—humans are only *accidental hosts* for the pathogen. The *reservoir* of the pathogen is the arthropod vector (Table 32.1). Nevertheless, the diseases can be devastating and often fatal.

32.3 Rickettsial Diseases

The **rickettsias** are small *Bacteria* that live an obligate intracellular existence and are associated with bloodsucking arthropods such as fleas, lice, or ticks. We discussed the biology of rickettsias in Section 16.1. Of the diseases that rickettsias can cause in humans and other vertebrates, the most important are *typhus fever, spotted fever rickettsiosis* (*Rocky Mountain spotted fever*), and *ehrlichiosis*. Rickettsias have not been cultured in artificial culture media but can be grown in laboratory animals, ticks and lice, mammalian tissue culture cells, and the yolk sac of chick embryos (see Figure 32.6*b*). In animals, growth takes place primarily in phagocytes, such as macrophages.

Rickettsias are divided into three groups based on the clinical diseases they cause: (1) the *typhus group*, such as *Rickettsia prowazekii*; (2) the *spotted fever group*, such as *Rickettsia rickettsii*; and (3) the *ehrlichiosis group*, characterized by *Ehrlichia chaffeensis*, and we examine the pathobiological features of each of these now.

The Typhus Group: *Rickettsia prowazekii*

Typhus is transmitted from person to person by the common body or head louse (**Figure 32.4*a***), and humans are the only known mammalian host. During World War I, a typhus epidemic spread throughout Eastern Europe and caused almost 3 million deaths. Typhus has historically been a problem among troops in wartime. Because of the unsanitary, cramped conditions characteristic of wartime military operations, infected lice can spread easily among soldiers with devastating results. Up until World War II, typhus caused more military deaths than did combat.

Cells of *R. prowazekii* are introduced through the skin when a puncture caused by a louse bite becomes contaminated with louse feces that contain the rickettsial cells. During an incubation period of 1–3 weeks, the organism multiplies inside cells lining the small blood vessels. Symptoms of typhus, such as fever, headache, and general body weakness, then begin to appear. Several days later, a characteristic rash is observed in the armpits and generally spreads over the body, except for the face, palms of the hands, and soles of the feet. Complications from untreated typhus include damage to the central nervous system, lungs, kidneys, and heart. Epidemic typhus has a mortality rate of as much as 30%. Tetracycline and chloramphenicol are most commonly used to control infections caused by *R. prowazekii*. *Rickettsia typhi*, the organism that causes murine (mouse) typhus, is another important pathogen in the typhus group and can also infect humans. A typhus vaccine is available but is typically only administered to those traveling to typhus endemic areas, such as certain parts of Africa and South America.

Mastering Microbiology
Art Activity: Figure 32.5 Spotted fever rickettsiosis (Rocky Mountain spotted fever) in the United States, 2010

The Spotted Fever Group: *Rickettsia rickettsii*

Spotted fever rickettsiosis (SFR), also called *Rocky Mountain spotted fever*, was first recognized in the western United States about 1900, but it is more prevalent today in the eastern and mid-South region (**Figure 32.5**). SFR is caused by *R. rickettsii* and is transmitted to humans by various ticks, most commonly the dog tick (Figure 32.4*b*) and wood ticks. The incidence of SFR has been increasing steadily for two decades, with reported cases in the United States increasing from just 500 in the year 2000 to over 6000 in 2017. The emergence of SFR is attributed to increased outdoor recreational activities, such as hiking and camping, in tick-infested areas. Fatalities in treated

CDC/PHIL, James Gathany

(a)

CDC/PHIL

(b)

Figure 32.4 Arthropod vectors of rickettsial diseases. *(a)* The female body louse, about 3 mm long, can carry *Rickettsia prowazekii*, the agent that causes typhus. In addition, the body louse can carry *Borrelia recurrentis*, the agent of relapsing fever, and *Bartonella quintana*, the agent of trench fever. *(b)* The American dog tick that carries *Rickettsia rickettsii*, the causative agent of spotted fever rickettsiosis, is about 5 mm long, but can expand to three times this size when engorged with blood.

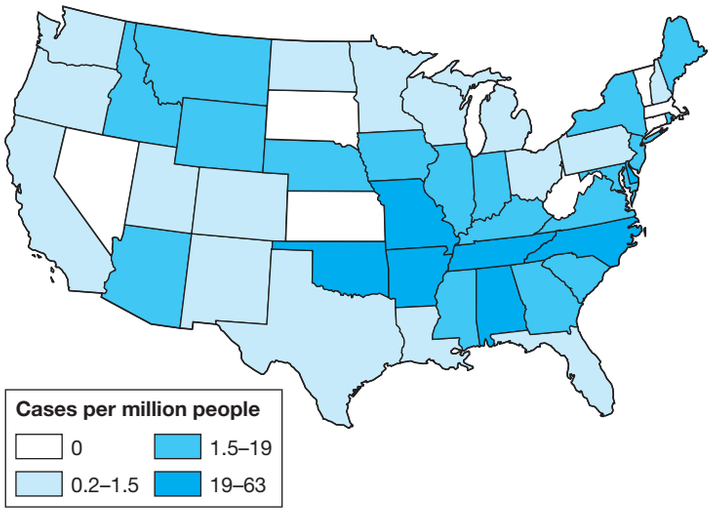


Figure 32.5 Spotted fever rickettsiosis (Rocky Mountain spotted fever) in the United States, 2017. Despite the name, cases of Rocky Mountain spotted fever are currently concentrated in the eastern and mid-South states from Oklahoma to North Carolina.

patients occur in less than 1% of those infected. Humans acquire the pathogen from the bite of an infected tick; rickettsial cells are present in the salivary glands of the tick and in the ovaries of female ticks.

Cells of *R. rickettsii*, unlike other rickettsias, grow within the nucleus of the host cell as well as in host cell cytoplasm (**Figure 32.6*a, c***). Following an incubation period of 3–12 days, characteristic symptoms, including fever and a severe headache, occur. A few days later, a systemic rash breaks out (Figure 32.6*d*) that is distinguished from typhus and viral rashes because it extends over the whole body, even the palms of the hands and soles of the feet. The rash is often accompanied by gastrointestinal complications, such as diarrhea and vomiting. The clinical symptoms of SFR persist for over 2 weeks if the disease is untreated. Tetracycline or chloramphenicol generally promotes a prompt recovery from SFR if administered early in the course of the infection. Mortality in untreated cases of SFR resembles that of typhus, up to 30%. No effective vaccine against SFR is currently available.

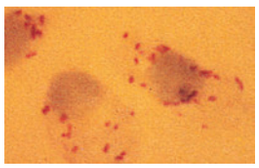
Ehrlichiosis and Tickborne Anaplasmosis

Ehrlichia and related genera (◀ Section 16.1) are responsible for two emerging tickborne diseases in the United States, *human monocytic ehrlichiosis* (*HME*) and *human granulocytic anaplasmosis* (*HGA*). The pathogens that cause HME are *Ehrlichia chaffeensis* and *Rickettsia sennetsu*, and those that cause HGA are *Ehrlichia ewingii* and *Anaplasma phagocytophilum*.

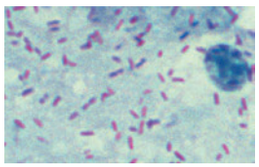
The onset of these clinically indistinguishable rickettsial diseases is characterized by flulike symptoms that can include fever, headache, malaise, changes in liver function, and a reduction in white blood cell numbers. Peripheral blood leukocytes such as monocytes have visible inclusions of rickettsial cells, a diagnostic indicator for the diseases (**Figure 32.7*a***). The symptoms, except for the inclusions, are similar to other rickettsial infections and can range from subclinical to fatal. Long-term complications for progressive untreated cases may include respiratory and renal insufficiency and serious neurological involvement.

HGA and HME are spread by ticks of various species, and mammalian reservoirs of the pathogens include deer, some rodents, and humans. In the United States, HGA occurs primarily in the upper Midwest and coastal New England, while HME is concentrated in the lower Midwest and the East Coast. Similar to SFR, the incidence of HME and HGA have steadily risen over the past 20 years, and together, about 6000 cases are reported each year, with cases of HGA predominating. Diagnosis of rickettsial syndromes is not straightforward because the rash observed can be mistaken for other diseases, such as scarlet fever, or even measles or syphilis. Confirmation of a rickettsial disease requires immunological tests, including fluorescent antibodies or immunoassays, or PCR-based analyses that detect pathogen DNA.

Prevention of HGA and HME is best achieved either by avoiding tick habitat or by wearing tick-proof clothing and applying insect repellents containing diethyl-*m*-toluamide (DEET). It is also good practice to examine yourself carefully for ticks after hiking in tick habitat and to remove any ticks immediately, taking care to remove all tick mouthparts if the tick has already attached. Doxycycline, a tetracycline antibiotic, is the drug of choice for the treatment of HGA and HME. Vaccines are currently unavailable for the prevention of HGA and HME.


Willy Burgdorfer

(a)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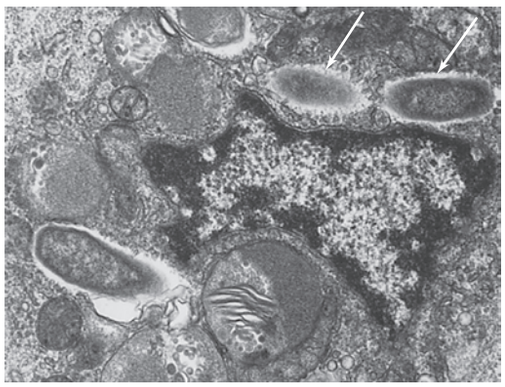
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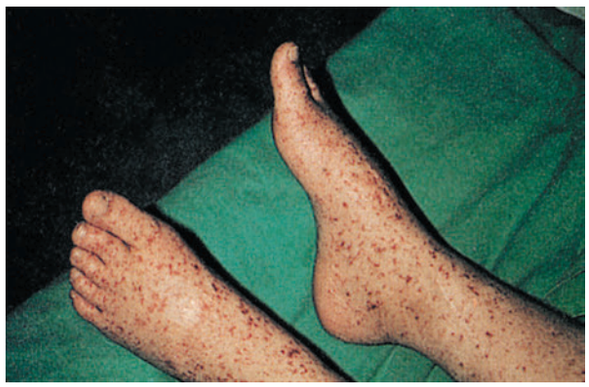
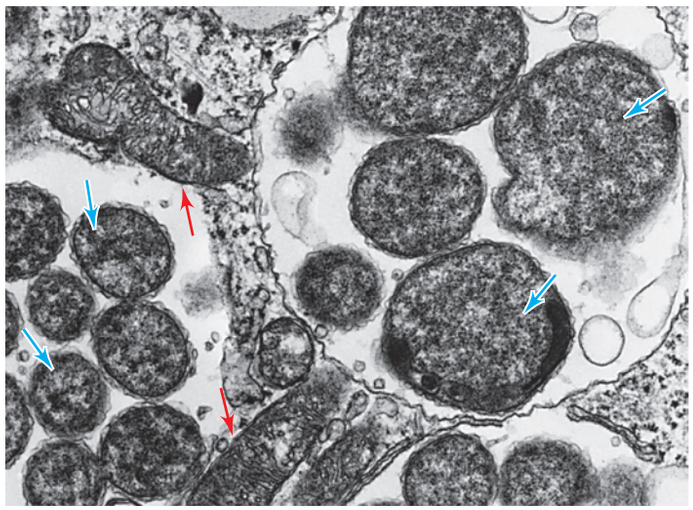

Kenneth E. Greer, Univ. of Virginia School of Medicine

(d)

Figure 32.6 *Rickettsia rickettsii* and spotted fever rickettsiosis. *(a)* Cells of *R. rickettsii*, growing in the cytoplasm and nucleus of tick hemocytes and *(b)* in chicken egg yolk sacs; cells are about 0.4 μm in diameter. *(c)* Transmission electron micrograph of *R. rickettsii* (arrows) in a granular hemocyte of an infected wood tick. *(d)* Rash of spotted fever rickettsiosis on the feet. The whole-body rash is indicative of spotted fever rickettsiosis and helps distinguish it from typhus, in which the rash does not cover the whole body.



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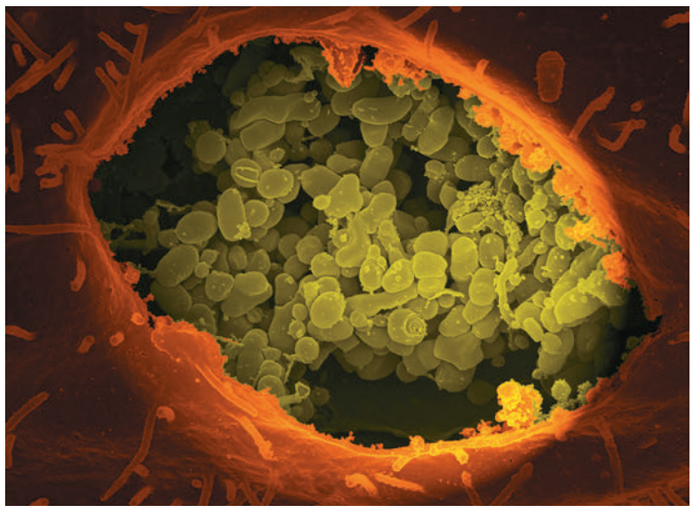


(b)

Figure 32.7 ***Ehrlichia* and *Coxiella*.** *(a) Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (HME). The electron micrograph shows inclusions in a human monocyte that contains large numbers of *E. chaffeensis* cells. The blue arrows indicate bacteria in each inclusion. The *E. chaffeensis* cells are about 0.3–0.9 μm in diameter. Mitochondria are indicated by red arrows. *(b)* Colorized scanning electron micrograph of cells of *Coxiella burnetii*, the causative agent of Q fever. The *Coxiella* cells were grown in animal cell culture and are shown inside a lysed host cell. A single *C. burnetii* cell is about 0.4 μm in diameter.

Q Fever

Q fever (the Q stands for "query") is a pneumonia-like infection caused by the intracellular parasite *Coxiella burnetii* (Figure 32.7*b*), a bacterium related to the rickettsias (◀ Section 16.1). Although not transmitted to humans by an insect bite, *C. burnetii* cells are transmitted to animals such as sheep, cattle, and goats by insect bites, and from these reservoirs are transmitted to humans. Domestic animals generally have asymptomatic infections but may shed large quantities of *C. burnetii* cells in their urine, feces, milk, and other body fluids. Infected animals or contaminated animal products such as wool, meat, and milk are potential sources for human infection. The resulting influenza-like illness can progress to include prolonged fever, headache, chills, chest pains, pneumonia, and endocarditis (inflammation of the inner lining of the heart). In the United States, Q fever is most prevalent in rural states with large farm or ranch animal populations, and about 150 cases have been reported annually in recent years.

As for rickettsial infections, laboratory diagnosis of *C. burnetii* infection is typically made by immunological tests designed to measure host antibodies to the pathogen. Q fever responds well to tetracycline, and therapy should be started quickly in any suspected case to prevent endocarditis and heart valve damage. *C. burnetii* is also a potential biological warfare agent (◀ Section 30.9).

Unlike its relatives in the *Rickettsia* group, the Q fever pathogen *C. burnetii* can now be grown in pure culture outside a host. This was accomplished by considering both the resources and conditions likely to exist in the intracellular host environment along with a careful analysis of the *C. burnetii* genome sequence to reveal the metabolic capacities and limitations of this pathogen. One major discovery gleaned from genomic analysis of the *C. burnetii* complement of cytochromes was the fact that the organism was likely to be microaerophilic (◀ Section 4.16). Indeed, one of the keys to its axenic (pure) culture was to incubate cultures under low oxygen tensions, which is somewhat surprising considering that host cells should be fully oxic.

Check Your Understanding

- What are the arthropod vectors and animal hosts for typhus, spotted fever rickettsiosis, ehrlichiosis, and anaplasmosis?
- What precautions can be taken to prevent rickettsial infections?

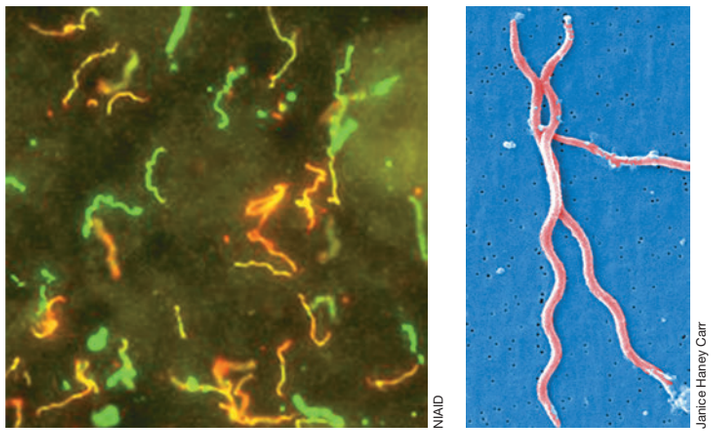
32.4 Lyme Disease and *Borrelia*

Lyme disease is a tickborne disease that affects humans and other animals. Lyme disease was named for Old Lyme, Connecticut, where cases were first recognized, and is currently the most prevalent arthropod-borne disease in the United States. Lyme disease is caused by infection with a spirochete, *Borrelia burgdorferi* (**Figure 32.8**; ◀ Section 15.17), transmitted by a tick bite. The ticks that carry *B. burgdorferi* feed on the blood of birds, domesticated animals, various wild animals, and humans. *B. burgdorferi* is of interest in a nonmedical way, as well, because it is one of only a handful of *Bacteria* that contain a linear (as opposed to a circular) chromosome (◀ Section 6.2).

Pathology, Diagnosis, and Treatment of Lyme Disease

Cells of *B. burgdorferi* are transmitted to humans while the tick is obtaining a blood meal (**Figure 32.9*a***). A systemic infection develops, leading to the acute symptoms of Lyme disease: headache, backache, chills, and fatigue. In about 75% of Lyme cases, a concentric circular or "bull's-eye" rash forms within a week at the site of the tick bite (Figure 32.9*b*). During this acute stage, Lyme disease is readily treatable with tetracycline or penicillin.

Untreated cases of Lyme disease may progress to a chronic stage weeks to months after the initial tick bite, causing arthritis in about half of those infected. Neurological problems such as palsy,



(a) (b)

Figure 32.8 The Lyme disease spirochete, *Borrelia burgdorferi*. *(a)* Fluorescent antibody staining (◀ Section 29.6) of cells of *B. burgdorferi* from a Lyme disease rash. Two different fluorescent antibodies were used, each specific for a different *B. burgdorferi* antigen and linked to either an orange or a green fluorescent tag. If both antibodies bind, cells appear yellow. *(b)* Colorized scanning electron micrograph of cells of *B. burgdorferi*. A single cell is approximately 0.4 μm in diameter and 5–20 μm long.

weakness in the limbs, and heart damage can also occur. In untreated cases, cells of *B. burgdorferi* infecting the central nervous system may lie dormant for long periods before causing additional chronic symptoms, including problems with vision and facial muscle movements, or seizures. Interestingly, the neurological symptoms of chronic Lyme disease mimic those of chronic syphilis, which is caused by a different spirochete, *Treponema pallidum* (◀ Sections 15.17 and 31.13). Unlike syphilis, however, Lyme disease is not spread person to person.

No toxins or other major virulence factors have been identified in Lyme disease pathogenesis, but the pathogen triggers a strong immune response. Antibodies to *B. burgdorferi* appear 4–6 weeks after infection and can be detected by various immunological assays. However, because antibodies to *B. burgdorferi* antigens persist for years after infection, the presence of these antibodies does not necessarily indicate a recent infection. A PCR assay (◀ Sections 12.1 and 29.8) is also in use to detect *B. burgdorferi* DNA in body fluids and tissues. In practice, however, Lyme disease is typically diagnosed from clinical symptoms and only confirmed later by laboratory assays. If a patient has had recent tick exposure and presents Lyme disease signs and symptoms, such as facial tics, arthritis, or the characteristic Lyme rash (Figure 32.9*b*), a presumptive diagnosis of Lyme disease is made and antibiotic treatment is initiated.

Treatment of early-stage Lyme disease is usually with doxycycline or amoxicillin for 20 to 30 days. For patients having neurological or cardiac symptoms, the antibiotic ceftriaxone is administered intravenously because this drug crosses the blood–brain barrier and thus can kill spirochetes in the central nervous system.

Epidemiology and Prevention of Lyme Disease

White-footed field mice and other small rodents are the major mammalian reservoir of *B. burgdorferi* in the northeastern United States, a hotbed of Lyme infection (see Figure 32.11). These animals become infected from bites by the deer tick, *Ixodes scapularis* (**Figure 32.10**), although some other ticks can transmit the Lyme spirochete as well. Deer themselves are not *B. burgdorferi* reservoirs but are major reproductive hosts for the tick. Lyme disease has also been identified in Europe and Asia. In these countries, both the tick vector and the species of *Borrelia* differ from those in the United States, which shows that Lyme disease has a broad geographic distribution. In all cases, however, Lyme disease is caused by related species of pathogenic *Borrelia* transmitted to humans by tick vectors.

Deer ticks are typically smaller than many other ticks (Figure 32.9*a*), making them easy to overlook. Moreover, unlike the case with ticks that carry other tickborne diseases (Figure 32.4*b*), a high percentage of deer ticks carry *B. burgdorferi*. Both of these factors—small vector size and high occurrence of the pathogen—undoubtedly contribute to the fact that Lyme is the most commonly reported vectorborne disease in the United States (**Figure 32.11**). Most cases of Lyme disease in the United States have been reported from the Northeast and upper Midwest—areas of the country where deer

(a) (b)

Figure 32.9 Lyme disease infection. *(a)* A deer tick (here, blood-engorged) obtaining a blood meal from a human is the route of disease transmission (see Figure 32.10 for photos of deer ticks). The tick is about the size of the head of a pin (about 1 mm in diameter). *(b)* Characteristic "bull's-eye" rash of Lyme disease. The rash starts at the site of a tick bite and grows in a concentric circular fashion over a period of several days. A typical rash is 5–8 cm in diameter.

Figure 32.10 Deer ticks, the major vector of Lyme disease. Left to right, male and female adult ticks, nymph, and larva forms. The length of an adult female is about 3 mm. Although all forms feed on humans, the female nymphal and adult ticks are principally responsible for transmitting *Borrelia burgdorferi*.

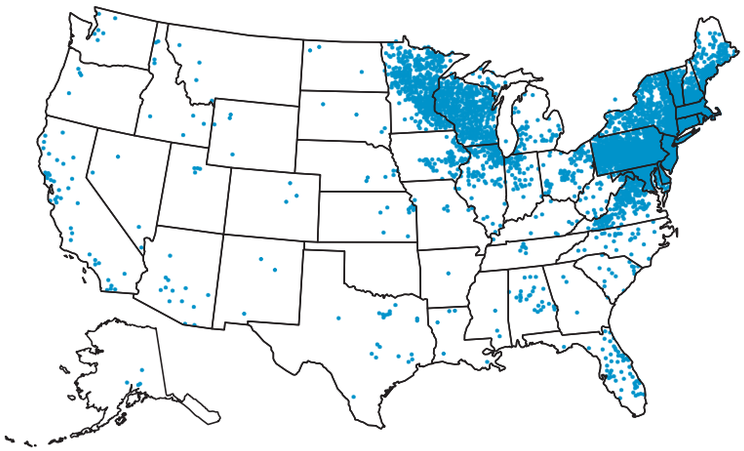


Figure 32.11 Lyme disease in the United States, 2017. Each dot represents a confirmed case. Confirmed and probable cases in 2017 totaled nearly 43,000, with about 95% of these localized to 13 states in the upper regions of the Midwest and East Coast. Lyme disease is reported through the National Notifiable Diseases Surveillance System of the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

and wooded areas are abundant—but cases have been observed throughout North America (Figure 32.11). The incidence of Lyme disease in the United States is significant, with 42,743 confirmed and probable cases reported in 2017.

As for any tickborne infection, prevention of Lyme disease begins by avoiding contact with the vector. Insect repellents containing DEET or the wearing of snug-fitting clothing is helpful, as is a thorough body exam following walks in tick-infested environments. Lyme disease vaccines are available for domestic animals, but no human Lyme disease vaccine is currently in use.

Check Your Understanding

- What are the primary symptoms of Lyme disease?
- In the United States, where is Lyme disease most prevalent?
- Outline methods for prevention of *Borrelia burgdorferi* infection.

32.5 Yellow Fever, Dengue Fever, Chikungunya, and Zika

Several arthropod-transmitted diseases are caused by flaviviruses. These are single-stranded plus-sense RNA viruses (◀ Section 11.8) transmitted by the bite of an infected arthropod. Because of this characteristic mode of transmission, these viruses are also called *arboviruses* (*ar*thropod-*bo*rne viruses).

Many serious human diseases are caused by arboviruses including various types of encephalitis and hemorrhagic fevers. Here we consider two potentially fatal flavivirus diseases common in some tropical and subtropical regions: yellow fever and dengue fever. Some of the symptoms of these diseases are similar, and both viruses are transmitted by the same vector, female mosquitoes of the genus *Aedes* (**Figure 32.12**). This insect vector infects nearly half a *billion* people each year with dangerous viral pathogens, which, in addition to yellow fever and dengue viruses, include Zika and Chikungunya viruses.

Yellow Fever

Yellow fever is an endemic disease of tropical and subtropical climates, especially in Latin America and Africa. Brazil, Colombia, Venezuela, and parts of Bolivia and Peru, along with most countries in sub-Saharan central Africa, experience the greatest incidence. Yellow fever is absent from the United States except in unvaccinated individuals who contract the disease through travel to an endemic area. Yellow fever virus is related to dengue virus (see later), West Nile virus (Section 32.6), and certain encephalitis viruses. Yellow fever is one of only a handful of infectious diseases for which isolation and quarantine are practiced (◀ Section 30.5). In the case of yellow fever, although the disease is not transmitted person to person, isolation of active cases prevents local mosquitoes from taking a blood meal from the infected individual and transmitting the disease to others.

Following a bite from an infected mosquito, the yellow fever virus replicates in lymph nodes and certain immune system cells and eventually travels to the liver. Once a person is infected, symptoms range anywhere from none to major organ failure and death. Most infected individuals display a mild fever with accompanying chills, a headache and back pains, nausea, and other symptoms that are not diagnostically useful. Presumably these are cases in which the immune system has the infection under control. However, in about one in five yellow fever cases, the disease enters its toxic phase, characterized by jaundice (thus the name *yellow fever*) and by hemorrhaging from the mouth, eyes, and gastrointestinal tract. This triggers the onset of bouts of bloody vomit, and if bleeding continues, it leads to toxic shock and multiple organ failure. About 20% of cases that reach this stage are fatal. Humans and nonhuman primates are the main reservoirs for the yellow fever virus.

Yellow fever is fully preventable by an effective vaccine. An attenuated yellow fever vaccine was developed in the 1930s (◀ Figure 25.10) and widely used by military and support personnel in tropical battlefields. Historically, the disease has been controlled by a combination of vaccination and elimination of both the vector (mosquito) population by chemical agents and vector breeding grounds by draining swamps and low-lying wetlands in endemic areas.

The yellow fever vaccine is highly recommended for those traveling to endemic areas, and many countries require proof of vaccination for anyone entering their country from a foreign country where yellow fever is endemic. In addition, the World Health Organization (WHO) has initiated a mass vaccination program in Africa. Despite the availability of a vaccine, the WHO estimates that each year nearly 200,000 cases of yellow fever occur, mostly unreported, and that about 30% of all cases are fatal. Other than palliative care, no treatment for yellow fever is available. Once the disease is diagnosed, typically by detecting anti–yellow fever virus antibodies in a blood sample, the patient is isolated and prescribed rest and drugs to control symptoms. Recovery without entering the toxemia stage is due to the immune response.

Dengue Fever

Like yellow fever, dengue (pronounced deng-gay) fever is transmitted by mosquitoes of the genus *Aedes* (Figure 32.12) and is a disease of tropical and subtropical regions. It is estimated that as much as *one-half* of the world's population lives in areas where dengue is endemic, and therefore it is no surprise that it is the most common vectorborne

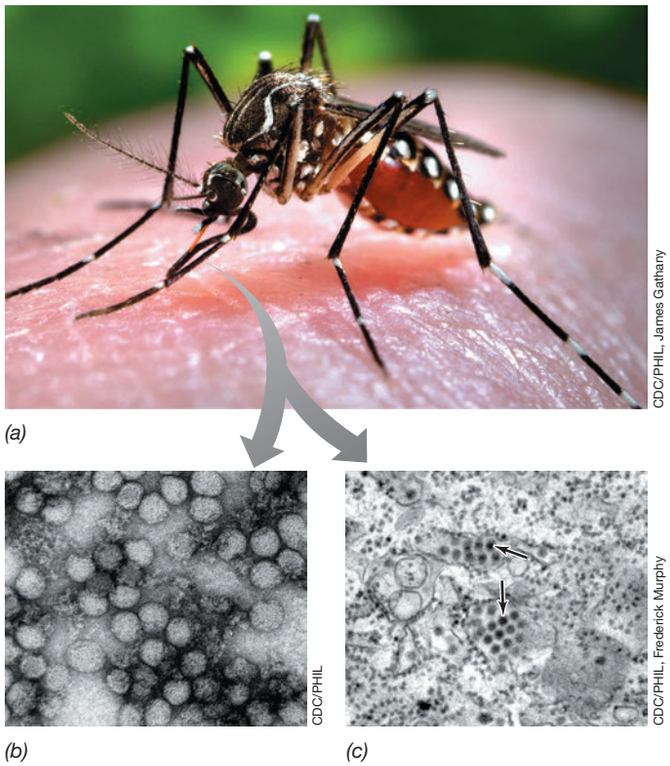


Figure 32.12 Yellow fever and dengue fever. *(a)* Yellow and dengue fever viruses are both transmitted by the bite of an infected *Aedes aegypti* mosquito. Transmission electron micrographs of *(b)* yellow fever virus and *(c)* dengue fever virus (arrows, in a tissue specimen). Both yellow and dengue fever viruses are about 50 nm in diameter and are plus-sense RNA viruses that replicate by way of polyprotein formation, as in poliovirus (◀ Figure 11.21).

disease in humans. About 100 million cases of dengue fever occur worldwide per year, with concentrations in Mexico, Central and South America, India, Indonesia, and Africa (◀ Figure 30.11).

Dengue begins with a high fever and headache or joint pains and in some patients, severe eye pain and a systemic rash. Most infected individuals show self-improvement within a week and no further symptoms, presumably because of an immune response to the dengue virus. But, as for yellow fever, a dengue infection can take a more severe course of events and proceed to *dengue hemorrhagic fever*. This condition is characterized by severe symptoms that can include bleeding from the nose and gums, bloody vomit and/or feces, intense abdominal pain, respiratory distress, and a general feeling of malaise. The blood pressure of a dengue patient can drop dramatically during the hemorrhagic fever stage, and a small percentage of these cases are fatal. Treatment for dengue is primarily to relieve symptoms, particularly dehydration from fever and loss of blood and other fluids.

A live recombinant vaccine (◀ Sections 12.8 and 28.3) marketed as Dengvaxia® is available for dengue in high-risk regions. However, because the vaccine has been shown to increase the risk of severe infection in those never before exposed to the virus, its use is restricted only to those that have already had a confirmed case of dengue fever (the same individual can be infected more than once with dengue virus). Because there is currently no point-of-care rapid diagnostic test to confirm prior exposure, use of the vaccine is significantly limited.

Dengue can be controlled by eliminating either the vector or contact with the vector. Extensive chemical spraying for mosquito eradication was widely practiced in urban centers of the southern United States and kept dengue in check in the twentieth century. Now, however, spraying programs are less common and global climate change is moving tropical temperature patterns northward. In response, the *Aedes* mosquito has become entrenched in southern and central regions of the United States. Besides *Aedes aegypti* (Figure 32.12*a*), the Asian tiger mosquito (*Aedes albopictus*), which is spreading quickly in the United States, also carries the dengue virus. Drainage of small sources of stagnant water, such as those in discarded tires or clogged gutters, removes mosquito breeding grounds and greatly reduces the opportunities for a dengue outbreak. Personal protection from mosquito bites by using effective insect repellents and clothing is also a proven means of preventing infection.

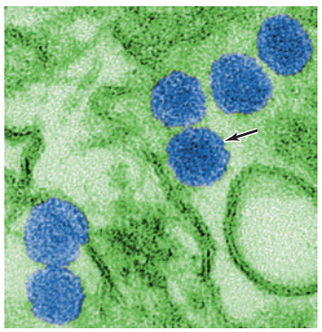
A recent strategy to control mosquito populations (and thus mosquito-borne disease transmission) is to employ the endosymbiotic bacterium *Wolbachia* (a close relative of *Rickettsia* and *Ehrlichia*) to control the reproductive success of the mosquito vector (◀ Section 23.7). Male mosquitoes infected with *Wolbachia* bacteria produce sperm that express proteins that render uninfected females infertile upon mating. Although not fully understood, the *Wolbachia*-derived proteins in the sperm activate a molecular switch that prevents mosquito reproduction by inhibiting embryo development. When *Wolbachia*-infected male mosquitoes are purposely released in areas where dengue is endemic, the incidence of dengue has decreased significantly, presumably because of reductions in the size of the local mosquito population.

The WHO estimates that nearly a half million cases of dengue hemorrhagic fever occur yearly with over 20,000 being fatal. Cases of dengue in the United States are rare, and many of them are imported from endemic areas. Dengue is a prime example of an emerging disease (◀ Section 30.7), as cases were geographically restricted until the mid-twentieth century when global commerce is thought to have transmitted species of the *Aedes* mosquito beyond their original range.

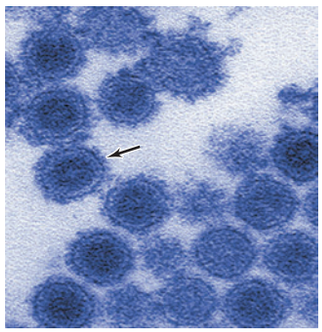
Zika and Chikungunya Disease

Zika and Chikungunya are both typically mild viral diseases transmitted by the same mosquito vector (Table 32.1). Zika virus disease is characterized by headache, fever, joint pain, and general malaise, and a rash is occasionally seen. The disease is caused by the Zika virus (**Figure 32.13*a***), a relative of the dengue virus. Zika first emerged over 65 years ago in the Zika forest (Uganda), and occasional small outbreaks occurred periodically in west central Africa and Indonesia. Zika appeared in Brazil in 2015, and by 2016, outbreaks of Zika virus disease were reported in the United States, primarily in individuals who had traveled to areas with endemic disease. However, a major outbreak in the U.S. territory of Puerto Rico has been linked to local mosquito-borne transmission, and the disease may spread northward within the range of the *Aedes* mosquito. Zika infections have been reported in Florida in recent years and may eventually spread to other regions of the southern United States.

Besides vectorborne transmission, Zika can be transmitted through sexual contact and by contaminated blood and, of most concern, from mother to fetus. The incidence of brain abnormalities, especially microcephaly, and other birth defects have been linked to



(a)



(b)

Figure 32.13 Zika and Chikungunya viruses. Both viruses contain single-stranded plus-sense RNA genomes and are surrounded by an envelope (◀ Sections 5.1, 5.2, and 11.8). *(a)* Colorized transmission electron micrograph of virions of the Zika virus embedded in a tissue sample. A single virion (shown in blue, arrow) is about 40 nm in diameter. *(b)* Colorized transmission electron micrograph of virions of Chikungunya virus (arrow). A virion is about 50 nm in diameter.

infants born to Zika-infected mothers, and so Zika in some way affects development of the fetus. It is known that the Zika virus readily infects a type of neural cell that eventually forms the cerebral cortex, a major part of the brain that governs intellectual capacity, and this likely leads to the brain defects observed in Zika-infected newborns. This danger has led to advisories for pregnant women to take great precautions to avoid contact with mosquitoes during the entire pregnancy period. Other than in pregnant women, Zika virus disease seems to be a rather mild, self-limiting infection. However, in rare instances, Zika infection may trigger Guillain–Barré syndrome, an autoimmune disease (◀ Section 28.1) caused by an infection with any of several different viruses and bacteria that results in the immune system attacking the peripheral nervous system.

Too little is known about the Zika virus and the various conditions it can or might cause to draw firm conclusions about the seriousness of this threat to public health, but it is clear that the virus is capable of causing explosive epidemics under certain conditions. Deaths directly attributable to a Zika infection have been rare, and thus the pathogen seems to be of only passing concern to the general public, even in the typical high-risk groups for infectious diseases, such as the very young or very old. However, the birth defect connection is extremely serious. Currently, it seems that pregnant women (or those trying to get pregnant, as Zika can also be transmitted by sexual intercourse) are the only group known to be at serious risk from Zika infection. No Zika vaccine is currently available, but several candidate vaccines are in development.

Like Zika, Chikungunya disease is caused by a single-stranded plus-sense RNA virus (Figure 32.13*b*), but the virus, of the family *Togaviridae*, is not closely related to the Zika, dengue, and yellow fever viruses (Table 32.1). Chikungunya virus is transmitted by species of *Aedes* mosquitoes and is currently endemic in South and Central America, Southeast Asia, central Africa, and Indonesia. Thus far, cases of Chikungunya in the United States have been limited to travelers returning from endemic areas, and in 2017, a total of 156 confirmed cases were reported. As for Zika, mortality in Chikungunya disease is rare, about 0.1% of all cases. The symptoms of Chikungunya are mild and self-limiting and the immune response to the virus is strong, conferring active immunity against reinfection. Unlike with Zika, no direct connection between Chikungunya viral infection and birth defects has been observed because Chikungunya virus is not transmitted from mother to fetus.

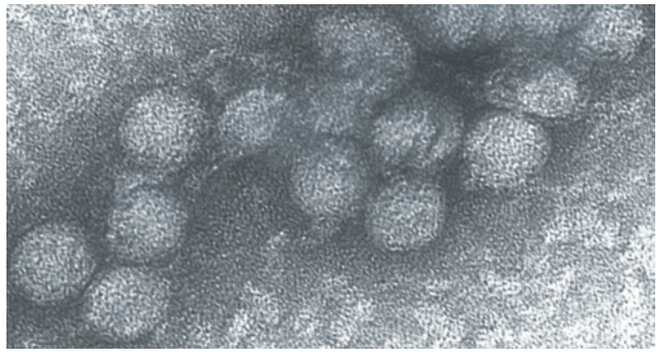
Check Your Understanding

- Identify the vector and reservoir for yellow fever and dengue viruses.
- Contrast the procedures for preventing infection in yellow and dengue fevers.
- Why is Zika virus disease considered dangerous even though it rarely kills?

32.6 West Nile Fever

West Nile virus (WNV) causes **West Nile fever**, a seasonal, human viral disease that is transmitted through a variety of mosquito vectors (**Figure 32.14*a***). Like the yellow fever, dengue, and Zika viruses, WNV is a flavivirus (Table 32.1) having an enveloped capsid (Figure 32.14*b*) and containing a plus-sense single-stranded RNA genome (◀ Section 11.8). The virus can invade the nervous system of its endothermic (warm-blooded) hosts, which include certain species of birds and mammals.

(a)



(b)

Figure 32.14 West Nile virus. *(a)* The mosquito *Culex quinquefasciatus*, shown here engorged with human blood, is a West Nile virus vector. *(b)* An electron micrograph of the West Nile virus. The icosahedral virion is about 40–60 nm in diameter.

WNV Transmission and Pathology

WNV causes active disease in over 100 species of birds and is transmitted to different hosts by over 40 species of mosquito, including *Culex quinquefasciatus* (Figure 32.14*a*), which is common in central and eastern portions of the United States and in urban centers throughout the country. Infected birds develop a systemic viral infection (viremia) that is often fatal. Mosquitoes feeding on viremic birds are infected and can then infect other susceptible birds, renewing the cycle. In contrast to birds, humans and other animals are dead-end hosts for the virus because they do not develop the viremia necessary to infect mosquitoes.

Mortality rates for WNV infection are species-specific. For example, the human mortality rate from WNV is about 4%, whereas that for horses is significantly higher at nearly 40%. Most human infections are asymptomatic or mild and are not reported. After an incubation period of 3–14 days, about 20% of infected individuals develop West Nile fever, a mild illness lasting 3–6 days. The fever may be accompanied by headache, nausea, muscle pain, rash, lymphadenopathy (swelling of lymph nodes), and malaise. Less than 1% of infected individuals develop serious neurological diseases such as *West Nile encephalitis* or *West Nile meningitis* from viral replication in neural tissues (**Figure 32.15**). These are more common in adults over age 50, and the neural effects may be permanent. About 5% of West Nile cases that progress to these forms are fatal. Diagnosis of WNV disease includes assessment of clinical symptoms followed by confirmation by immunological tests that detect WNV antibodies in blood.

Control and Epidemiology of WNV

Human WNV disease was first identified in the West Nile region of Africa in 1937 and spread from there to Egypt and Israel. In the 1990s there were WNV outbreaks in horses, birds, and humans in African and European countries. The first North American cases of WNV were reported in the northeast United States in 1999, and in the years since, the disease has spread to every state in the continental U.S. (**Figure 32.16**). In the 1999–2017 period, the number of reported WNV cases per year has fluctuated wildly, from as few as about 20 to as many as 9800. The major U.S. foci of infection appear to be in the south-central states and the Great Plains from the Southwest to the Canadian border (Figure 32.16). West Nile disease is now enzootic in the bird population in the United States and in only low incidence in the human population, its accidental host.

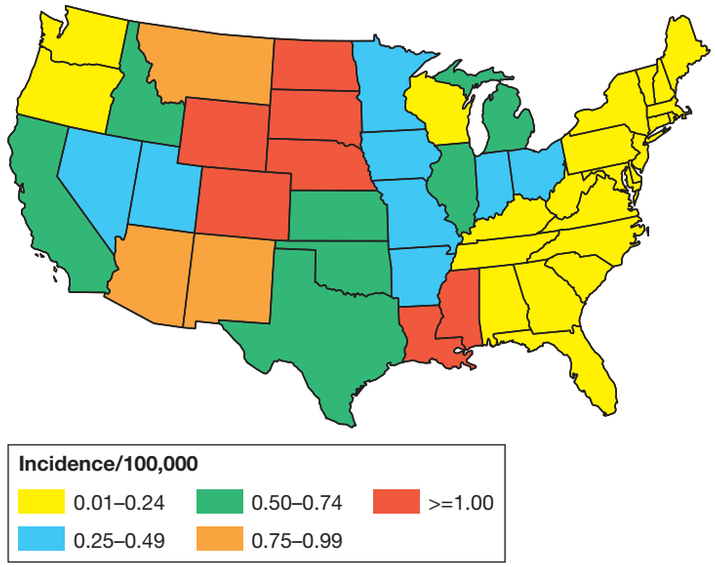


Figure 32.16 Average annual incidence of West Nile disease in the United States, 1999–2017. In 2017, 2097 cases of human West Nile disease were reported, resulting in 146 deaths (7% mortality). West Nile virus is now endemic in mosquitoes and birds throughout the United States. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Control of WNV illness is much the same as for other vectorborne diseases: Limit exposure to mosquitoes by using insect repellents or wearing tight-fitting, lighter-colored clothing. Spraying for mosquitoes has limited effectiveness, but removal of mosquito breeding grounds, particularly sources of standing water, helps control mosquito populations. A veterinary WNV vaccine is widely used in horses where the mortality risk demands it, but no human WNV vaccine is currently available.

Check Your Understanding

- Identify the vector and reservoir for West Nile virus.
- Trace the progress of West Nile virus in the United States since 1999.

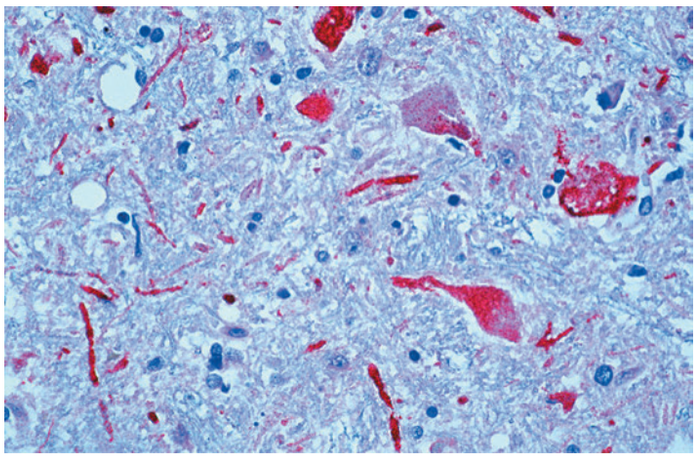
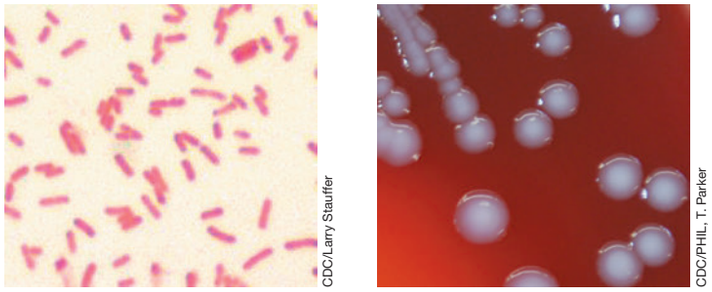


Figure 32.15 West Nile encephalitis. Brain section from a West Nile encephalitis victim. Red areas in the tissue are neurons containing West Nile virus as detected using an immunofluorescent staining technique (◀ Section 29.6).

32.7 Plague

Plague has caused more human deaths than any other bacterial disease in recorded history; only smallpox and measles have killed more among all infectious diseases. Plague is primarily a zoonosis of wild rodents, but humans can become accidental hosts when rodent populations experience a die-off and the disease vector, the rat flea, seeks alternative sources of a blood meal. Plague is caused by *Yersinia pestis*, a gram-negative, facultatively aerobic, rod-shaped, and encapsulated enteric bacterium (*Gammaproteobacteria*, ◀ Section 16.3) that is easily grown in laboratory culture (**Figure 32.17**).



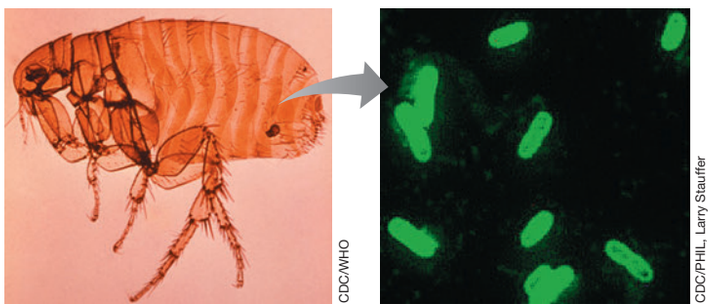
(a) (b)

Figure 32.17 ***Yersinia pestis.*** *(a)* Gram stain of cells of *Yersinia pestis*. The cells are about 0.8 μm in diameter. *(b)* Colonies of *Y. pestis* grown on blood agar.

Pathology and Treatment of Plague

The pathogenesis of plague is not clearly understood, but cells of *Y. pestis* produce several virulence factors that contribute to the disease process. The V and W antigens in *Y. pestis* cell walls are protein–lipoprotein complexes that inhibit immune cell phagocytosis. *Murine toxin*, an exotoxin (◀ Section 25.6) that is lethal for mice, is produced by virulent strains of *Y. pestis*. Murine toxin is a respiratory inhibitor that causes systemic shock, liver damage, and respiratory distress in mice. The toxin likely plays a role in human plague as well, because these symptoms are common in plague patients. *Y. pestis* also produces a highly immunogenic endotoxin (◀ Section 25.8) that may play a role in the disease process.

Plague can occur in several forms (see Figures 32.19 and 32.20). *Sylvatic plague* is enzootic among wild rodents. Plague is transmitted by several species of fleas, a main one being the rat flea *Xenopsylla cheopis* (**Figure 32.18*a***). Fleas ingest *Y. pestis* cells in a blood meal, and the bacterium (Figure 32.18*b*) multiplies in the flea's intestine. From there, the infected flea transmits the disease to rodents or humans in the next bite. The most common form of plague in humans is *bubonic plague*. In this case, cells of *Y. pestis* travel to the lymph nodes, where they replicate and cause swelling. The regional and pronounced swollen lymph nodes are called *buboes*, and the disease gets its name from these structures (**Figure 32.19*a***). The buboes become filled with *Y. pestis* cells, and the bacterium's capsule prevents phagocytosis and



(a) (b)

Figure 32.18 **The rat flea, a major vector of plague.** *(a)* The rat flea *Xenopsylla cheopis* carries cells of *Yersinia pestis*. The bacterium replicates in the flea gut and *(b)* cells of *Y. pestis* are transmitted to a host in a flea bite. The rat flea was the major vector for the pandemics of plague that ravaged medieval Europe in the fourteenth century. Cells in part *b* were stained with a fluorescent antibody prepared against *Y. pestis* cell surface antigens.

(a) (b)

Figure 32.19 **Plague in humans.** *(a)* A bubo (arrow) formed in the groin. *(b)* Gangrene in the hand of a plague victim.

destruction by cells of the immune system. Secondary buboes form in peripheral lymph nodes, and cells eventually enter the bloodstream, causing septicemia. Multiple local hemorrhages produce dark splotches on the skin and eventual tissue necrosis, giving plague its historical name, the "Black Death" (Figure 32.19*b*). If the infection is not treated quickly, the symptoms of plague (lymph node swelling and pain, prostration, shock, gangrene, and delirium) usually progress quickly, causing death within 3–5 days.

Pneumonic plague occurs when cells of *Y. pestis* are either inhaled directly or reach the lungs via the blood or lymphatic circulation. Significant symptoms are usually absent until the last day or two of the disease when large amounts of bloody sputum are produced. Greater than 90% of untreated cases of pneumonic plague result in death within 48 h. Moreover, pneumonic plague is highly contagious and can spread rapidly from person to person if those infected are not immediately isolated. *Septicemic plague* is the rapid spread of *Y. pestis* throughout the body via the bloodstream without the formation of buboes and is so severe that it usually causes death before a diagnosis can be made.

Bubonic plague can be successfully treated with streptomycin or gentamicin, administered by injection. Alternatively, doxycycline, ciprofloxacin, or chloramphenicol may be given intravenously. If treatment is started promptly, mortality from bubonic plague can be reduced to less than 5%. Pneumonic and septicemic plague can also be treated, but these forms progress so rapidly that antibiotic therapy, even if begun when symptoms first appear, is usually too late.

Mastering Microbiology
Art Activity: Figure 32.20 Plague epidemiology

Plague Epidemiology and Control

Sylvatic plague is enzootic in a variety of rodents, including ground squirrels, prairie dogs, chipmunks, and mice; rats are the primary hosts in urban communities and were typically the hosts in episodes of sylvatic plague that triggered human pandemics during the Middle Ages. Fleas are intermediate hosts and vectors for plague, spreading the disease between rodent hosts and humans (**Figure 32.20**). Most infected rats or other rodents die soon after symptoms appear, but a small proportion of survivors develop a chronic infection, providing a persistent reservoir of *Y. pestis* cells to fuel new outbreaks.

Plague is endemic in developing countries in Africa, Asia, the Americas, and in south-central Eurasia; most cases occur in sub-Saharan

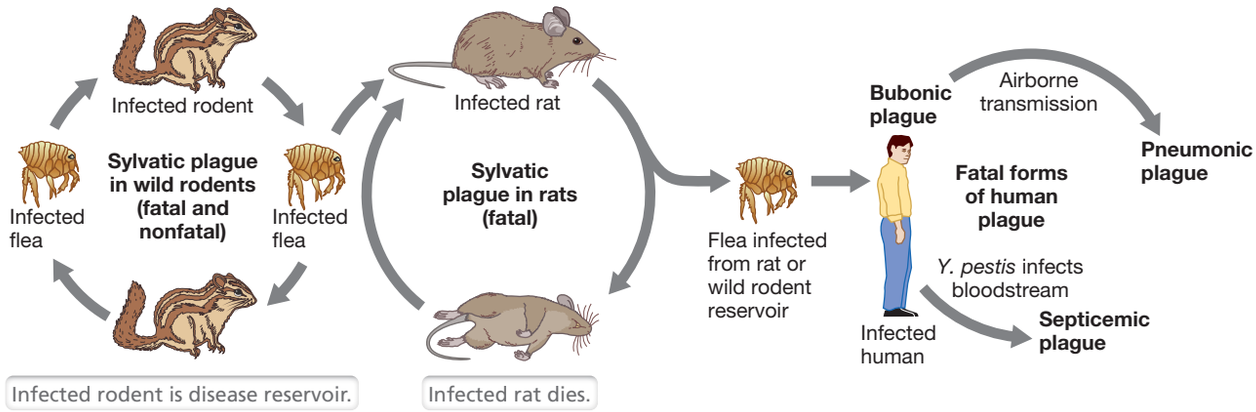


Figure 32.20 Plague epidemiology. In some wild rodents, sylvatic plague causes only a mild infection, but diseased animals remain a reservoir of *Yersinia pestis*. In rodents such as rats that act as disseminating hosts, as well as in humans, plague is often fatal. When the domestic rodent reservoir dies off in an epidemic, infected fleas seek alternate hosts in humans.

Africa. Pandemic plague was historically associated with unsanitary surroundings, a major factor supporting large rat populations. In sparsely populated rural areas, this is not so great a problem as the disease runs its course when the rodent population dies off, leaving a shortage of hosts. But in urban centers where alternative hosts (humans) are plentiful, an outbreak of sylvatic plague can set the stage for a human plague epidemic. In the United States only a handful of cases of plague are diagnosed annually, mostly in southwestern states (New Mexico, Colorado, and Arizona in particular) where sylvatic plague is enzootic among wild rodents (Figure 32.20). In 2017, only five cases of human plague were reported, with no deaths. From 2000 through 2016 exactly 100 cases were reported, including 12 fatalities.

Plague control is accomplished through good sanitation practices, surveillance and control of rodent reservoirs and vectors (fleas), isolation of active cases, and imposing quarantine on those who have had contact with diseased individuals. Improved public health practices and the control of rodent populations are the major reasons that outbreaks of plague are extremely rare in developed countries.

Check Your Understanding

- Distinguish among sylvatic, bubonic, septicemic, and pneumonic plague.
- Describe the insect vector, the natural host reservoir, and the treatment for plague.

III • Soilborne Bacterial Diseases

Soil is a reservoir for several formidable bacterial pathogens of humans, including certain species of the genera *Bacillus* and *Clostridium*. Although humans are only accidental hosts, these bacteria produce potent exotoxins that cause life-threatening illnesses.

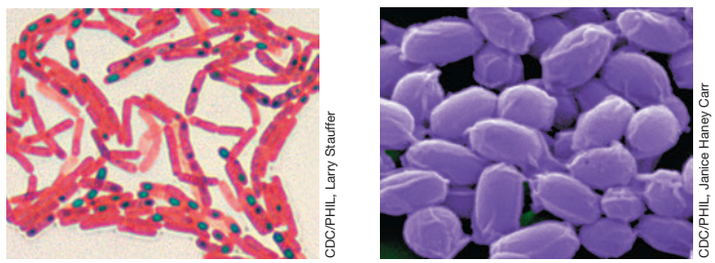
32.8 Anthrax

Some human diseases are caused by microorganisms whose major habitat is soil (Table 32.1), and anthrax is an excellent example. We covered some aspects of the disease anthrax in Section 30.9 in the context of its use as a potential bioterrorism or biological warfare agent. Here we focus more on the biology of the organism and the disease process.

Discovery and Properties of Anthrax

The famous pioneering medical microbiologist Robert Koch (◀ Section 1.12) first isolated the causative agent of the disease anthrax, the endospore-forming bacterium *Bacillus anthracis* (**Figure 32.21**). Using mice caught in the wild as experimental animals, Koch used the disease anthrax to develop his principles for linking cause and effect in infectious disease—Koch's postulates (◀ Figure 1.33). Anthrax is quickly fatal in mice, but in humans, anthrax can take on several different forms, from mild to severe skin infections to respiratory failure and death.

Anthrax is an enzootic disease (an endemic disease of animals) of worldwide occurrence. *B. anthracis* lives a saprophytic existence in soils, growing as an aerobic chemoorganotroph and forming endospores (◀ Section 2.8, Figure 32.21) when conditions warrant. From soil, cells or endospores of *B. anthracis* can become embedded in animal hair, hides, or other animal materials, or can be ingested, and from here the disease can develop, allowing *B. anthracis* to be transmitted to humans. Anthrax is primarily seen



(a) (b)

Figure 32.21 *Bacillus anthracis*. The anthrax pathogen produces endospores. *(a)* Light micrograph of a malachite green–stained smear of *B. anthracis* cells showing greenish-blue endospores. *(b)* Colorized scanning electron micrograph of *B. anthracis* endospores. Cells of *B. anthracis* are about 1.2 μm in diameter.

in farm animals, particularly in cattle, sheep, and goats, and is transmitted from them to humans.

Forms of Human Anthrax

The disease anthrax can manifest itself in one of three forms: cutaneous (on the skin), intestinal, and respiratory (inhalation anthrax). In all forms, disease symptoms are due to two major toxins called *lethal toxin* and *edema toxin*. The different forms of anthrax show increasing severity, which is primarily a function of where in the body these toxins are excreted. Growth and toxin production by *B. anthracis* in the lymph nodes and lymphatic tissues leads to progressively worsening symptoms, beginning with a sore throat, muscle aches, and fever, and escalating to respiratory distress and systemic shock. In addition to anthrax toxins, the unusual protein capsule that surrounds cells of *B. anthracis* (**Figure 32.22*a***) is also an important virulence factor of this pathogen, as it prevents destruction of the bacterium following phagocytosis by immune cells such as macrophages. Instead, cells of *B. anthracis* grow within the macrophage, eventually killing it and giving the bacterium access to the bloodstream.

Virtually all cases of human anthrax are *cutaneous anthrax*, where endospores of *B. anthracis* have entered through a break in the skin, germinate, and form a painless, black, and swollen pustule of necrotic tissue called an *eschar* (Figure 32.22*b*); the eschar is highly characteristic of the disease and allows for a firm diagnosis even though human anthrax is rarely seen in clinical medicine. In cutaneous anthrax the bacterium usually remains localized and the disease is readily treatable. Although cutaneous anthrax is fatal for about 20% of those untreated, most cases are treated because of the obvious symptoms, and thus fatalities are rare. *Intestinal anthrax* is very uncommon and is triggered by the ingestion of endospores of *B. anthracis* (Figure 32.21*b*) in undercooked meat from diseased animals. Symptoms of intestinal anthrax include abdominal pain, bloody diarrhea, and ulcer-like lesions throughout the intestinal tract. The disease is still treatable at this stage, but because of its rarity, diagnoses are easily missed. As a result, about half of all cases of intestinal anthrax are fatal.

Inhalation anthrax is the most severe form of the disease and is fatal in almost every case (Figure 32.22*c*, *d*). Inhalation anthrax occurs from the inhalation of endospores of *B. anthracis* and, along with cutaneous anthrax, is an occupational hazard for farm workers that process wool and hides (inhalation anthrax is also known as "woolsorter's disease"). In inhalation anthrax, the organism enters the bloodstream from inhaled dust or animal dander and multiplies to become systemic. The mounting toxemia from this runaway growth of *B. anthracis* triggers septic shock and fluid accumulation in the lungs (Figure 32.22*c*) that can kill a patient in less than a day.

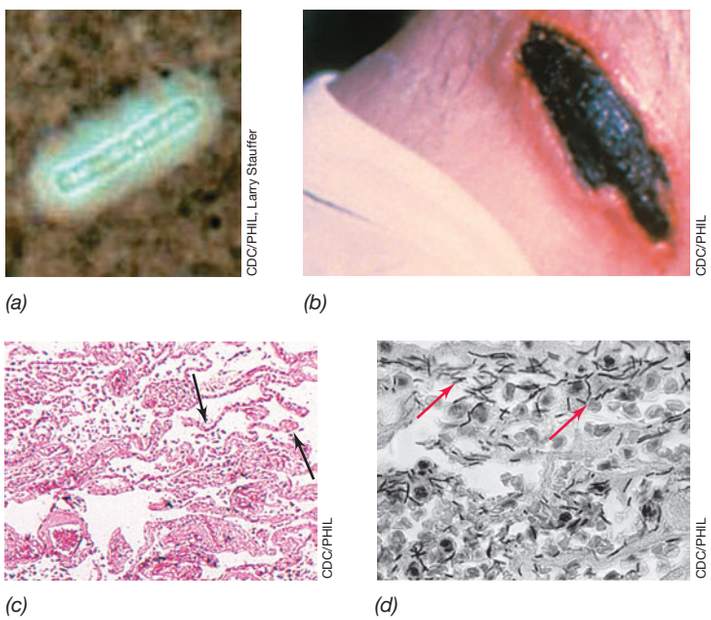


Figure 32.22 Anthrax pathology. *(a)* The protein capsule of *Bacillus anthracis* cells is a major virulence factor because it prevents killing by macrophages. *(b)* Cutaneous anthrax, with its characteristic black scabby appearance on the neck of a patient. *(c, d)* Inhalation anthrax. *(c)* The lung fills with bacterial cells (arrows) and fluids (cleared zones). *(d)* From the systemic infection, *B. anthracis* cells can be found almost anywhere, including the lining of the central nervous system (arrows point to *B. anthracis* cells).

Prevention and Vaccines

Complete prevention of anthrax is impossible because the reservoir of the organism is soil. However, anthrax is avoidable by limiting close exposure to farm animals and is easily treatable with antibiotics. For the cutaneous form this is a routine treatment, but antibiotic therapy is less effective in intestinal anthrax and especially in inhalation anthrax. By the time the latter is diagnosed, the disease has progressed to the point where it is usually too late to save the patient. An anthrax vaccine is available but because the disease is so rare, it is recommended only for high-risk individuals, such as scientists working with the organism, slaughterhouse or livestock workers, and military personnel (for biowarfare reasons). An effective and inexpensive anthrax vaccine is available for vaccinating livestock and is commonly used in cattle, sheep, goats, and horses.

Check Your Understanding

- What are the major virulence factors of *Bacillus anthracis*?
- What are the three forms of anthrax, and which is most dangerous?

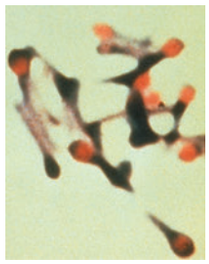
32.9 Tetanus and Gas Gangrene

Tetanus is a serious, life-threatening disease. Although tetanus is completely preventable through immunization, it still causes over 150,000 deaths per year, mostly in countries in Africa and Southeast Asia. *Gas gangrene* is caused by the growth in dead tissues of bacteria related to the tetanus pathogen, leading to a gassy putrefaction and loss of an infected limb or death from systemic shock. Both diseases are caused by clostridia.

Biology and Epidemiology of Tetanus

Tetanus is caused by an exotoxin produced by *Clostridium tetani*, an obligately anaerobic, endospore-forming rod (**Figure 32.23*a***; ◀ Section 16.8). The natural reservoir of *C. tetani* is soil, where it is a ubiquitous resident, although it is occasionally found in the gut of healthy humans, as are other *Clostridium* species.

Cells of *C. tetani* normally gain access to the body through a soil-contaminated wound, typically a deep puncture. In the wound, anoxic conditions develop around the dead tissue and allow



(a)

(b)

Figure 32.23 **Tetanus.** *(a) Clostridium tetani* showing the "drumstick" appearance of sporulating cells with their terminal endospores. Cells of *C. tetani* are about 0.8 μm in diameter. *(b)* A tetanus patient showing the rigid paralysis characteristic of tetanus. Tetanus paralysis typically begins with the facial muscles ("lockjaw") and descends to lower body regions.

germination of endospores, growth of the organism, and production of a potent exotoxin, the *tetanus toxin* (also called *tetanospasmin*). *C. tetani* is essentially noninvasive; its sole ability to cause disease is through toxemia, and thus tetanus is observed only as the result of untreated deep tissue injuries. The onset of tetanus symptoms may take from 4 days to several weeks, depending on the number of endospores inoculated at the time of the injury.

Pathogenesis of Tetanus

We have already examined the activity of tetanus toxin at the cellular and molecular level (◀ Section 25.6). The toxin directly affects the release of inhibitory signaling molecules in the nervous system. These inhibitory signals control the "relaxation" phase of muscle contraction. The absence of inhibitory signaling molecules results in rigid paralysis of the voluntary muscles, often called *lockjaw* because it is usually observed first in the muscles of the jaw and face. Preceding actual lockjaw, tetanus symptoms typically include mild spasms of facial muscles and muscles of the neck and upper back. Later, the paralysis extends to the torso and lower body (Figure 32.23*b*). When tetanus is fatal, death is usually due to respiratory failure. Mortality is relatively high, occurring in about 10% of all reported cases, and up to 50% of cases in which treatment is delayed until generalized full body tetanus has set in.

Diagnosis, Control, Prevention, and Treatment of Tetanus

Diagnosis of tetanus is based on exposure, clinical symptoms (Figure 32.23*b*), and, rarely, identification of the toxin in the blood or tissues of the patient. The natural reservoir of *C. tetani* is soil and thus control measures must focus on disease prevention rather than pathogen removal. The widely used tetanus toxoid vaccine is highly effective, and thus virtually all tetanus cases occur in individuals who either were never immunized or were vaccinated more than 10 years prior to exposure to the pathogen.

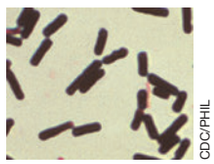
A second line of tetanus protection is to administer appropriate medical care to serious cuts, lacerations, and punctures. Even though vaccination against tetanus is widely practiced, any serious wound should be thoroughly cleaned and the damaged tissue removed. If the vaccination status of the individual is unclear or the last tetanus booster was more than 10 years ago, revaccination is highly recommended. If a deep wound is severe or heavily contaminated by soil, treatment might also include administration of a tetanus antitoxin preparation, especially if the patient's immunization status is unknown or is out of date.

Acute symptomatic tetanus (Figure 32.23*b*) is treated with antibiotics, usually penicillin, to stop growth and toxin production by *C. tetani*, and antitoxin is injected intramuscularly (or into the sheath surrounding the spinal cord if necessary) to prevent binding of newly released toxin to cells. Supportive therapy such as sedation, administration of muscle relaxants, and mechanical respiration may be necessary to control the effects of paralysis. Treatment cannot provide a quick reversal of symptoms because toxin that is already bound to tissues cannot be neutralized. Even with antitoxin, antibiotic administration, and supportive therapy, tetanus patients show significant morbidity and mortality. A complete recovery from tetanus often takes many months.

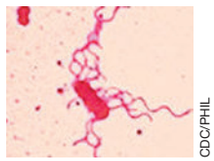
Gas Gangrene

Tissue destruction due to the growth of proteolytic and gas-producing clostridia is called **gas gangrene**. In this life-threatening condition, amino acids obtained from the breakdown of muscle proteins are fermented to the gases H_2 and CO_2 plus a variety of foul-smelling organic compounds, including short-chain fatty acids and other putrid molecules; ammonia released during amino acid fermentation (◀ Section 14.19) adds to the stench. In addition, a variety of bacterial toxins are produced that accelerate tissue destruction.

Although *C. tetani* is a proteolytic *Clostridium* species, it does not cause gangrene but can be associated with cases of gangrene triggered by a deep tissue wound. The most common causes of gangrene are *Clostridium perfringens* (**Figure 32.24*a***), which is also a common cause of foodborne illness unrelated to gangrene; *C. novyi*; and *C. septicum*. These organisms reside in soil and are also part of the normal human intestinal microbiota. When these species reach deep into tissues, typically from traumatic tissue invasion such as a war wound or other puncture wound, or occasionally from gastrointestinal tract surgery, endospores and vegetative cells of proteolytic clostridia are inserted into what are now dead tissues. As the bacteria grow, they release enzymes that destroy collagen and tissue proteins



(a)



(b)

(c)

Figure 32.24 **Gas gangrene.** *(a)* Gram stain of cells of *Clostridium perfringens*, the most common cause of gangrene. *(b)* Flagellum stain showing a cell of *Clostridium novyi*, also an agent of gangrene. Cells of both *C. perfringens* and *C. novyi* are about 1.2 μm in diameter. *(c)* A case of gas gangrene on the foot.

and also excrete a series of toxins. *C. perfringens* (Figure 32.24*a*) α-*toxin*, which is distinct from the toxins the bacterium produces in perfringens food poisoning (▶ Section 33.9), is a major virulence factor in gangrene, as is the general ability of the pathogens to grow rapidly in the warm, moist, and protein-rich environment created by an invasive injury. *C. perfringens* α-toxin is a phospholipase that hydrolyzes the membrane phospholipids of host cells, leading to cell lysis and the typical accumulation of gas and fluids that accompanies gas gangrene (Figure 32.24*c*).

In severe cases of gas gangrene, the toxemia can become systemic and cause death. Antibiotic treatment is taken as a preventive measure in cases of gangrene in addition to the typical though dramatic treatment: amputation of the infected limb. Gangrenous tissues are dead and will not regenerate, and amputation prevents the infection from reaching healthy tissues.

Hyperbaric oxygen treatment of the infected limb is attempted in some cases to try to save it, with the high levels of O_2 inhibiting growth of the obligately anaerobic clostridia. In hyperbaric treatment, the patient sits in an enclosed chamber containing 100% O_2 at about twice atmospheric pressure. This enriches the blood in O_2 and helps still-living blood vessels seed the formation of new tissue. Several hyperbaric treatments are administered and may be accompanied by surgical removal of some of the dead tissue. If an adequate blood supply can be established in damaged tissues, a skin graft may also be done to help connect regenerating tissues with damaged ones.

Check Your Understanding

- Describe infection by *Clostridium tetani* and the effects of tetanus toxin. How does the mode of action of tetanus toxin differ from that of α-toxin produced by *Clostridium perfringens*?
- Describe the steps necessary to prevent tetanus in an individual who has sustained a puncture wound.
- How does the physiology of *C. perfringens* make it suitable for growing in puncture wounds?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Animal-Transmitted Viral Diseases

32.1 Rabies occurs primarily in wild animals but can be transmitted to humans and domestic animals. In the United States, rabies is transmitted primarily from the wild animal reservoir to domestic animals or, very rarely, to humans. Vaccination of domestic animals is key to the control of rabies. Most human deaths from rabies occur in developing countries.

Q Which pathogen causes rabies? How does it enter the body? What are the factors that determine the incubation period before the onset of the symptoms of rabies?

32.2 Hantaviruses are present worldwide in rodent populations and cause zoonotic diseases such as hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome in humans. Hantavirus is a highly dangerous hemorrhagic fever virus related to Lassa fever virus. In the Americas, hantavirus infections have mortality rates of over 30%.

Q Describe the conditions that may cause emergence of hantavirus pulmonary syndrome (HPS). How can HPS be prevented?

II • Arthropod-Transmitted Bacterial and Viral Diseases

32.3 Rickettsias are obligate intracellular parasitic bacteria transmitted to hosts by arthropod vectors. The incidence of spotted fever rickettsiosis and other rickettsial syndromes is increasing as a result of several factors. Most rickettsial infections can be controlled by antibiotic therapy, but prompt recognition and diagnosis of these diseases remains difficult.

Q Identify the three major categories of organisms that cause rickettsial diseases. For typhus, spotted fever rickettsiosis, and ehrlichiosis, identify the most common reservoir and vector.

32.4 Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is the most prevalent arthropod-borne disease in the United States today. The pathogen is transmitted from several mammalian host reservoirs to humans by tick vectors. Prevention and treatment of Lyme disease are straightforward, but accurate and timely diagnosis of infection is essential.

Q Identify the most common reservoir and vector for Lyme disease in the United States. How can the spread of Lyme disease be controlled? How can Lyme disease be treated?

32.5 Yellow, dengue, and Zika fevers are caused by related flaviviruses transmitted to humans by mosquito bites; Chikungunya fever shows similar disease symptoms but is caused by a togavirus. These diseases are widespread in tropical and subtropical countries and can show mild to severe symptoms, including hemorrhagic fevers. An effective vaccine for yellow fever is in use. A vaccine for dengue is in restricted use. No vaccine is currently available to prevent Zika or Chikungunya infections, but vaccines against both viruses are in development.

Q Why are people with active cases of yellow fever put in isolation?

32.6 West Nile fever is a mosquito-borne viral disease. In the natural cycle of the pathogen, birds are infected with West Nile virus by the bite of infected mosquitoes. Humans and other vertebrates are occasional terminal hosts. Most human infections are asymptomatic and undiagnosed, but complications of some infections cause about 5% mortality.

Q Describe the spread of West Nile virus infections in the United States. Which animals are the primary hosts? Are humans productive alternate hosts?

32.7 Plague can be transmitted to individuals who have had contact with rodent populations and their parasitic fleas, the enzootic reservoirs for the plague bacterium, *Yersinia pestis*. A systemic infection or a pneumonic infection usually leads to rapid death, but the bubonic form is treatable with antibiotics.

Q For a potentially serious disease like bubonic plague, vaccines are not routinely recommended for the general population; why not? Identify the public health measures used to control plague.

III • Soilborne Bacterial Diseases

32.8 Anthrax is caused by the endospore-forming bacterium *Bacillus anthracis* and can take on three different forms: cutaneous, intestinal, or inhalation. Cutaneous anthrax is most common and along with inhalation anthrax is an occupational hazard for livestock workers, where *B. anthracis* endospores can be transmitted from animal hides to humans.

Q Which key feature of the bacterium *Bacillus anthracis* allows this organism to persist for extended periods on animal hides or other environments where growth may not occur? Which form of anthrax is the most serious?

32.9 *Clostridium tetani* is a soil bacterium that causes tetanus, a potentially fatal disease characterized by a toxemia and rigid paralysis. Treatment for acute tetanus includes antibiotics and active and passive immunization, and the disease is preventable by toxoid immunization. Gas gangrene occurs from the growth of various proteolytic clostridia in traumatic wounds, leading to gas and toxin formation.

Q Discuss the major mechanism of pathogenesis for tetanus and define measures for prevention and treatment. Why is it possible that a traumatic puncture wound could end up causing both tetanus and gas gangrene?

APPLICATION QUESTIONS

1. Describe the sequence of steps you would take if your child received a bite (provoked or unprovoked) from a stray dog with no collar and record of rabies immunization. Present one scenario in which you were able to capture and detain the dog and another for a dog that escaped. How would these procedures differ from a situation in which the child was bitten by a dog that had a collar and rabies tag with documented, up-to-date rabies immunizations?
2. Contrast the modes of transmission of the following diseases: rabies, Lyme disease, yellow fever, West Nile disease, anthrax, and plague. Which of these diseases could be virtually eliminated in humans by control of the disease vector and which could not, and why?
3. Devise a plan to prevent the spread of West Nile virus to humans in your community. Identify the relative costs involved in such a plan, both at the individual level and at the community level. Find out if a mosquito abatement program is active in your community. What methods, if any, are used in your area for the reduction of mosquito populations? What is a simple way to limit mosquito numbers around your residence?

CHAPTER GLOSSARY

Enzootic an endemic disease present in an animal population

Epizootic an epidemic disease present in an animal population

Gas gangrene tissue destruction due to the growth of proteolytic and gas-producing clostridia

Hantavirus pulmonary syndrome (HPS) an emerging, acute disease characterized by pneumonia and caused by rodent hantavirus

Hemorrhagic fever with renal syndrome (HFRS) an emerging, acute disease characterized by shock and kidney failure and caused by rodent hantavirus

Lyme disease a tick-transmitted disease caused by the spirochete *Borrelia burgdorferi*

Plague an enzootic disease in rodents that is caused by *Yersinia pestis* and can be transferred to humans through the bite of a flea

Rabies a usually fatal (if untreated) neurological disease caused by the rabies virus, which is typically transmitted by the bite or saliva of an infected animal

Rickettsias obligate intracellular bacteria of the genus *Rickettsia* and related genera responsible for diseases including typhus, spotted fever rickettsiosis, and ehrlichiosis

Spotted fever rickettsiosis (SFR) a tick-transmitted disease caused by *Rickettsia rickettsii*, characterized by fever, headache, rash, and gastrointestinal symptoms; also called Rocky Mountain spotted fever

Tetanus a disease characterized by rigid paralysis of the voluntary muscles, caused by an exotoxin produced by *Clostridium tetani*

Typhus a louse-transmitted disease caused by *Rickettsia prowazekii*, characterized by fever, headache, weakness, rash, and damage to the central nervous system and internal organs

West Nile fever a neurological disease caused by West Nile virus, a virus transmitted by mosquitoes from birds to humans

Zoonosis any disease that occurs primarily in animals but can be transmitted to humans

Waterborne and Foodborne Bacterial and Viral Diseases 33

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II Waterborne Diseases 1040

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IV Food Poisoning 1048

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MICROBIOLOGYNOW

Reverse Zoonosis in the Southern Ocean

A zoonosis is an animal disease that is transmissible to humans, usually by direct contact (including bites) or airborne particles. Rabies, for example, is a classic zoonotic disease. But does the opposite ever happen? That is, do humans ever transmit pathogens to animal populations?

The answer is yes, "reverse zoonotic" transmission occurs regularly when humans and animals are in close proximity, but, with the exception of a few key pathogens (for example, influenza A), this transmission generally poses no significant threat to animal populations as a whole. But is this also true for animal species that have historically been isolated from contact with humans? Not having coevolved with humans, are these animals more at risk from novel exposures to anthropogenic opportunistic pathogens?

This question has come to the forefront in Antarctica, where vibrant multinational research programs and a burgeoning tourism industry have led to more traffic—tens of thousands of visitors every year—on and around the Southern Ocean than ever before.

Ever since centuries-old murmurings of an unseen, mythical land to the far south of the globe were confirmed in the 1820s, research scientists and adventurous travelers have found the allure of the frozen continent compelling. However, the steadily increasing presence of humans—and in particular, their gut microbiota—may negatively impact Antarctic and subantarctic animal populations.

A recent study identified human-derived enteric pathogens, including antibiotic-resistant strains of *Salmonella* and *Campylobacter* spp., in fecal samples from Antarctic marine birds such as various penguins (photo, an Adélie penguin rookery in Cape Bird, Ross Island, Antarctica), brown skuas, and gulls. Although the long-term ramifications of reverse zoonosis in Antarctica are unclear, the study shows that stricter biosecurity regulations to limit the potential for widespread reverse zoonotic outbreaks among sensitive animal populations may be a prudent and perhaps overdue course of action.

Source: Cerdà-Cuéllar, M., et al. 2019. Do humans spread zoonotic enteric bacteria in Antarctica? *Sci. Total Environ. 654*: 190. doi: 10.1016/j.scitotenv.2018.10.272.

In this chapter we consider microbial pathogens whose mode of transmission is either water or food. The diseases caused by these pathogens are called "common-source" diseases because they occur only in those who have consumed the same contaminated water or eaten the same contaminated food (◀ Section 30.4). Waterborne and foodborne illnesses are common infectious diseases worldwide. While foodborne illnesses are most commonly of bacterial or viral origin, waterborne illnesses can have bacterial, viral, or parasitic causes. Waterborne diseases caused by eukaryotic pathogens will be considered in Chapter 34.

I • Water as a Disease Vehicle

Whether potable or recreational, water is a potential source of a variety of pathogenic microorganisms that can cause serious disease. Because of this, rapid and accurate methods to test water quality are essential to maintain public health standards.

Water is used in enormous quantities, and its microbiological safety rests in the hands of wastewater and drinking water engineers and microbiologists. Indeed, water quality is the single most important factor for ensuring public health. In Chapter 22 we examined the microbiology of wastewater and examined how highly polluted water can be cleaned by microbial activities and reused for many purposes, including for drinking. Here we see what can happen when water intended for human use becomes a vehicle for disease.

Waterborne diseases begin as infections (or occasionally as toxemias), and contaminated water may cause an infection even if only small numbers of the particular pathogen are present. Whether exposure causes disease or not is a function of the virulence of the pathogen and the ability of the host to resist infection.

33.1 Agents and Sources of Waterborne Diseases

Mastering Microbiology
Art Activity: Table 33.1 Major waterborne pathogens

Many different microorganisms can cause waterborne infectious diseases, and some of the major ones are summarized in **Table 33.1**. Many different microbes can be waterborne pathogens, but here we will consider bacterial pathogens with a major focus on cholera, a waterborne disease of pandemic proportions (◀ Section 30.8). We consider a few viral pathogens that can be either foodborne or waterborne later in this chapter.

We begin by considering the disease vehicle itself—water. Waterborne illnesses can be transmitted through untreated or improperly treated water used for drinking or food preparation or from water used for swimming and bathing (recreational water sources). The major waterborne illnesses traced to drinking water and recreational waters are typically quite different, and these different disease patterns are shown in **Table 33.2**.

Potable Water

Water supplies in developed countries typically meet rigid quality standards, greatly reducing the spread of waterborne diseases. Drinking water in particular undergoes extensive treatment that includes both filtration and chlorination. Although filtration removes turbidity and many microorganisms, it is *chlorination* that makes drinking water safe. Chlorine gas (Cl_2) is a strong oxidant and oxidizes both organic matter dissolved in the water and microbial cells themselves. Drinking water chlorination facilities add sufficient chlorine to allow a residual level to remain in the water all the way to the consumer. Water suitable for human consumption is called **potable** water (◀ Sections 22.7–22.10).

Despite filtration and chlorination, waterborne disease outbreaks from potable water occasionally occur. In the United States an average of 40 outbreaks of disease associated with drinking water are recorded in a year (a waterborne outbreak is defined as two or more human illnesses specifically linked to the consumption of the same water at the same time). About 60% of drinking water disease outbreaks are due to *bacterial* pathogens, most notably *Legionella*, the causative agent of legionellosis (Table 33.2 and Section 33.4).

TABLE 33.1 Major waterborne pathogens

Pathogen	Disease
Bacteria[a]	
Vibrio cholerae	Cholera
Legionella pneumophila	Legionellosis
Salmonella enterica (typhi)	Typhoid fever
Escherichia coli	Gastrointestinal illness
Pseudomonas aeruginosa	Nosocomial pneumonia, septicemia, and skin infections
Campylobacter jejuni	Gastrointestinal illness
Viruses	
Norovirus	Gastrointestinal illness
Hepatitis A virus	Viral hepatitis
Parasites[b]	
Cryptosporidium parvum	Cryptosporidiosis
Giardia intestinalis	Giardiasis
Schistosoma	Schistosomiasis

[a]Except for *S. enterica* (*typhi*), these bacteria have been associated with major outbreaks of waterborne illness in the United States in recent years, as have the bacteria *Shigella sonnei* and *Leptospira* sp.
[b]See Chapter 34. *C. parvum* and *G. intestinalis* are unicellular microbial parasites; *Schistosoma* is a microscopic worm 10–20 mm long (see MicrobiologyNow in the Chapter 28 opener).

Recreational Waters

Recreational waters include freshwater aquatic systems such as ponds, streams, and lakes, as well as public swimming and wading pools. Recreational waters can be sources of waterborne disease, and on average they cause more disease outbreaks than those due to drinking water (Table 33.2). Moreover, in contrast to drinking water, where *bacterial* pathogens are most common, disease outbreaks from recreational waters are more frequently linked to *parasitic* (eukaryotic) pathogens. In addition, recreational waters transmit a significant number of gastrointestinal illnesses that are of unknown origin (Table 33.2).

In the United States, the operation of public swimming pools is regulated by state and local health departments. The United States

TABLE 33.2 Sources of outbreaks of acute gastrointestinal illness in drinking and recreational waters, 2013–2014[a]

	Drinking water (n = 42) (%)	Recreational water (n = 493) (%)
Bacteria	60	26
Legionella pneumophila	57	12
Other	3	14
Parasites	19	45
Viruses	7	3
Chemical/toxin	10	4
Multiple causes[b]	2	0
Unknown[c]	2	22

[a]Numbers are rounded to the nearest percent and were obtained from the Centers for Disease Control and Prevention Waterborne Disease and Outbreak Surveillance System.
[b]Outbreak linked to more than one cause.
[c]Suspected to be caused by one or more microbes or chemicals but not confirmed.

Environmental Protection Agency (EPA) establishes limits for bacterial numbers in both potable and public recreational water sources, but local and state governments can set standards above or below these guidelines for nonpotable sources. By contrast, the water quality of *private* recreational waters, such as swimming pools, spas, and hot tubs, is totally unregulated, and these are therefore prime vehicles for waterborne disease transmission.

Check Your Understanding

- What is potable water?
- Contrast the major pathogens responsible for disease outbreaks in drinking water versus recreational waters.

33.2 Public Health and Water Quality

Water that looks perfectly transparent may still be contaminated with pathogenic microorganisms and thus pose a risk of disease. It is impractical to screen water for every pathogen that may be present (Table 33.1), and so both potable and recreational waters are routinely tested for specific *indicator organisms*, the presence of which signals the potential for waterborne disease.

Coliforms and Water Quality

A widely used indicator for microbial water contamination is the **coliform** group of bacteria. Many coliforms inhabit the intestinal tract of humans and other animals, and therefore their presence in water indicates likely fecal contamination. Coliforms are defined as facultatively aerobic, gram-negative, rod-shaped, nonsporulating bacteria that ferment lactose with the production of gas within 48 h at 35°C. However, this definition includes several bacteria that are not necessarily restricted to the intestine; for this reason, it is *fecal coliforms* that are important in water safety assessments. *Escherichia coli*, a coliform whose only habitat is the intestine and that survives only a relatively short time outside the intestine, is the key fecal coliform of interest. The presence of cells of *E. coli* in a water sample is taken as evidence of fecal contamination and means that the water is unsafe for human consumption. Conversely, however, the absence of *E. coli* does not ensure that a water source is potable, because other pathogenic bacteria or pathogenic viruses or protists may still be present.

Testing for Fecal Coliforms and the Importance of *Escherichia coli*

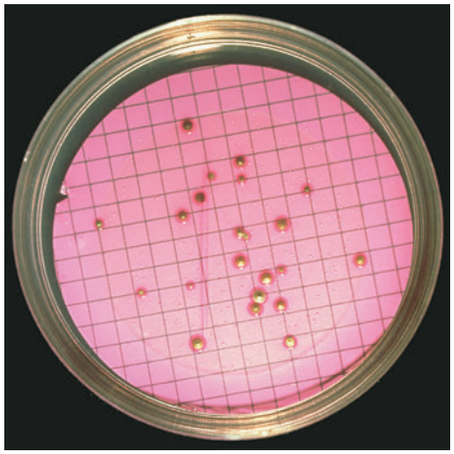
Well-developed and standardized methods are in routine use for detecting coliforms and fecal coliforms in water samples. A common method is the *membrane filter* (*MF*) procedure where at least 100 ml of freshly collected water is passed through a sterile membrane filter, trapping any bacteria on the filter surface. The filter is placed on a plate of eosin–methylene blue (EMB) medium, which is selective for gram-negative, lactose-utilizing bacteria. EMB medium is also differential, allowing strongly fermentative species such as *E. coli* (**Figure 33.1*a***) to be distinguished from weakly fermentative species such as *Proteus*.

Selective media are also available that not only detect total coliforms but also specifically identify *E. coli* simultaneously. These *defined substrate tests* are typically faster and more accurate than EMB-based assays. One popular plate-based test is based on the ability of *E. coli* but not other enteric bacteria to metabolize a combination of two specific chemicals to form a fluorescent blue compound (Figure 33.1*b*). A commonly used liquid method reveals whether coliforms are present and also specifically detects *E. coli* in the water sample (Figure 33.1*c*). In addition to these colorimetric tests, dipstick assays have been developed that detect adenosine triphosphate (ATP) in a water sample. There is a strong correlation between the total number of bacteria in a sample and its ATP content, and the latter can be measured using the luciferase enzyme system in which a flash of light is emitted when ATP is hydrolyzed (◀ Figure 10.4*b*). Using this system, the total microbial load in a water sample can be quickly assessed, and portable kits are available commercially to carry out these analyses in water purification facilities as well as remote field sites.

Reporting Water Purity Data

In properly regulated drinking water supply systems, total coliform and *E. coli* fecal coliform tests should be negative. A positive test indicates that a breakdown has occurred in either the purification or distribution system (or both). In the United States, microbiological standards for drinking water are specified in the *Safe Drinking Water Act* and are administered by the Environmental Protection Agency (EPA). Water utilities must report coliform test results to the EPA monthly, and if they do not meet the prescribed standards, the utilities must notify the public and take steps to correct the problem.

Major improvements in public health in the United States beginning in the early twentieth century were largely due to the adoption of water filtration and chlorination procedures in large-scale wastewater and drinking water treatment plants (◀ Sections 22.8 and 22.9). Where drinking water standards have not reached this level, especially in developing countries, a variety of waterborne diseases are common. We turn our attention to these diseases now, beginning with cholera, the most widespread and devastating of all waterborne diseases.



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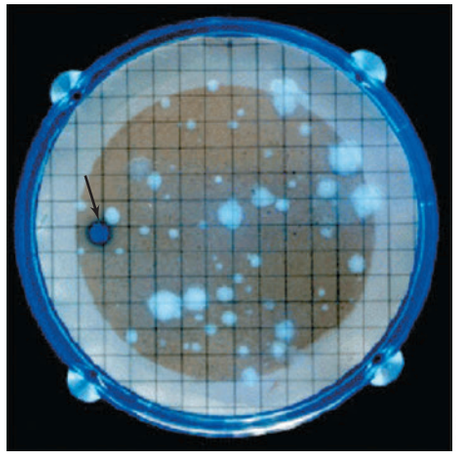


US EPA

(a) (b) (c)

Figure 33.1 Fecal coliforms and their detection in water samples. *(a)* Colonies growing on a membrane filter. A drinking water sample was passed through the filter and the filter placed on an eosin–methylene blue (EMB) agar plate (EMB is both selective and differential for coliforms; more strongly fermentative species [such as *Escherichia coli*] form colonies with darker centers). *(b)* Total coliforms and *E. coli*. A filter exposed to a drinking water sample was incubated at 35°C for 24 hours on a medium containing special compounds that fluoresce when metabolized. The filter was then examined under ultraviolet light. The single *E. coli* colony in the sample fluoresces dark blue (arrow). Coliforms that do not metabolize the compound form colonies that fluoresce white to light blue. *(c)* The IDEXX Colilert® water quality test system. When specific reagents are added to water samples and incubated for 24 h, they develop a yellow color if they contain coliforms (right). Samples containing *Escherichia coli* develop a yellow color but also fluoresce blue (left). Samples negative for coliforms remain clear (center).

Check Your Understanding

- Why is *Escherichia coli* used as an indicator organism in microbial analyses of water?
- What procedures are used to ensure the safety of potable water supplies?

II • Waterborne Diseases

Waterborne diseases can result from ingestion of contaminated water, causing serious gastrointestinal distress, or inhalation of contaminated aerosols, causing respiratory illness and pneumonia-like symptoms. Cholera is a prime example of a dangerous waterborne disease.

Waterborne infectious diseases are a constant threat to humanity, especially in areas where proper sanitation and supplies of potable drinking water are either unavailable or subject to intermittent disruption. Cholera leads the list here for its potential to initiate large-scale and rapidly developing disease outbreaks.

33.3 *Vibrio cholerae* and Cholera

Cholera is a severe gastrointestinal diarrheal disease that is now largely restricted to countries in the developing world. Cholera is caused by ingestion of contaminated water containing cells of *Vibrio cholerae*, a gram-negative and motile curved species of *Proteobacteria* (**Figure 33.2**). Cholera can also be contracted from contaminated food, especially improperly cooked shellfish.

The ingestion of a large number ($> 10^8$) of *V. cholerae* cells is required to cause disease. The ingested cells attach to epithelial cells in the small intestine where they grow and release *cholera toxin*, a potent enterotoxin (◀ Figure 25.16). Studies with human volunteers have shown that normal stomach acidity (about pH 2) is why the large inoculum of *V. cholerae* cells is needed to initiate disease. In these studies, those given bicarbonate to neutralize gastric acidity develop cholera when given as few as 10^4 cells. Even lower cell numbers can initiate infection if *V. cholerae* is ingested with food, presumably because the food protects the vibrios from destruction by stomach acidity.

Cholera enterotoxin causes severe diarrhea that can rapidly result in dehydration and death unless the patient is given fluid and electrolyte therapy. The enterotoxin causes fluid losses of up to 20 liters (20 kg or 44 lb) per person per day, causing severe dehydration. The mortality rate from *untreated* cholera is 25–50% and can be even higher under conditions of severe crowding and malnutrition as often occurs in refugee camps or in areas that have experienced natural disasters, such as floods and earthquakes. In these situations, there is often a near-complete breakdown in sanitation, leading to the contamination of drinking water with feces and the rapid transmission of cholera.

Diagnosis, Treatment, and Prevention of Cholera

At treatment facilities in large outbreaks of cholera, each cholera patient is placed on a "cholera cot," which is a conventional folding cot containing an opening into which feces can be voided (Figure 33.2). The feces of a cholera patient are more liquid than solid, and confirmation of the disease is straightforward because the pathogen is easily cultured on selective agar media (Figure 33.2). Cholera treatment is simple and effective. An oral (or in severe cases, intravenous) liquid and electrolyte replacement therapy is the most effective means of treatment. Oral treatment is preferred because no special equipment or sterile precautions are necessary.

The rehydration solution is a mixture of glucose, salt (NaCl), sodium bicarbonate ($NaHCO_3$), and potassium chloride (KCl). If the solution is administered quickly during an outbreak, cholera mortality can be greatly reduced, as rehydration allows patients the time necessary to mount an immune response.

Antibiotics may shorten the course of cholera infection and the shedding of viable cells, but antibiotics are of little health benefit to the patient without simultaneous fluid and electrolyte replacement. Public health measures such as adequate sewage treatment and a reliable source of safe drinking water are the keys to preventing cholera. *V. cholerae* is eliminated from wastewater during proper sewage treatment and drinking water purification procedures (Chapter 22). For individuals traveling in cholera-endemic areas, attention to personal hygiene and avoidance of untreated water or ice, raw food,

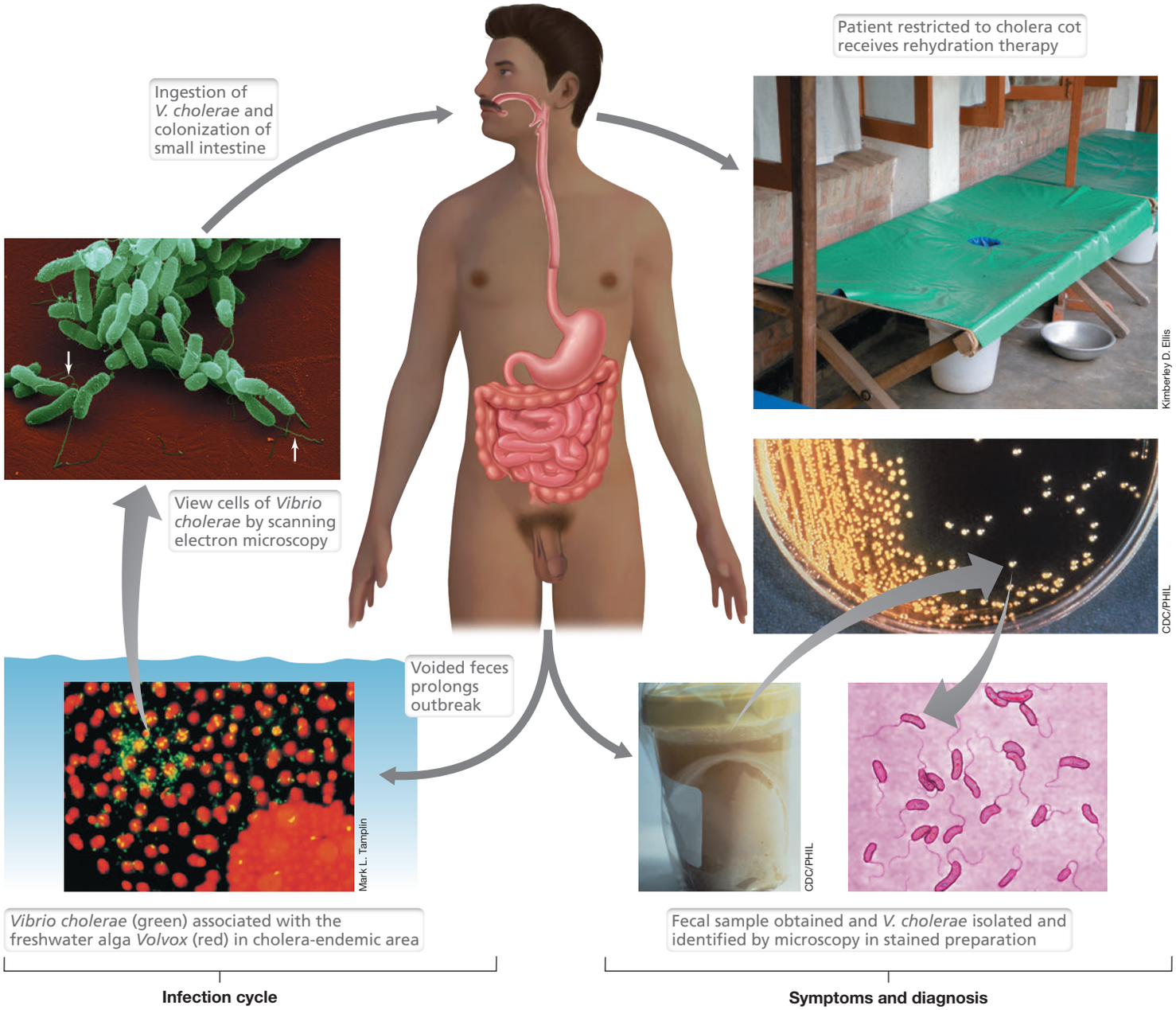


Figure 33.2 Infection cycle, symptoms, and diagnosis of cholera. Highly motile cells of *Vibrio cholerae*, the causative agent of cholera (upper left, scanning electron micrograph, note flagella), may associate with algae in freshwaters (lower left) in cholera endemic areas and be inadvertently ingested by a human host. Subsequent colonization of the small intestine and enterotoxin secretion by *V. cholerae* (◀ Figure 25.16) causes severe diarrhea in the form of "rice water" stools, so called because they are extremely watery and contain flecks of whitish-colored mucus. While being treated for cholera, patients lie prostrate on "cholera cots" and void feces directly into a bucket below (top right). Any contamination of natural waters by patient stools (for example, by emptying or rinsing cholera buckets in local streams) magnifies and prolongs the cholera outbreak. Patient diagnosis is confirmed by recovering cells of *V. cholerae* from a stool sample using selective culture media, such as medium TCBS (middle right), which contains bile salts and citrate to inhibit enterobacteria and gram-positive bacteria, as well as thiosulfate and sucrose, which cells of *V. cholerae* (bottom right) use as a sulfur and carbon/energy source, respectively.

and raw or undercooked fish or shellfish that can feed on phytoplankton contaminated with *V. cholerae* (Figure 33.2) can prevent cholera.

Since 1817, cholera has swept the world in seven major pandemics with an eighth pandemic likely already started (◀ Section 30.8 and Figure 30.15). The World Health Organization estimates that only 5–10% of cholera cases are reported, so the total worldwide incidence of cholera probably exceeds 1 million cases per year. Only a handful of cases of cholera are reported each year in the United States, typically from imported shellfish that are eaten raw or after only minimal cooking.

Check Your Understanding

- What organism causes cholera, and what are the symptoms of the disease?
- Why does transmission of cholera usually require a large inoculum? Under what conditions can cholera be transmitted by fewer cells?
- Describe how cholera can be prevented and how it is treated.

33.4 Legionellosis

Legionella pneumophila, the bacterium that causes *legionellosis*, is an important waterborne pathogen whose transmission was originally linked to aerosols from evaporative cooling devices. However, *L. pneumophila* (**Figure 33.3**) is now known to be a major pathogen in residential water systems as well, where the organism persists in biofilms that form on interior surfaces of water distribution pipes and also within the cells of certain microbial parasites. In these sites, *L. pneumophila* is protected from the chlorine present in potable waters, and thus biofilms and infected parasites are reservoirs for transmitting legionellosis by a waterborne route (◀ Section 22.10 and Figure 22.25).

Pathogenesis, Diagnosis, and Treatment

Cells of *L. pneumophila* invade the lungs and grow within macrophages and monocytes. Infections are often asymptomatic or produce only a mild cough, sore throat, mild headache, and fever; these self-limiting cases typically resolve themselves in 2–5 days. However, the elderly, whose resistance may be naturally reduced, and those with compromised immune systems often acquire more serious *Legionella* infections resulting in pneumonia. Prior to the onset of pneumonia, intestinal disorders, followed by high fever, chills, and muscle aches, are common. These symptoms precede the dry cough and chest and abdominal pains typical of legionellosis. Up to 10% of cases that reach this stage are fatal, usually as a result of respiratory failure.

Clinical detection of *L. pneumophila* infection is usually done by culture of the organism from bronchial washings, pleural fluid, or other body fluids or tissues (Figure 33.3*d*). Various serological tests can detect anti-*Legionella* antibodies or *Legionella* cells in these samples and also from patient urine, and this is used to confirm a diagnosis (Figure 33.3*b*). Legionellosis can be treated with antibiotics, in particular rifampin and erythromycin, and intravenous administration of erythromycin is the treatment of choice for life-threatening cases.

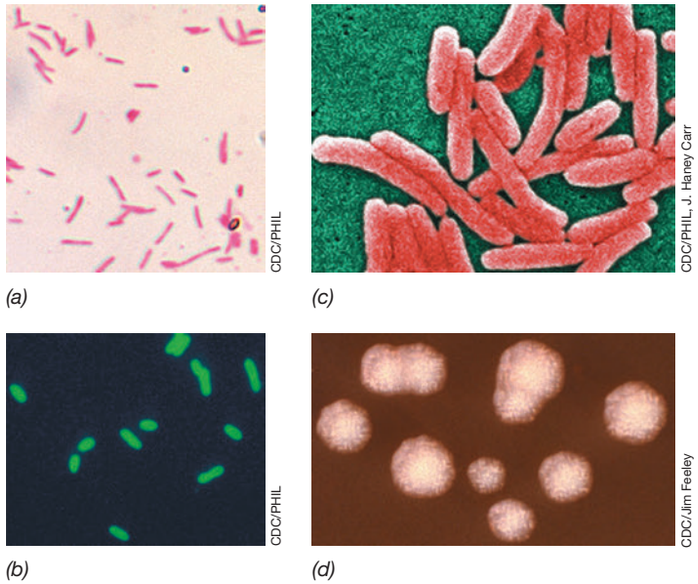


Figure 33.3 ***Legionella pneumophila.*** *(a)* Gram-stained cells of *L. pneumophila* from lung tissue of a legionellosis victim. *(b)* Cells of *L. pneumophila* can be positively identified using fluorescent anti–*L. pneumophila* antibodies (◀ Section 29.6). *(c)* Colorized scanning electron micrograph of *L. pneumophila* cells. Cells are about 0.5 × 2 μm. *(d)* Colonies of *L. pneumophila* grown on a complex enrichment medium showing their typical textured surface.

Epidemiology

L. pneumophila is a gram-negative, obligately aerobic rod-shaped species of the *Gammaproteobacteria* (Figure 33.3) and shows complex nutritional requirements, including an unusually high requirement for iron. The organism can be isolated from terrestrial and aquatic habitats as well as from legionellosis patients. *Legionella pneumophila* was first recognized as the pathogen that caused an outbreak of fatal pneumonia at an American Legion convention (thus the name legionellosis) in Philadelphia (USA) in 1976. Besides legionellosis, the same bacterium can also cause a milder syndrome called *Pontiac fever*.

L. pneumophila is present in freshwaters and in soil. It is relatively resistant to heating and chlorination, so it can spread through drinking water distribution systems (◀ Section 22.10). The pathogen is often found in large numbers in improperly sanitized cooling towers and evaporative condensers of large air conditioning systems. The pathogen grows in the water and is disseminated in humidified aerosols. Human infection is by way of airborne droplets, but the infection does not spread from person to person.

Besides its presence in evaporative coolers and domestic water systems, *L. pneumophila* has also been detected in hot water tanks and spas; in the latter, it can reach high cell numbers in warm (35–45°C), stagnant water, especially if chlorine (or other sanitizer) levels are not maintained. Many outbreaks of legionellosis have been linked to swimming pools. *L. pneumophila* can be eliminated from water supplies by hyperchlorination or by heating water to greater than 63°C. Although incidence peaks in the summer months, epidemiological studies indicate that *L. pneumophila* infections can occur at any time of year, primarily as a result of aerosols generated from heating and cooling systems and contaminated premise water

(◀ Section 22.10) used for showering or bathing. In the United States, a few thousand cases of legionellosis are typically reported each year.

Check Your Understanding

- How is legionellosis transmitted?
- Identify specific measures for control of *Legionella pneumophila*.

33.5 Typhoid Fever and Norovirus Illness

Although cholera and legionellosis are among the more lethal waterborne diseases, other waterborne pathogens also cause serious disease. We focus on two major ones here, the causative agents of typhoid (a bacterium) and norovirus gastrointestinal illness (an RNA virus).

Typhoid Fever

On a global scale, probably the most important waterborne bacterial pathogens are *Vibrio cholerae* (Section 33.3) and *Salmonella enterica* (*typhi*), the organism that causes typhoid fever. *S. enterica* (*typhi*) is a gram-negative, peritrichously flagellated bacterium related to *Escherichia coli* and other enteric bacteria (**Figure 33.4*a***). The organism is transmitted in feces-contaminated water, and thus typhoid fever, like cholera, is primarily restricted to areas where sewage treatment and general sanitation are either absent or poorly maintained. Typhoid today is a well-entrenched endemic disease in sub-Saharan Africa, the Indian subcontinent, and Indonesia, but appears only sporadically in North America, Europe, northern Asia, and Australia.

Typhoid fever progresses in several stages. Cells of the pathogen (Figure 33.4*a*) ingested in contaminated water (or occasionally food) reach the small intestine where they grow and enter the lymphatic system and the bloodstream; from here, the pathogen can travel to many different organs. One to two weeks later, the first symptoms of typhoid appear and include a mild fever, headache, and general malaise. During this period, the liver and spleen of the typhoid patient become heavily infected. About a week later, the fever becomes more intense (up to 40°C), and the patient typically becomes delirious; diarrhea can occur in this stage and abdominal pain can be severe. Complications can follow, including intestinal bleeding and perforation of the small intestine. The latter releases large numbers of bacterial cells into the abdomen, leading to a condition called *sepsis* (systemic infection and inflammation) and possibly also to septic shock. Both of these conditions are potentially fatal, and up to 40% of sepsis cases are fatal. After about a week in this crisis stage, the symptoms of typhoid begin to diminish, and recovery occurs.

Treatment for typhoid consists of antibiotic therapy and fluid replacement to ward off dehydration. In some cases, surgery may be necessary to repair perforated intestines. Although a variety of antibiotics can kill *S. enterica* (*typhi*), resistance to many of these has developed. Isolation of the causative strain and assessment of its antibiotic sensitivity (◀ Section 29.4) is often necessary to ensure that antibiotics will cure the infection.

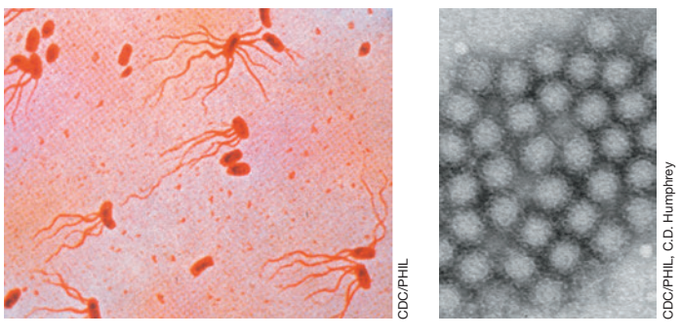
In the United States, fewer than 400 cases of typhoid occur per year, but typhoid fever used to be a major public health threat before drinking water was routinely filtered and chlorinated (◀ Figure 30.7). However, breakdown of water treatment methods, contamination of water during floods, earthquakes, and other disasters, or contamination of water supply pipes with leakage from sewer lines can propagate epidemics of typhoid fever, even in developed countries.

In some typhoid patients, the gallbladder becomes infected with the pathogen. If these individuals also have gallstones, these can become colonized with *S. enterica* (*typhi*) cells and serve as a long-term reservoir of the pathogen from which it is continuously shed into feces and urine. Such individuals are otherwise healthy "carriers" of typhoid and can transmit the disease over long periods. The notorious "Typhoid Mary," who as a cook for hire spread typhoid throughout the New York City area for nearly 15 years beginning in the early twentieth century, was the classic example of a typhoid carrier.

Norovirus Illness

Viruses can be transmitted in water and cause human disease. Norovirus (Figure 33.4*b*) is one example and a common cause of gastrointestinal illness due to contaminated water (or food, Section 33.14). Norovirus is a single-stranded plus-sense RNA virus (◀ Section 11.8) and is the leading cause of gastrointestinal illnesses worldwide (see Table 33.5). The virus specifically attacks *tuft cells* found in the intestinal epithelium. Normally quite rare, tuft cells proliferate during parasitic worm infections, and this likely explains why parasite-infected mice, having more tuft cells, experience more severe symptoms from exposure to norovirus than mice that do not have a worm infection. Presumably, humans would show similar responses to these infections.

Norovirus infection causes symptoms of vomiting, diarrhea, and malaise of relatively short duration. The disease is rarely fatal, although in compromised individuals (very young, elderly, or immune deficient), the significant dehydration that accompanies repeated bouts of norovirus-triggered vomiting and diarrhea can be life-threatening. A clinical diagnosis of norovirus gastrointestinal illness is made by a combination of observing symptoms and the direct



(a) (b)

Figure 33.4 Bacterial and viral agents of severe gastrointestinal water borne diseases. *(a)* Flagella-stained cells of *Salmonella enterica* (*typhi*) showing peritrichous flagellation (◀ Section 2.9). A single cell measures about 1 × 2 μm. *(b)* Transmission electron micrograph of virions of norovirus. A single virion is about 30 nm in diameter.

detection of either viral RNA by RT-PCR (◀ Sections 12.1 and 29.8) or viral antigens by enzyme immunoassay (◀ Section 29.7) in samples of feces or vomit.

Norovirus disease is easily transmitted person to person or to food by the fecal–oral route. The infectious dose is very small, as exposure to as few as 10–20 norovirus virions (Figure 33.4*b*) is sufficient to initiate disease. The most common sources of waterborne norovirus outbreaks are well water or recreational waters that have been contaminated with sewage. Norovirus is also often the culprit when mass common-source gastrointestinal illnesses strike people on cruise ships or in long-term care facilities or other group settings. In these situations, the virus can be transmitted person to person, by contaminated food or water (usually food, such as shellfish, deli meats, and fruits and vegetables), or by any combination of these.

Check Your Understanding

- Contrast the causative agents of typhoid and norovirus gastrointestinal disease.
- What public health conditions allow for outbreaks of typhoid fever?

III • Food as a Disease Vehicle

Mastering Microbiology
Art Activity: Table 33.3 Microbial spoilage of fresh food

By their very nature, foods are nutritious substances. Proper food storage and preservation practices are thus necessary to prevent food contamination and common-source foodborne outbreaks.

The foods we eat, whether they are fresh, prepared, or preserved, are rarely sterile. Instead, they are almost always contaminated with spoilage microorganisms of various kinds and occasionally with pathogens. Microbial activities are key to the production of some foods, such as fermented foods, but most of the microorganisms in or on food are unwelcome and diminish either food quality or safety (or both). In the next two sections, we explore the contrasting processes of food spoilage and food preservation, how food safety is assessed, and the transmission of pathogens in food. In the remainder of the chapter we focus on major foodborne diseases.

33.6 Food Spoilage and Food Preservation

Many foods provide an excellent medium for the growth of bacteria and fungi. Properly stored food can still undergo food spoilage but is usually not a vehicle for disease, assuming that it was free of pathogens to begin with. This is because with rare exception, organisms responsible for food spoilage are not the same as those that cause foodborne illnesses.

Food Spoilage

Food spoilage is any change in the appearance, smell, or taste of a food product that makes it unacceptable to the consumer, regardless of whether the change is due to microbial growth. Foods are rich in organic matter, and the physical and chemical characteristics of a food determine its susceptibility to microbial activity. With respect to spoilage, a food or food product falls into one of three categories: (1) **Perishable foods** include many fresh food items such as meats and many fruits and vegetables; (2) **semiperishable foods** include foods such as potatoes, some apples, and nuts; and (3) **nonperishable foods** include items such as sugar and flour. The foods in these categories differ primarily with regard to their *moisture content*, as measured by their water activity (a_w, ◀ Section 4.15). Nonperishable foods have low moisture levels and can generally be stored for long periods without spoilage. Perishable and semiperishable foods, by contrast, typically have higher moisture levels and hence these foods must be stored under conditions that inhibit microbial growth.

Fresh foods are typically spoiled by a wide variety of bacteria and fungi (Table 33.3). The chemical properties of foods vary widely, and each food is characterized by its moisture level and the nutrients it contains as well as other factors, such as its acidity or alkalinity. As a result, each susceptible food is typically spoiled by a specific group of microorganisms. The time required for a microbial population to reach a significant level in a given food product depends on both the size of the initial inoculum and the rate of growth during the exponential phase. Microbial numbers in a food product may initially be so low that no measurable effect can be observed, with only the last few cell doublings leading to observable spoilage. Hence, an unconsumed portion of a food product that is palatable and eaten one day can be badly spoiled the next.

The type of food spoilage and the microbial composition of the spoilage community (Table 33.3) are functions of both the food product and the storage temperature. Food spoilage microorganisms

TABLE 33.3 Microbial spoilage of fresh food[a]

Food product	Type of microorganism	Common spoilage organisms, by genus
Fruits and vegetables	Bacteria	*Erwinia, Pseudomonas, Corynebacterium* (mainly vegetable pathogens; rarely spoil fruit)
	Fungi	*Aspergillus, Botrytis, Geotrichum, Rhizopus, Penicillium, Cladosporium, Alternaria, Phytophthora*, various yeasts
Fresh meat, poultry, eggs, and seafood	Bacteria	*Acinetobacter, Aeromonas, Pseudomonas, Micrococcus, Achromobacter, Flavobacterium, Proteus, Salmonella, Escherichia, Campylobacter, Listeria*
	Fungi	*Cladosporium, Mucor, Rhizopus, Penicillium, Geotrichum, Sporotrichum, Candida, Torula, Rhodotorula*
Milk	Bacteria	*Streptococcus, Leuconostoc, Lactococcus, Lactobacillus, Pseudomonas, Proteus*
High-sugar foods	Bacteria	*Clostridium, Bacillus, Flavobacterium*
	Fungi	*Saccharomyces, Torula, Penicillium*

[a]The organisms listed are the most commonly observed spoilage agents of fresh, perishable foods. Many of these genera include species that are human pathogens (Chapters 31–34).

are often *psychrotolerant*, meaning that although they grow best at temperatures above 20°C, they can also grow at refrigeration temperatures (3–5°C) (◀ Section 4.12). However, at any given storage temperature, some species grow faster than others, and thus the composition of the microbial spoilage community of the same food product stored at different temperatures can vary significantly.

Food Preservation and Fermentation

Food storage and preservation methods are designed to slow or stop the growth of microorganisms that spoil food or that can cause foodborne disease. The major methods of food preservation include altering the temperature, acidity, or moisture level of the food, or treating it with radiation or chemicals that prevent microbial growth.

Refrigeration slows microbial growth, but a remarkable number of microorganisms, particularly bacteria, can grow at refrigeration temperatures. Storage of foods in a freezer reduces growth considerably, but slow growth still occurs in pockets of liquid water trapped within the frozen food. In general, a lower storage temperature results in less microbial growth and slower spoilage, but storage at temperatures below −20°C is too expensive for routine use and also can negatively affect food appearance, consistency, and taste.

Heat reduces the bacterial load and can even sterilize a food product, and it is especially useful for the preservation of liquids or high-moisture foods. The limited heat treatment of **pasteurization** (◀ Section 4.17) does not sterilize liquids but reduces microbial numbers and eliminates pathogens. *Canning*, by contrast, typically sterilizes the food but requires careful processing in a sealed container at the correct temperature for the correct length of time. If viable microorganisms remain in a can or glass jar, their growth can produce gas, causing the vessel to bulge or even explode (**Figure 33.5**). The environment inside a can or sealed jar is anoxic, and an important genus of anaerobic bacteria that can grow in canned foods is the endospore-forming *Clostridium*, one species of which causes botulism (Section 33.9 and ◀ Section 25.6).

Foods can be made drier by either physically removing the water or by adding solutes, such as salt or sugar. Extremely dry or solute-loaded foods help prevent bacterial growth, but spoilage can still occur and when it does, it is typically from fungi. Many foods are preserved by the addition of small amounts of antimicrobial chemicals. These chemicals, which include nitrites, sulfites, propionate, and benzoate along with a few others, find wide application in the food industry for enhancing or preserving food texture, color, freshness, or flavor. However, one of these flavor-enhancing additives, the sugar trehalose, stimulates the growth of *Clostridioides difficile*, a dangerous emerging pathogen (◀ Section 30.7). Therefore, the benefit of any chemical food additive must be weighed against its potential to cause more harm than good by inadvertently promoting the growth of unwanted microorganisms.

Perhaps the fastest growing form of food preservation is *irradiation* with ionizing radiation, especially that generated by electron beam (eBeam) technology. The application of eBeam irradiation to foods causes the oxidation—and therefore deactivation—of key macromolecules in microbial cells. Such treatment has been shown to be an effective means for reducing microbial contamination of foods without adding anything to the product itself and potentially changing its form or flavor.

Many common foods and beverages are preserved through the metabolic activities of microorganisms; these are *fermented foods* (**Figure 33.6**, **Table 33.4**, and ◀ Figure 1.16). The fermentation process (Chapters 3 and 14) yields large amounts of natural preservative chemicals. The major bacteria important in the fermented foods industry produce organic acids or fatty acids; prime examples are the lactic acid bacteria (in fermented milk products), the acetic acid bacteria (in pickling), and the propionic acid bacteria (in certain cheeses) (Table 33.4). The yeast *Saccharomyces cerevisiae* produces alcohol as the preservative in the production of alcoholic beverages.

Mastering Microbiology
Art Activity: Table 33.4 Fermented foods and fermentation microorganisms

The high level of organic acids or alcohol generated from these fermentations prevents the growth of both spoilage organisms and pathogens in the fermented food product. But the fermentation process yields more than just preservatives. Depending on the product and the microbes that carry out the fermentation, many other chemicals are released that add to the overall nutritional value, taste, and aroma of a given fermented food.

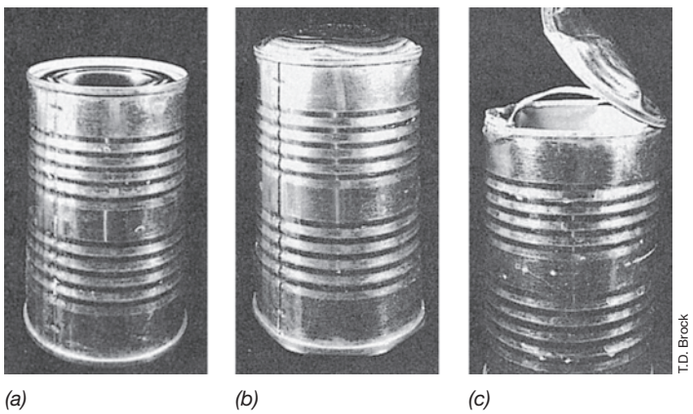


Figure 33.5 Changes in sealed cans as a result of microbial spoilage. *(a)* A normal can. The top of the can is pulled in a bit because of the normal slight vacuum inside. *(b)* Swelling due to gas production. *(c)* The can shown in *b* was dropped, and the gas pressure resulted in a violent explosion, tearing the lid apart.

Figure 33.6 Examples of fermented foods. Bread, sausage meats, cheeses and many other dairy products, and fermented and pickled vegetables are food products that are produced or enhanced by fermentation reactions catalyzed by microorganisms (see also Table 33.4).

TABLE 33.4 Fermented foods and fermentation microorganisms

Food category/ Preservative	Primary fermenting microorganisms[a]
Dairy foods/Lactic acid, propionic acid	
Cheeses	*Lactococcus, Lactobacillus, Streptococcus thermophilus, Propionibacterium* (Swiss cheese)
Fermented milk products	
Buttermilk and sour cream	*Lactococcus*
Yogurt	*Lactobacillus, Streptococcus thermophilus*
Alcoholic beverages/Ethanol	*Zymomonas, Saccharomyces*[b]
Yeast breads/Baking	*Saccharomyces cerevisiae*[b]
Meat products/Lactic and other acids	
Dry sausages (pepperoni, salami) and semidry sausages (summer sausage, bologna)	*Pediococcus, Lactobacillus, Micrococcus, Staphylococcus*
Vegetables/Lactic acid	
Cabbage (sauerkraut)	*Leuconostoc, Lactobacillus*
Cucumbers (pickles)[c]	Lactic acid bacteria
Vinegar/Acetic acid	*Acetobacter*
Soy sauce/Lactic acid and many other substances	*Aspergillus*,[d] *Tetragenococcus halophilus*, yeasts

[a]Unless otherwise noted, these are all species of *Firmicutes* except for *Micrococcus*, which is in the *Actinobacteria*, and *Zymomonas* and *Acetobacter*, which are in the *Alphaproteobacteria*. For some of these fermented food products, see Figure 1.16 and Figure 33.6.
[b]Yeast. Various *Saccharomyces* species are used in alcohol fermentations. *S. cerevisiae* is the common baker's yeast. To make sourdough bread, species of *Lactobacillus* are used.
[c]Nonfermented pickles are cucumbers marinated in vinegar (5–8% acetic acid).
[d]A mold.

Check Your Understanding

- List the major food groups as categorized by their susceptibility to spoilage.
- Identify physical and chemical methods used for food preservation. How does each method limit growth of microorganisms?
- List some dairy, meat, beverage, and vegetable foods produced by microbial fermentation. What is the preservative in each case?

33.7 Foodborne Diseases and Food Epidemiology

Foodborne illnesses resemble waterborne illnesses in being *common-source* diseases (◀ Section 30.4). Most foodborne disease outbreaks are due to improper food handling and preparation by domestic consumers; these typically affect only a few people and are rarely reported. However, occasional disease outbreaks due to breakdowns in safe food handling and preparation at restaurants or food-processing and distribution plants can affect large numbers of people in geographically widespread regions.

Foodborne Diseases and Microbial Sampling

Foodborne diseases are of two types: *food infections* and *food poisonings*; some foodborne diseases fall into both categories. **Food poisoning**, also called **food intoxication**, results from ingestion of foods containing preformed microbial toxins. The microorganisms that produced the toxins do not have to grow in the host and may not even be alive at the time the contaminated food is consumed; ingestion and activity of the toxin is what causes the illness. We previously discussed some of these toxins, notably the exotoxin of *Clostridium botulinum* and the superantigen toxins of *Staphylococcus* and *Streptococcus* (◀ Sections 25.6 and 28.2). In contrast to food poisoning, **food infection** occurs from the ingestion of food containing sufficient numbers of viable pathogens to cause colonization and growth of the pathogen in the host, ultimately resulting in disease.

Food infections are the most common foodborne illnesses in the United States and account for four of the top five leading foodborne illnesses. Table 33.5 lists the major microorganisms that cause food infections and food poisonings in the United States.

Eight microorganisms account for the great majority of foodborne illness, hospitalizations, and deaths in the United States: *Salmonella* species, *Clostridium perfringens, Campylobacter jejuni, Staphylococcus aureus, Listeria monocytogenes,* and *Escherichia coli* (all bacteria); norovirus; and *Toxoplasma* (a protist) (Table 33.5). Four of these—norovirus, *Salmonella, C. perfringens*, and *Campylobacter*—account for nearly 90% of all foodborne illness, with norovirus (Sections 33.5 and 33.14) being the most common culprit (60%).

Rapid diagnostic methods that do not require culturing an organism have been developed to detect important food pathogens, and many of these were described in Chapter 29. Isolation of pathogens from foods usually requires preliminary treatment of the food to suspend microorganisms embedded or entrapped within. A standard method for this purpose employs a blender called a paddle blender, or Stomacher® (Figure 33.7), a device to process food samples sealed in sterile bags. Paddles in the Stomacher crush, blend, and homogenize the samples in a fashion resembling the peristaltic action of the stomach but under conditions that prevent contamination. The homogenized samples are then analyzed for specific pathogens or their products.

In addition to identifying pathogens in the food itself, disease investigators must also recover the foodborne pathogen from the diseased patient in order to establish a cause-and-effect relationship between the pathogen and the illness. In fact, identification of the *same strain* (not just the *same species*) of a particular pathogen in patients and the suspected contaminated food is the "gold standard" for linking cause and effect in a foodborne disease outbreak, and a variety of microbiological, immunological, and molecular techniques are available for these purposes (Chapter 29).

Foodborne Disease Epidemiology

An outbreak of foodborne disease can occur in a home, a school cafeteria, a college dining hall, a restaurant, a military mess hall, or anywhere a contaminated food is consumed by many individuals. In addition, central food-processing plants and distribution centers provide opportunities for contaminated foods to cause disease outbreaks far from where the food was originally processed. It is the job

TABLE 33.5 Major foodborne pathogens[a]

Organism	Disease[b]	Foods
Bacteria		
Bacillus cereus	FP and FI	Rice and starchy foods, high-sugar foods, meats, gravies, pudding, dry milk
Campylobacter jejuni	FI (2)[c]	Poultry, dairy
Clostridium botulinum	FP	Improperly heat-processed nonacidic foods such as home-canned vegetables (beans, potatoes, corn, asparagus)
Clostridium perfringens	FP and FI (4)[c]	Meat and vegetables held at improper storage temperature
Escherichia coli O157:H7	FI	Meat, especially ground beef, raw vegetables
Other enteropathogenic *Escherichia coli*	FI	Meat, especially ground meat, raw vegetables
Listeria monocytogenes	FI	Refrigerated "ready to eat" foods
Salmonella spp.	FI (3)[c]	Poultry, meat, dairy, eggs
Staphylococcus aureus	FP (5)[c]	Meat, desserts
Streptococcus spp.	FI	Dairy, meat
Yersinia enterocolitica	FI	Pork, milk
All other bacteria	FP and FI	
Protists[d]		
Cryptosporidium parvum	FI	Raw and undercooked meat
Cyclospora cayetanensis	FI	Fresh produce
Giardia intestinalis	FI	Contaminated or infected meat
Toxoplasma gondii	FI	Raw and undercooked meat
Viruses		
Norovirus	FI (1)[c]	Shellfish, many other foods
Hepatitis A	FI	Shellfish and some other foods eaten raw

[a]Data from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.
[b]FP, food poisoning; FI, food infection.
[c]The number in parentheses is the rank of the top five foodborne pathogens in the United States.
[d]All of these protists are discussed in Chapter 34.

of the food epidemiologist to track disease outbreaks and determine their source, often down to the precise location in which the food was contaminated.

A good example of effective foodborne disease tracking is the outbreak caused by *Escherichia coli* O157:H7 (see Section 33.11 and Figure 33.12*b*) in the United States in 2006. Through culturing and molecular studies, this outbreak was linked to the consumption of contaminated packaged spinach and was quickly traced to a food-processing facility in California. The contaminated spinach was distributed nationwide from the California plant, but most disease cases were in the Midwest. In the summer of 2013, another "packaged" outbreak occurred in the Midwest but in this case was linked to lettuce instead of spinach and to the parasite *Cyclospora cayetanensis* (Section 34.4) instead of to the bacterium *E. coli*.

Figure 33.7 A paddle blender (Stomacher®). Paddles in this specialized blender homogenize the food sample contained in a sealed and sterile bag (arrow) to mimic the peristaltic action of the human stomach. The sample is first suspended in a sterile solution to form a uniform mixture.

To be effective, foodborne disease trackers must work quickly. For example, when the first case in the *E. coli* spinach outbreak appeared in late August, a link to the specific spinach product was made less than a month later. Because *E. coli* O157:H7 has been well studied, public health officials were able to quickly identify the strain contaminating the bagged spinach. Authorities then traced this strain back to the processing plant and eventually identified a specific agricultural field near the processing plant as the source of the pathogen. Although it remains unclear how the spinach was contaminated, domestic animal manure was the likely source. During the outbreak, two foodborne disease surveillance networks, *FoodNet* (Centers for Disease Control and Prevention) and *PulseNet* (an international molecular typing network for foodborne diseases), played important roles in exposing and ending the outbreak.

The spinach *E. coli* epidemic, although serious and even deadly for some, was discovered, contained, and stopped very quickly. However, this incident shows how centralized food-processing facilities can quickly spread disease to distant populations. Because of this, food hygiene standards and surveillance must be maintained at the highest possible level at all times in restaurants and central food-processing and distribution facilities.

Check Your Understanding

- Distinguish between food infection and food poisoning.
- Describe microbial sampling procedures for solid foods such as meat.
- Describe how a foodborne disease outbreak is tracked.

IV • Food Poisoning

Food poisoning occurs from the ingestion of food contaminated by bacterial or fungal toxins and does not require the producing organism to grow in the host. Food poisoning symptoms range from short-duration, mild or severe afflictions to life-threatening conditions that can be fatal.

Food poisoning can be caused by various bacteria and a few fungi. Here we consider the gram-positive bacteria *Staphylococcus aureus, Clostridium botulinum*, and *Clostridium perfringens*, the most common causes of bacterial food poisoning. Two of these microbes—*S. aureus* and *C. perfringens*—are among the "top five" causes of foodborne illness (Table 33.5).

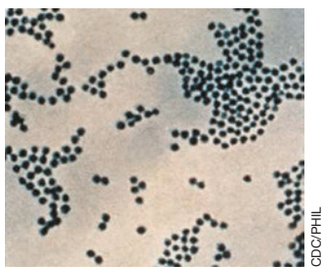
33.8 Staphylococcal Food Poisoning

A powerful form of food poisoning is caused by enterotoxins (◀ Section 25.6) produced by the gram-positive bacterium *Staphylococcus aureus* (**Figure 33.8**; ◀ Section 16.7). This organism is commonly associated with the skin and upper respiratory tract and is a frequent cause of pus-forming wounds (◀ Section 31.9 and Figure 31.29). *S. aureus* can grow aerobically or anaerobically in many common foods and produces a suite of enterotoxins. When consumed, the toxins cause gastrointestinal symptoms characterized by one or more of nausea, vomiting, diarrhea, and dehydration. The onset of symptoms is rapid, within 1–6 h of ingestion depending on the amount of enterotoxin consumed, but the symptoms usually pass within 48 h.

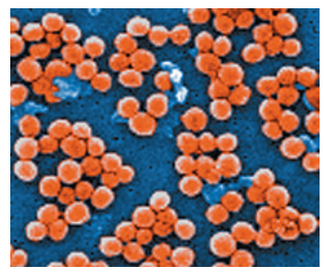
Staphylococcal Enterotoxins

Many *S. aureus* enterotoxins are heat-stable and all are stable to stomach acidity. Most strains of *S. aureus* produce only one or two of these toxins, and some strains are nonproducers. However, any one of the staphylococcal ("staph") enterotoxins can cause food poisoning. The toxins pass through the stomach to the small intestine and trigger disease symptoms from there. Besides their normal gastrointestinal activities, staph enterotoxins are also *superantigens* and can lead to potentially lethal toxic shock syndrome (◀ Sections 25.7 and 28.2).

S. aureus enterotoxins are given acronyms beginning with "SE" (for "staphylococcus *e*nterotoxin"): SEA, SEB, SEC, and SED, which are encoded by the genes *sea, seb, sec*, and *sed*. Not all of these genes are on the *S. aureus* chromosome, but their sequences show them to be highly related. The genes *seb* and *sec* are encoded on the bacterial chromosome, *sea* on a lysogenic bacteriophage (◀ Section 5.6), and *sed* on a plasmid. The phage- and plasmid-encoded genes can transfer the ability to make toxin to nontoxigenic strains of *Staphylococcus* by horizontal gene transfer (Chapter 9). SEA is the most common cause of staph food poisoning worldwide.



(a)



(b)

Figure 33.8 ***Staphylococcus aureus.*** *(a)* Gram-stained light micrograph showing the typical "cluster of grapes" morphology of staphylococci. *(b)* Colorized scanning electron micrograph of cells of *S. aureus*. A single cell is about 0.8 μm in diameter.

Disease Properties, Treatment, and Prevention

Foods may contain cells of *S. aureus* for several reasons. The organism may have been present on the food source itself, for example, on a meat product. More commonly, however, cells of *S. aureus* are introduced to the food by contamination from the food preparer or by contamination of the food product with raw meat or a contaminated sauce or dressing. A common scenario for a staph food poisoning incident is when a food preparer introduces *S. aureus* from nasal secretions or from an uncovered skin wound or leaking bandage into the food during its preparation. If the contaminated food is then stored at room temperature or above, the stage is set for the rapid growth of *S. aureus* and the production of staph enterotoxins.

Each year there are an estimated nearly quarter million cases of staphylococcal food poisoning in the United States. The foods most commonly implicated are custard- and cream-filled baked goods, poultry, eggs, raw and processed meat, puddings, and creamy salad dressings. Salads prepared with mayonnaise-based dressings or those that contain shellfish, chicken, pasta, tuna, potato, egg, or meat, are also common vehicles. Salted foods such as ham can be vehicles because of the ability of *S. aureus* to grow quickly in salty environments (◀ Section 31.9). If any of these foods are contaminated with *S. aureus* but are refrigerated immediately after preparation, they usually remain safe because the organism grows poorly at low temperatures. But if enterotoxin has already been produced, mild heating may not make the food safe, as staph enterotoxins are stable to 60°C.

Treatment of staph food poisoning with antibiotics is not useful because any ingested cells of *S. aureus* have already been killed by the acidity in the stomach and antibiotics have no effect on the enterotoxins. Rest, drinking plenty of fluids, and using anti-nausea drugs are the best prescription for a rapid recovery. As for any foodborne illness, staphylococcal food poisoning can be prevented by proper sanitation and hygiene in food production, preparation, and storage. In this regard, food preparers should practice thorough and frequent hand washing, prevent foods from coming into contact with nasal tissues and secretions, and routinely wear and frequently change disposable gloves when handling food products, especially if they have a bandaged hand wound.

In addition, thorough cleaning of plastic (polyethylene) cutting boards is essential but notoriously difficult, as the scratches and cuts that accumulate on these items tend to harbor significant numbers of microbes. Unlike wooden cutting boards, whose fibers tend to self-seal, and glass and granite boards that are not scored by cutting utensils, plastic cutting boards that retain deep cuts and scratches may be nearly impossible to clean satisfactorily. Despite this, these items are still used routinely in the food industry, even though the

United States Food and Drug Agency Food Code dictates that food preparation surfaces must be smooth to ensure that sanitation protocols are effective.

Check Your Understanding

- Identify the symptoms and mechanism of staphylococcal food poisoning.
- Why does antibiotic treatment not affect the outcome or the severity of disease with staph food poisoning?

33.9 Clostridial Food Poisoning

The endospore-forming anaerobic bacteria *Clostridium perfringens* and *Clostridium botulinum* (◀ Section 16.8) cause serious food poisoning. Canning and cooking procedures kill vegetative cells of these species but may not kill all endospores. If this occurs, viable endospores in the food can germinate, and the resulting cells produce toxins.

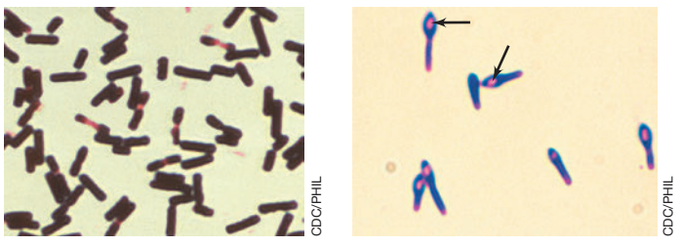
There is a clear distinction in the disease process between perfringens food poisoning and botulism. In the case of botulism, the toxin is a neurotoxin and only the toxin is required for disease. Botulism does not require the growth of *C. botulinum* in the human body, but growth may nevertheless occur, particularly in cases of infant botulism. By contrast, with perfringens food poisoning, a large number of cells must be ingested in order for the toxin—in this case, an enterotoxin—to be produced.

Clostridium perfringens Food Poisoning

Clostridium perfringens (**Figure 33.9*a***) is commonly found in soil but can also be found in sewage, primarily because it lives in small numbers in the intestinal tract of humans and other animals. *C. perfringens* is the fourth most often reported cause of foodborne disease in the United States behind norovirus illnesses (Sections 33.5 and 33.14), *Campylobacter* infections (Section 33.12), and *Salmonella* infections (Section 33.10 and Table 33.5). About 1 million cases of perfringens food poisoning occur in the United States every year.

C. perfringens is a proteolytic bacterium; proteins are catabolized by fermentation (◀ Section 14.19). Perfringens food poisoning requires the ingestion of a large dose ($>10^8$) of *C. perfringens* cells in contaminated cooked or uncooked foods, especially high-protein foods such as meat, poultry, and fish. *C. perfringens* can grow in meat dishes cooked in bulk where heat penetration is often insufficient. *C. perfringens* grows quickly in the food, especially if left to cool at room temperature. It is when endosporulation (◀ Section 2.8 and Figures 2.26–2.29) begins that the perfringens enterotoxin is produced. The toxin alters the permeability of the intestinal epithelium, leading to nausea, diarrhea, and intestinal cramps. The onset of perfringens food poisoning typically begins 6–24 h after consumption of the contaminated food and usually resolves within 24 h; for this reason, the disease is sometimes written off as a "stomach flu" or "24-hour flu." Fatalities from perfringens food poisoning are rare, and no specific treatment is necessary other than replacing fluids lost from diarrhea or vomiting (if it occurs).

A diagnosis of perfringens food poisoning is made from isolation of *C. perfringens* from the feces or, more reliably, by an immunoassay that can detect *C. perfringens* enterotoxin in feces. Prevention of perfringens food poisoning requires that cooked foods not be contaminated with raw foods and that all foods be properly heated during cooking and home canning. The perfringens enterotoxin is heat-labile and thus any toxin that may have formed in a food product is destroyed by proper heating (75°C). Cooked foods not eaten immediately should be refrigerated as soon as possible to rapidly lower temperatures and inhibit the growth of any *C. perfringens* that may have been present.



(a) (b)

Figure 33.9 Food poisoning clostridia. *(a)* Gram stain of a growing culture of *Clostridium perfringens*, the bacterium that causes perfringens food poisoning. A cell measures about 1 × 3 μm. *(b)* Gram stain of a sporulating culture of *Clostridium botulinum*, the agent of botulism. A cell measures about 1 × 5 μm and endospores (arrows) appear red.

Botulism

Botulism is a severe and potentially fatal food poisoning caused by the consumption of food containing the exotoxin produced by *C. botulinum* (Figure 33.9*b*). This bacterium normally inhabits soil or water, but its cells or endospores may contaminate raw and processed foods. If viable endospores of *C. botulinum* remain in the food, they may germinate and produce botulinum toxin; ingesting even a small amount of this highly poisonous substance can cause severe illness or death.

Botulinum toxin is a neurotoxin that affects autonomic nerves that control key body functions such as respiration and heartbeat; the typical result is a flaccid paralysis (◀ Section 25.6 and Figure 25.14). At least seven distinct botulinum toxins are known. Because the toxins are destroyed by heat (80°C for 10 minutes), *thoroughly* cooked food, even if contaminated with toxin, is harmless. Much foodborne botulism is from improperly processed home-canned foods, especially nonacidic foods such as corn, potatoes, and beans. Any viable *C. botulinum* endospores that remain in the sealed (and now anoxic) jar may germinate during storage and produce toxin. Many of these foods are used without cooking when making cold salads, and hence any botulinum toxin present is not destroyed. Prevention of foodborne botulism thus requires careful attention to canning and related food preservation practices.

Although infants can be poisoned by toxin-contaminated food, the majority of infant botulism cases occur from toxin produced following actual *infection* of the infant with *C. botulinum*. This occurs most commonly in newborns up to about 2 months of age because they lack a well-developed intestinal microbiota that can outcompete *C. botulinum*. Ingested *C. botulinum* endospores germinate in the infant's intestine, triggering growth and toxin production. Wound botulism can also occur from infection, presumably from endospores in contaminating material introduced via a parenteral route. Wound botulism is most commonly associated with illicit injectable drug use.

In 2017, 182 cases of botulism were confirmed in the United States, most of which (141 cases; 77%) were infant botulism; cases of foodborne and wound botulism occurred less often and with equal frequency. Although rare, botulism is always serious because of the high mortality associated with untreated disease. However, because most cases are diagnosed and treated, less than 5% of all botulism cases result in death.

Botulism is diagnosed when either botulinum toxin or *C. botulinum* cells are detected in the patient (or in the contaminated food) coupled with clinical observations of localized paralysis (for example, impaired vision and speech) beginning 18–36 h after ingestion of the contaminated food. Treatment for botulism is by administration of botulinum antitoxin if the diagnosis is early, and mechanical ventilation if signs of respiratory paralysis have already appeared. If the dose of toxin is not too high, infant botulism is usually self-limiting, and most infants recover with only supportive therapy, such as assisted ventilation.

Mastering Microbiology
Art Activity: Figure 33.12 Some sources of *Salmonella*

We move on now to consider food infections—afflictions distinct from food poisonings—with the "salmonella problem" taking stage front and center.

Check Your Understanding

- Compare and contrast toxin production and toxemia in botulism and perfringens food poisoning.
- Describe differences in the transmission of botulism in adults versus infants.

V • Food Infection

Food infections result from the ingestion of pathogens that colonize and multiply in the host and trigger symptoms that usually require medical intervention. *Salmonella* is one of the most notorious bacterial culprits in food infections, but viruses and parasites can also cause these maladies.

Recall that food *infection* is not the same thing as food *poisoning* (Section 33.7). Food infection results from ingestion of food containing sufficient numbers of viable pathogens to allow growth of the pathogen and disease in the host. Food infections are common, and in the United States, the sum total of food infections outnumbers cases of food poisoning by nearly 10-fold. Sections 25.1 and 25.2 reviewed the infection process, summarizing the steps by which microorganisms—friend and foe alike—attach and become established in host tissues.

33.10 Salmonellosis

Salmonellosis is a gastrointestinal disease typically caused by ingesting food contaminated with *Salmonella* or by handling *Salmonella*-contaminated animals or animal products (**Figure 33.10**). Only *Campylobacter* (Section 33.12) produces more bacterial food infections than *Salmonella* (Table 33.5). Symptoms of salmonellosis begin after the pathogen—a gram-negative, facultatively aerobic rod related to *Escherichia coli* (◀ Section 16.3 and see Figure 33.11)—colonizes the intestinal epithelium. *Salmonella* species normally inhabit the gastrointestinal tract of endothermic (warm-blooded) and many ectothermic (cold-blooded) animals (Figure 33.10) and are common in sewage. Thus, some cases of salmonellosis are waterborne rather than foodborne infections, and this is especially the case for typhoid fever (Section 33.5).

The accepted species epithet for pathogenic *Salmonella* is *enterica*, and there are seven subspecies of *S. enterica*. Most human-associated salmonellas fall into the *S. enterica* subspecies *enterica* group. Each subspecies is also divided into *serovars* (serological variants). Thus, there are *Salmonella enterica* serovar Typhi, or *Salmonella enterica* (*typhi*) for short, and *Salmonella enterica* serovar Typhimurium, and so on. *S. enterica* serovars Typhimurium and Enteritidis are most frequently associated with foodborne salmonellosis.

Pathogenesis and Epidemiology

The most common form of salmonellosis is *enterocolitis*. Ingestion of food containing viable cells of *Salmonella* results in colonization of both the small and large intestines. From here, cells of *Salmonella* invade phagocytic cells and grow intracellularly, spreading to adjacent cells as host cells die. After invasion, pathogenic *Salmonella* deploy several virulence factors, including endotoxins, enterotoxins, and cytotoxins that damage and kill host cells (Chapter 25). Symptoms of enterocolitis typically appear 12–72 h after ingestion and include a headache, chills, nausea, vomiting, and diarrhea, followed

CDC/PHIL, Eric Grafman

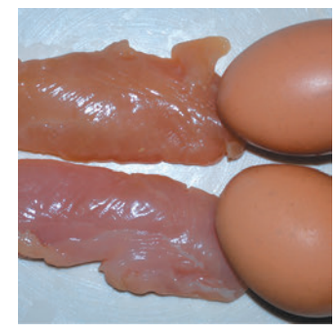
(a)

CDC/PHIL, James Gathany

(b)

CDC/PHIL, James Gathany

(c)


Michael T. Madigan

(d)

Figure 33.10 Some sources of *Salmonella*. *(a)* Poultry contain *Salmonella* in their intestines and droppings. *Salmonella* can also be transferred to humans from both *(b)* reptiles and *(c)* amphibians. *(d)* Fresh chicken breasts and eggs.

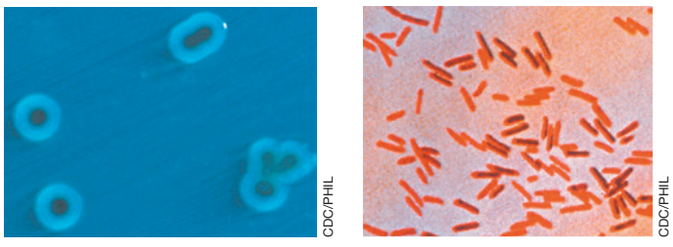
by a fever that can last for several days. The disease normally resolves without intervention in 2–5 days. After recovery, however, patients may shed *Salmonella* in their feces for several weeks and some become healthy carriers. A few serovars of *S. enterica* may also cause septicemia (a blood infection) and enteric or typhoid fever, a potentially fatal disease characterized by systemic infection and high fever lasting several weeks (Section 33.5).

The incidence of salmonellosis in the United States has been steady over the last decade, with about 1.2 million estimated cases each year. There are several routes by which *Salmonella* may enter the food supply. The bacteria may reach food through fecal contamination from food handlers. Food production animals such as chickens, pigs, and cattle harbor *Salmonella* serovars that are pathogenic to humans, and these may be carried through to fresh foods such as eggs, meat, and dairy products (Figure 33.10*d*). *Salmonella* food infections are often traced to products such as custards, cream cakes, meringues, pies, and eggnog made with uncooked eggs. Other foods commonly implicated in salmonellosis outbreaks are meats and meat products, especially poultry, cured but uncooked sausages and other meats, milk, and milk products. The simple handling of *Salmonella*-contaminated animals, especially reptiles (Figure 33.10*b*), can also lead to salmonellosis.

Diagnosis, Treatment, and Prevention

Foodborne salmonellosis is diagnosed from a combination of clinical symptoms, a history of recent consumption of high-risk foods, and culturing of the organism from feces. Selective, differential media are used to isolate *Salmonella* and discriminate it from other gram-negative enteric bacteria (**Figure 33.11**). Tests for the presence of *Salmonella* are commonly carried out on foods of animal origin, such as raw meat, poultry, eggs, and powdered milk. Tests include several rapid tests (Chapter 29), but even rapid tests usually rely on enrichment procedures to increase cell numbers of *Salmonella* to testable levels.

Treatment of enterocolitis is usually unnecessary, and antibiotic treatment does not typically shorten the course of the disease or eliminate the carrier state. Foods containing *Salmonella* but heated to at least 70°C are generally safe if consumed immediately, held at 50°C or above, or quickly refrigerated. Any foods that become contaminated by an infected food handler can support the growth of *Salmonella* if the food is held for a long enough period, especially if it is not kept very warm or refrigerated. A recently developed anti-*Salmonella* bacteriophage preparation, which is sprayed directly onto foods susceptible to *Salmonella* contamination, may help minimize the risk of salmonellosis, especially if refrigeration is unavailable.



(a) (b)

Figure 33.11 Isolation of *Salmonella*. *(a)* Colonies of *S. enterica* (*typhimurium*) on Hektoen agar, which contains inhibitors of gram-positive bacteria and both lactose and peptone as carbon sources. Thiosulfate in the medium is reduced to H_2S by *Salmonella* and complexes with iron to form black FeS. *Salmonella* thus forms white colonies with black FeS centers, a pattern unique among enteric bacteria. The blue color results from the medium turning alkaline because *Salmonella* species do not ferment lactose and instead consume the amino acids in peptone. *(b)* Gram stain of cells of *Salmonella*; an average cell is about 1 × 3 μm.

Check Your Understanding

- Describe salmonellosis food infection. What foods and disease symptoms are typically associated with salmonellosis?
- How might *Salmonella* contamination of food production animals be contained?

33.11 Pathogenic *Escherichia coli*

Most strains of *Escherichia coli* are common microbiota in the human colon and are not pathogenic. However, a few strains are potential foodborne (and occasionally waterborne) pathogens (**Figure 33.12**) and produce potent enterotoxins (◀ Section 25.6). These pathogenic strains are grouped on the basis of the type of toxin they produce and their specific disease syndromes. We focus here on Shiga toxin–producing *E. coli* and briefly consider some other toxigenic *E. coli* strains.

Although not in the "top five" in terms of foodborne infection pathogens (Table 33.5), pathogenic *E. coli* strains cause disease symptoms so severe that they often require hospitalization. Indeed, infections with pathogenic *E. coli* may cause life-threatening diarrheal disease and urinary tract distress.

Shiga Toxin–Producing *Escherichia coli* (STEC)

Shiga toxin–producing *Escherichia coli* (STEC) strains produce *verotoxin*, an enterotoxin similar to the Shiga toxin produced by *Shigella dysenteriae*, a close relative of *E. coli*. This toxin inhibits protein synthesis and induces a bloody diarrhea (dysentery) and kidney failure. STEC strains of *E. coli* are also called *enterohemorrhagic E. coli* (EHEC). The most widely distributed STEC is *E. coli* O157:H7 (Figure 33.12*b*). Following ingestion of food or water containing STEC (as few as 10 cells can trigger the disease), the bacteria infect the small intestine where they grow and produce verotoxin, which both causes a bloody diarrhea and initiates signs of kidney failure.

Nearly half of STEC infections are caused by the consumption of contaminated uncooked or undercooked meat, particularly mass-processed ground beef. *E. coli* O157:H7 is normally present in the intestines of healthy cattle and enters the human food chain if meat is contaminated with the animal's intestinal contents during slaughter and processing. STEC strains have also been implicated in food infection outbreaks caused by dairy products (especially raw milk products), fresh fruit, and raw vegetables. Contamination of the fresh foods by fecal material, typically from cattle carrying STEC strains, has been implicated in several of these cases (Section 33.7).

Other Pathogenic *Escherichia coli*

Children in developing countries often contract diarrheal disease caused by *E. coli*, and *E. coli* can also be the cause of "traveler's diarrhea," a common infection causing watery diarrhea (as opposed to the bloody diarrhea of STEC strains) in travelers to developing

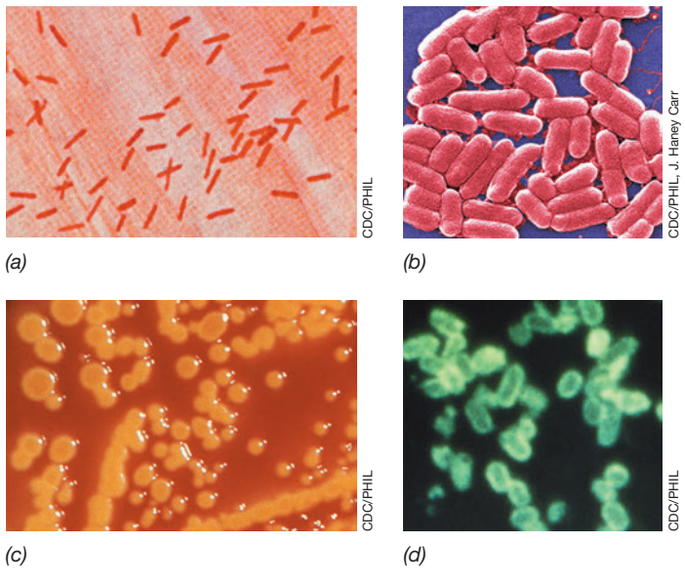


Figure 33.12 **Pathogenic *Escherichia coli*.** *(a)* Gram-stained cells showing the typical gram-negative, rod-shaped morphology of *E. coli*. *(b)* Colorized scanning electron micrograph of cells of *E. coli* O157:H7. Cells measure about 1 × 3 μm. *(c) E. coli* can be easily isolated on various selective and differential culture media such as Hektoen agar, where colonies of *E. coli* turn yellow because this bacterium ferments lactose and acidifies the medium (compare these with colonies of *Salmonella* on Hektoen agar in Figure 33.11*a*). *(d)* Enteropathogenic strains of *E. coli* can be detected, as in this fecal smear, using a specific fluorescent antibody (◀ Section 29.6).

countries. The primary causal agents here are *enterotoxigenic E. coli* (ETEC, Figure 33.12*d*). These strains infect the small intestine and produce one of two heat-labile, diarrhea-producing enterotoxins.

In studies of United States citizens traveling in Mexico, the infection rate with ETEC is often greater than 50%. The major vehicles are perishable foods, such as fresh vegetables (for example, lettuce in salads), and public water supplies. The local population is typically resistant to the ETEC strains because of long-term contact with the organism. Other pathogenic *E. coli* strains include *enteropathogenic E. coli* (EPEC) strains that cause diarrheal diseases in infants and small children but do not cause invasive disease or produce toxins, and *enteroinvasive E. coli* (EIEC) strains, which invade the colon and cause watery and sometimes bloody diarrhea.

Diagnosis, Treatment, and Prevention

The general pattern established for the diagnosis, treatment, and prevention of STEC infection reflects current procedures used for all pathogenic *E. coli* strains. Laboratory diagnosis can be accomplished by culturing the pathogen from a fecal sample (Figure 33.12*c*) and identifying the O (lipopolysaccharide) and H (flagellar) antigens and toxins by immunological methods (Figure 33.12*d*). Identification and typing can also be done using various molecular analyses, and rapid tests to detect Shiga toxin are also now available (◀ Section 29.7).

Treatment of STEC infections includes supportive care for dehydration and monitoring of renal function, blood hemoglobin, and platelets. Bactericidal antibiotics may actually be harmful because they may trigger the release of large amounts of verotoxin from dying *E. coli* cells that would otherwise be voided intact in feces. For other pathogenic *E. coli* infections, treatment includes supportive therapy and, for severe cases and invasive disease, antimicrobial drugs to shorten and eliminate infection.

The most effective way to prevent infection with pathogenic *E. coli* of any type is to wash raw foods vigorously and make sure that meat, especially ground beef, is cooked thoroughly, which means that it should appear gray or brown with clear juices and have attained a temperature of greater than 70°C. In general, proper food handling, water purification, and appropriate hygiene also prevent the spread of pathogenic *E. coli*. Travelers can avoid diarrhea from pathogenic *E. coli* by drinking only properly sealed bottled water and avoiding any uncooked foods.

Check Your Understanding

- How do STEC strains of *Escherichia coli* differ from other pathogenic *E. coli*?
- Why are meats prime vehicles for pathogenic *E. coli*? How can contaminated meat be rendered safe to eat?

33.12 *Campylobacter*

Causing an estimated 1.3 million cases every year, *Campylobacter* infection has usurped salmonellosis (Section 33.10) as the most prevalent bacterial foodborne disease in the United States in recent years (Table 33.5). Cells of *Campylobacter* are gram-negative and motile spiral-shaped *Epsilonproteobacteria* (◀ Section 16.5) that grow best at reduced oxygen tension (microaerophilic). Several species of *Campylobacter* are recognized, but *C. jejuni* and *C. fetus* (**Figure 33.13**) are the most commonly linked to human foodborne illnesses.

Epidemiology and Pathology

Campylobacter is transmitted to humans via contaminated food, most commonly in undercooked poultry or pork, raw shellfish, or occasionally in feces-contaminated water from surface sources. *C. jejuni* is a normal resident of the intestinal tract of poultry, and according to the United States Department of Agriculture, up to 90% of turkey and chicken carcasses are contaminated with *Campylobacter*. Pork can also carry *Campylobacter*, while beef is rarely a vehicle. *Campylobacter* species also infect domestic animals such as dogs, causing a milder form of diarrhea in the animal than that observed in humans. *Campylobacter* infections in infants in particular are often traced to infected domestic animals, especially puppies and kittens, from which the bacteria are transmitted to children through the fecal–oral route.

After cells of *Campylobacter* are ingested, the organism multiplies in the small intestine, invades the epithelium, and causes inflammation. Because *C. jejuni* is sensitive to gastric acid, cell numbers as high as 10^4 may be required to initiate infection. However, this number may be reduced to fewer than 500 cells if the pathogen is ingested in food or if the person is taking medication to reduce stomach acid production. *Campylobacter* infection causes a high fever (usually greater than 40°C), headache, malaise, nausea, abdominal cramps, and diarrhea with watery, frequently bloody emissions; symptoms subside in about a week.

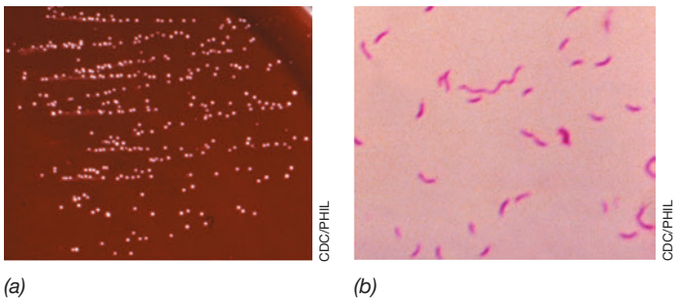


Figure 33.13 ***Campylobacter.*** *(a)* Colonies of *C. jejuni* grown on *Campylobacter* agar, a selective medium. The medium contains several antibiotics to which *Campylobacter* species are naturally resistant. *(b)* Gram stain and *(c)* scanning electron micrograph of cells of a *Campylobacter* species. Single cells average 0.4 × 2 μm in size.

Diagnosis, Treatment, and Prevention

Diagnosis of *Campylobacter* food infection requires isolation of the organism from feces and identification by growth-dependent tests, immunological assays, or genomic analyses. Culture media containing multiple antibiotics to which campylobacters are naturally resistant have been developed for selective isolation of this organism (Figure 33.13*a*). Various immunological methods are also available for diagnosing a campylobacter infection.

Antibiotic treatment with the drug azithromycin is widely practiced if a confirmed diagnosis is made from culture or culture-independent evidence. In addition, severe cases of dehydration from a *Campylobacter* infection may require intravenous perfusion and hospitalization. Rigorous personal hygiene, especially by those in food preparation facilities, proper washing of uncooked poultry (and any kitchenware coming in contact with uncooked poultry, such as cutting boards), and thorough cooking of meat are the major means of preventing *Campylobacter* infections.

Check Your Understanding

- Describe the pathology of *Campylobacter* food infection. What are the major vehicles for this pathogen?
- How might *Campylobacter* contamination of food production animals be controlled?

33.13 Listeriosis

Listeria monocytogenes causes **listeriosis**, a gastrointestinal food infection that may lead to bacteremia (bacteria in the blood) and meningitis. *L. monocytogenes* is a gram-positive, nonsporulating coccobacillus (phylum *Firmicutes*) that is acid-, salt-, and cold-tolerant and facultatively aerobic (**Figure 33.14**; ◀ Section 16.7). Although *Listeria* is a minor foodborne pathogen in terms of the number of cases observed per year, infections can be severe and cause up to 25% of all deaths from foodborne illness in the United States. Listeriosis is primarily seen in the elderly, pregnant women, newborns, and adults with weakened immune systems. Every year, *L. monocytogenes* is estimated to cause 1600 cases of invasive listeriosis (infection beyond the gastrointestinal tract), leading to about 260 deaths (16% mortality).

Epidemiology

L. monocytogenes is present in soil and water and although it is not common in foods, virtually no food source is safe from possible *L. monocytogenes* contamination. Food can become contaminated at any stage during production or processing. Ready-to-eat meats, fresh soft cheeses, unpasteurized dairy products, and inadequately pasteurized milk are the major food vehicles for *Listeria*, even when these foods are properly stored at refrigerator temperature (4°C). Food preservation by refrigeration, which ordinarily prevents the growth of other foodborne pathogens, is ineffective in the case of *Listeria* because the organism is psychrotolerant. Cells of *L. monocytogenes* produce a series of branched-chained fatty acids that keep the cytoplasmic membrane functional at cold temperatures (◀ Section 4.12).

Pathology

Immunity to *L. monocytogenes* is normally conferred by cell-mediated Th1 inflammatory cells (◀ Section 27.8). However, if cells of *Listeria* evade these immune cells, as they can in hosts with compromised immune systems, the organism is taken up by intestinal phagocytic cells. Although one might think that this is good from the standpoint of host defense, it is actually not, because phagocytic uptake initiates the *Listeria* infection cycle.

Listeria cells are taken up by host phagocytic cells into a vacuole called the *phagosome*. This triggers production of a major *Listeria* virulence factor, the exotoxin *listeriolysin O*, and this protein lyses the phagosome and releases *L. monocytogenes* into the cytoplasm (**Figure 33.15*a***; see also MicrobiologyNow in Chapter 1). Here the bacterium multiplies and produces a second major virulence factor,

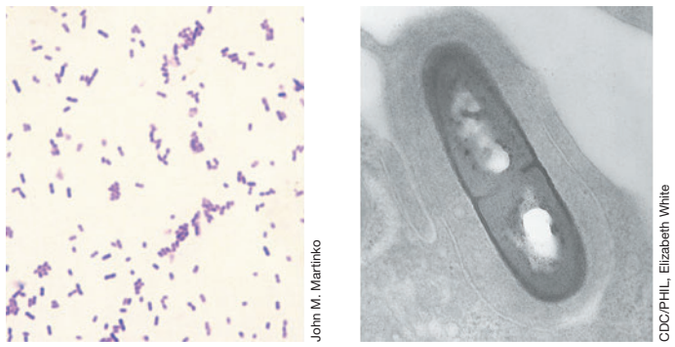


Figure 33.14 ***Listeria monocytogenes.*** *(a)* Gram stain and *(b)* transmission electron micrograph of cells of *L. monocytogenes*, the cause of listeriosis. Cells measure about 0.5 × 1 μm. The *Listeria* cell in *b* is within host tissues (see Figure 33.15).

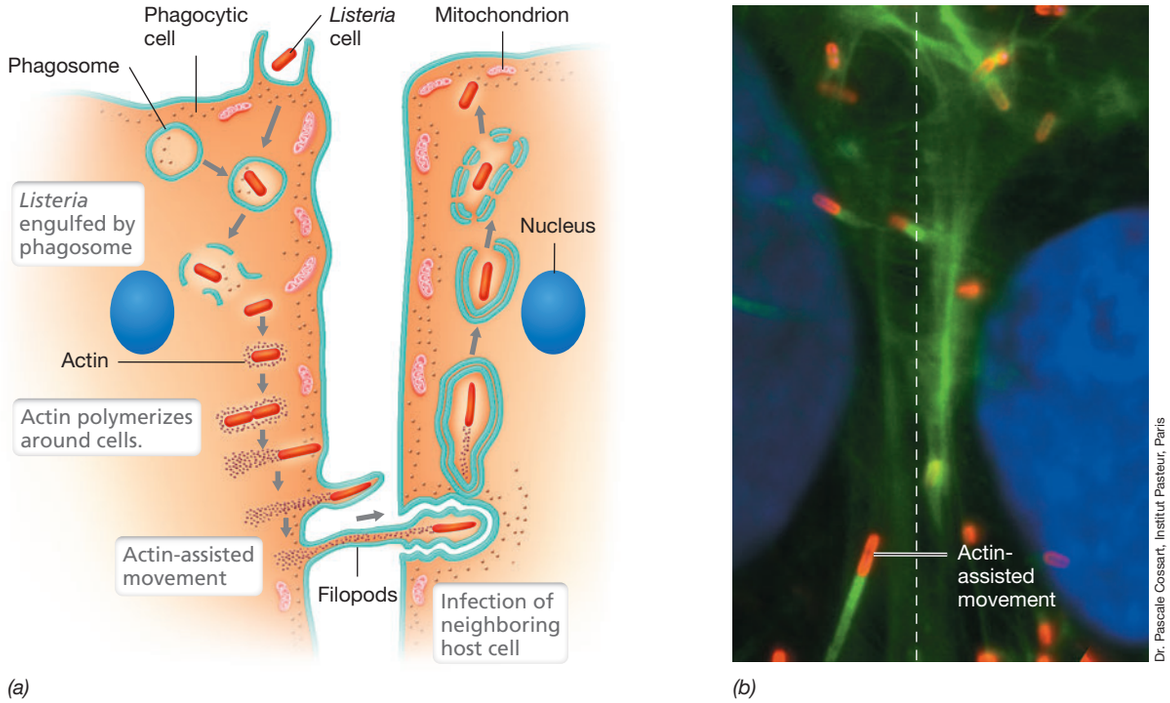


Figure 33.15 Transmission of *Listeria* during listeriosis. *(a)* Cells of *Listeria* are taken up in phagosomes of phagocytic cells. These are eventually lysed by the virulence factor listeriolysin O to release *Listeria* cells into the cytoplasm. The bacterial cells then become covered with host cell actin that assists in their movement to the cell periphery. Filopods facilitate transfer of *Listeria* cells to neighboring host cells, where the cycle repeats. *(b)* Fluorescently stained cells of *Listeria monocytogenes* (red) showing actin-assisted motility inside a eukaryotic cell (actin stains green, and the host cell nucleus is blue). The dashed white line labels the boundary between the two eukaryotic cells.

Mastering Microbiology
Art Activity: Figure 33.15 Transmission of *Listeria* during listeriosis

ActA, a protein that induces host cell actin polymerization; the actin coats the bacterial cell and assists in moving the pathogen to the host cell cytoplasmic membrane (Figure 33.15*b*). Once there, the bacterium–actin complex pushes out, forming protrusions called *filopods*, which are then taken up by surrounding phagocytic cells (Figure 33.15*a*). Filopod formation allows cells of *L. monocytogenes* to move about host tissues without exposure to the major weapons of the immune system: antibodies, complement, and neutrophils (Chapters 26 and 27).

Cells of *Listeria* in the intestine cross the intestinal barrier and are carried by the lymph and blood to other organs, in particular the liver, and multiply there as they do in intestinal phagocytes. From here cells of *L. monocytogenes* can infect the central nervous system, where they grow in neurons and lead to inflammation of the meninges (the tissues covering the brain and spinal cord), causing meningitis. In addition to listeriolysin O, which also allows *Listeria* to establish chronic infections in many host tissues, other major virulence factors include phospholipases that can destroy host cell membranes, antioxidants that counter phagocytic cell oxidants, and an array of "stress proteins" common in many bacteria (◀ Section 7.9).

Diagnosis, Treatment, and Prevention

Listeriosis is diagnosed by culturing *L. monocytogenes* (Figure 33.14) from the blood or cerebrospinal fluid. *L. monocytogenes* can be identified in foods by direct culture or by several molecular methods. The latter methods are also used to subtype clinical isolates in order to track the source(s) of infection. Intravenous antibiotic treatment with penicillin, ampicillin, or trimethoprim plus sulfamethoxazole (Bactrim®) is used to treat invasive listeriosis.

Prevention measures include recalling contaminated food and taking steps to limit *L. monocytogenes* contamination at the food-processing site. Because *L. monocytogenes* is susceptible to heat and radiation, raw food and food-handling equipment can be readily decontaminated. However, unless the finished food product is pasteurized (◀ Section 4.17) or cooked, the risk of contamination cannot be eliminated because of the widespread distribution of the pathogen.

Check Your Understanding

- What is the likely outcome of *Listeria monocytogenes* exposure in normal healthy individuals?
- Which populations are most susceptible to serious disease from *L. monocytogenes* infection?

33.14 Other Foodborne Infectious Diseases

Over 200 microorganisms, viruses, and other infectious agents can cause foodborne diseases, and we have thus far summarized the major ones. Here we consider a few other bacterial pathogens that are rather uncommon compared with the "top five" (Table 33.5), and we take a second look at norovirus (previously considered as a waterborne pathogen, Section 33.5) in its more frequent context as a foodborne pathogen and overall number one cause of gastrointestinal illness in the United States.

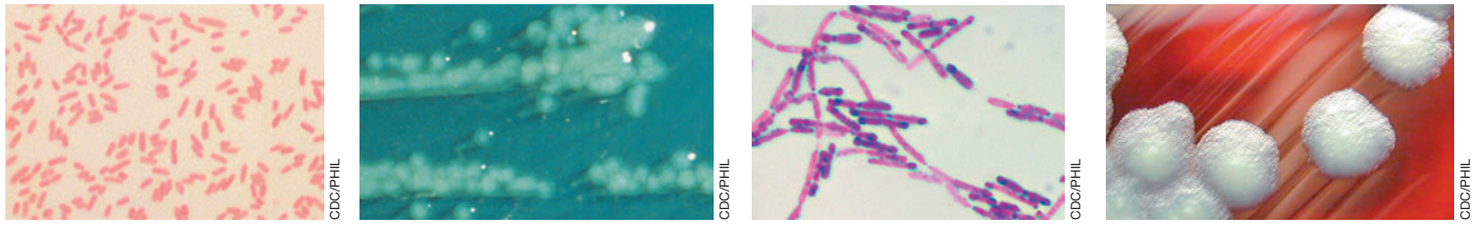


Figure 33.16 Less common foodborne bacterial pathogens: *Yersinia enterocolitica* and *Bacillus cereus*. *(a)* Gram-stained cells of *Y. enterocolitica*. *(b)* Colonies of *Y. enterocolitica* on Hektoen agar, a selective and differential medium. *Y. enterocolitica* forms white colonies because this bacterium does not ferment lactose and does not produce sulfide (compare with colonies of *Salmonella* on Hektoen agar in Figure 33.11*a* and colonies of *Escherichia coli* on Hektoen agar in Figure 33.12*c*). *(c)* Gram-stained cells of a sporulating culture of *B. cereus*. *(d)* Large crystalline-like colonies of *B. cereus* formed on blood agar. Foodborne illness due to *Y. enterocolitica* or *B. cereus* is much less common than illness due to *Salmonella*, *Campylobacter*, or *Clostridium perfringens*.

Bacteria

Besides the major bacterial foodborne pathogens we have already considered, several other bacteria cause human gastrointestinal illnesses. *Yersinia enterocolitica* is an enteric bacterium commonly found in the intestines of domestic animals and causes foodborne infections from contaminated meat and dairy products. The most serious consequence of *Y. enterocolitica* infection is *enteric fever*, a severe, life-threatening infection. *Y. enterocolitica* can be isolated on the same selective, differential medium used to isolate *Salmonella* (**Figure 33.16*a, b***) but is easily distinguished from this organism on plates (compare Figures 33.11*a* and 33.16*b*).

Bacillus cereus is responsible for a relatively small number of food poisoning cases. This endospore-producing bacterium (◀ Sections 2.8 and 16.8) produces two enterotoxins that cause different symptoms. In the *emetic form*, symptoms are primarily nausea and vomiting. In the *diarrheal form*, diarrhea and gastrointestinal distress are observed. *B. cereus* grows in foods such as rice, pasta, meats, or sauces that are cooked and left at room temperature to cool slowly. When endospores of this bacterium germinate, toxin is produced. Reheating may kill the *B. cereus* cells, but the toxin is heat-stable and may remain active. *B. cereus* is readily culturable and can be tentatively identified by a combination of microscopy and its typically large, grainy, and spreading colonies (Figure 33.16*c, d*).

The enteric bacterium *Shigella* causes the food infection *shigellosis*, and species of *Vibrio* can also cause food poisoning, primarily from consumption of contaminated shellfish. Most *Shigella* infections are the result of fecal to oral contamination, but food and water are occasional vehicles. We discussed the Shiga-like toxin produced by some pathogenic strains of *Escherichia coli* in Section 33.11.

Viruses

About 70% of annual foodborne infections in the United States are caused by norovirus (**Figure 33.17*a***; Section 33.5). The virus is also known as *Norwalk virus* and is a single-stranded plus-sense RNA virus related to poliovirus (◀ Section 11.8). In general, noroviral foodborne illnesses are characterized by diarrhea, often accompanied by nausea and recurrent vomiting. Recovery from norovirus infections is typically spontaneous and rapid, usually within 24–48 h (thus the disease is often nicknamed "the 24-hour bug").

Rotavirus, astrovirus, and hepatitis A make up the bulk of the remaining foodborne *viral* infections. These viruses inhabit the gut and are often transmitted in food or water contaminated with feces. Hepatitis A virus (HAV, Figure 33.17*b*) is an RNA virus that, like norovirus, is related to poliovirus, but HAV replicates in liver cells. We considered hepatitis viruses transmitted primarily by blood in Section 31.11, but HAV is mainly a foodborne virus. HAV usually triggers mild, and in many cases subclinical, symptoms, but rare cases of severe liver disease from HAV can occur. The most significant food vehicles for HAV are shellfish, usually oysters or clams harvested from water polluted by human feces and then eaten raw. In recent years, HAV has also been seen in fresh produce served without cooking.

Despite a slight uptick in incidence in recent years, the general trend for both foodborne and bloodborne hepatitis has moved steadily downward and is now at low levels, partly due to the availability of effective vaccines against both hepatitis A and hepatitis B (HBV) viruses (◀ Figure 31.32), but also because of heightened awareness of the potential danger of eating raw shellfish. Nevertheless, widespread and likely mild HAV infections continue to occur because surveys have shown that over 30% of individuals in the United States have circulating antibodies to HAV, indicating past subclinical infections. In 2016, 2007 cases of hepatitis A were reported in the United States.

Protists and Other Agents

Important foodborne protist diseases are listed in Table 33.5. The major pathogens here include *Giardia intestinalis, Cryptosporidium*

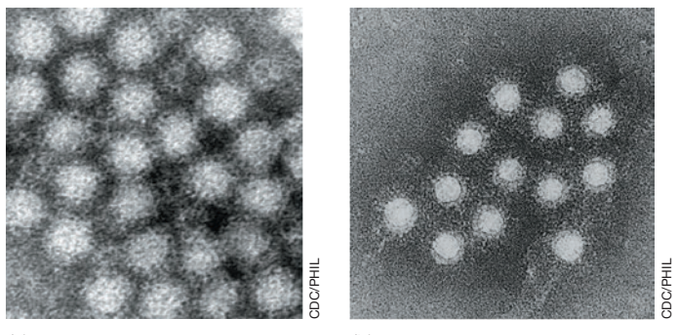


Figure 33.17 Viruses transmitted in contaminated foods. *(a)* Transmission electron micrograph of norovirus; an individual virion is about 30 nm in diameter. *(b)* Transmission electron micrograph of hepatitis A virus; a virion is 27 nm in diameter.

parvum, *Cyclospora cayetanensis*, and *Toxoplasma gondii*. *G. intestinalis* and *C. parvum* are spread in foods when contaminated water is used to wash, irrigate, or spray crops. Fresh foods such as fruits are often implicated as vehicles for these protists. *Toxoplasma gondii* is a protist spread primarily through cat feces, but it can also be found in raw or undercooked meat, especially pork. The incidence of foodborne transmission of *C. cayetanensis* has remained low (fewer than 20 cases per year) in recent years, and fresh cilantro and related produce have been the major vehicles of this pathogen in the majority of outbreaks. We discuss the diseases giardiasis, cryptosporidiosis, cyclosporiasis, and toxoplasmosis in Section 34.4.

At least one type of foodborne disease agent is neither cellular nor viral; these are the prions. *Prions* are proteins that adopt novel conformations, inhibiting normal protein function and causing degeneration of host neural tissues (◀ Section 11.13). Human prion diseases are characterized by neurological symptoms including depression, loss of motor coordination, and eventual dementia. A foodborne human prion disease called *variant Creutzfeldt–Jakob disease* (vCJD) has been linked to consumption of meat products from cattle suffering from *bovine spongiform encephalopathy* (*BSE*), a disease caused by a prion. Although several thousand cases of vCJD were diagnosed in Great Britain in the mid-1990s, bans on cattle feeds containing rendered cattle and sheep parts and bone meal have greatly diminished the incidence of BSE in Europe and have kept the incidence of this disease extremely low in the United States.

Check Your Understanding

- In what two forms can *Bacillus cereus* food poisoning manifest itself?
- Compared with all other foodborne or waterborne pathogens, what is unique about prions?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Water as a Disease Vehicle

33.1 Contaminated drinking and recreational waters are sources of waterborne pathogens. In the United States, the number of disease outbreaks due to these sources is relatively small in relation to the large exposure the population has to water. Worldwide, lack of adequate water treatment facilities and access to clean water contribute significantly to the spread of infectious diseases.

Q What determines whether or not a pathogen present in a water source will lead to a person being infected with a waterborne disease?

33.2 Drinking water quality is determined by counting coliform and fecal coliform bacteria using standardized techniques. Filtration and chlorination of water significantly decreases microbial numbers. Water purification methods in developed countries have been a major factor in improving public health, although in developing countries, waterborne illness is still a significant source of infectious disease.

Q Describe how ATP can be used as a tool for estimating total bacterial numbers in a given water sample.

II • Waterborne Diseases

33.3 The bacterium *Vibrio cholerae* causes cholera, an acute diarrheal disease associated with severe dehydration. Cholera occurs in epidemics, primarily in developing countries where sewage treatment and sanitation is lacking. Oral rehydration and electrolyte replacement can effectively treat cholera and greatly reduce disease mortality.

Q Describe the mode of action of *V. cholerae* on the small intestine in the body.

33.4 *Legionella pneumophila* is a respiratory pathogen that causes Pontiac fever and legionellosis, a more serious infection that may result in pneumonia. *L. pneumophila* grows to high numbers in warm waters and is spread via cooling tower aerosols and in domestic water distribution systems where the bacterium develops in biofilms.

Q How is *L. pneumophila* usually detected, and what are the various means of treating legionellosis in an infected patient?

33.5 Typhoid fever, caused by a *Salmonella* species, and norovirus illness are important waterborne diseases. Typhoid is common in developing countries, while norovirus illness is seen worldwide. Both of these diseases can be controlled by good sanitation practices and effective water treatment.

Q How is *S. enterica*, the causative agent of typhoid fever, transmitted? What are the stages of typhoid fever?

III • Food as a Disease Vehicle

33.6 The potential for microbial food spoilage depends on the nutrients and moisture levels of the food. Growth of microorganisms in perishable foods can be controlled by refrigeration, freezing, canning, pickling, dehydration, chemicals, and irradiation. Microbial fermentations can be used to naturally preserve many foods, including dairy products, meats, fruits and vegetables, and alcoholic beverages.

Q Identify and define the three major categories of food perishability. Why is milk more perishable than sugar even though both are rich in organic matter? Identify the major methods used to preserve food and the major categories of fermented foods.

33.7 Food poisoning results from the activities of microbial toxins, whereas food infections are due to the growth of the pathogen within the body. Identification of shared characteristics of foodborne pathogens from seemingly isolated foodborne outbreaks can pinpoint the origin of foodborne contamination and track the spread of the disease. The top five most prevalent foodborne pathogens in the United States are (1) norovirus, (2) *Campylobacter jejuni*, (3) *Salmonella* spp., (4) *Clostridium perfringens*, and (5) *Staphylococcus aureus*.

Q Distinguish between food infection and food poisoning, and give two examples of each.

IV • Food Poisoning

33.8 Staphylococcal food poisoning results from the ingestion of a preformed staphylococcal enterotoxin, a superantigen produced by cells of *Staphylococcus aureus* as they grow in food. Proper food preparation, handling, and storage can prevent staphylococcal food poisoning.

Q What causes the symptoms of staphylococcal food poisoning? Why are cases of staph food poisoning often linked to a food preparer?

33.9 *Clostridium* food poisoning results from ingestion of toxins produced by microbial growth in foods or from microbial growth followed by toxin production in the body. Perfringens food poisoning is quite common and is usually a self-limiting gastrointestinal disease. Botulism is a rare but serious disease having significant mortality.

Q Identify the two major types of clostridial food poisoning. Which is most prevalent? Which is most dangerous and why?

V • Food Infection

33.10 About 1.2 million cases of salmonellosis occur every year in the United States. Infection results from ingestion of cells of *Salmonella* introduced into food primarily from animal-derived food products or food handlers.

Q What are the possible sources of *Salmonella* spp. that cause food infections?

33.11 Toxigenic *Escherichia coli* strains cause many food infections, and of these, STEC strains are the most severe. Contamination of foods from animal feces spreads these pathogenic strains of *E. coli*, but good hygiene practices and specific antibacterial measures, such as irradiation or thorough cooking of ground beef, a major vehicle, can control disease outbreaks.

Q How does *Escherichia coli* O157:H7 end up in ground beef? To what class of pathogenic *E. coli* does this strain belong? How does this class differ from other classes?

33.12 With an incidence of 1.3 million cases per year, *Campylobacter* infection is the most prevalent foodborne bacterial disease in the United States. Poultry is a major vehicle for *Campylobacter* illness, whereas beef and pork are not. Proper poultry preparation and cooking can prevent *Campylobacter* illness.

Q Name a food product that could transmit both *Salmonella* and *Campylobacter* simultaneously. How could this food product be rendered safe to eat?

33.13 *Listeria monocytogenes* is a ubiquitous bacterium, and in healthy individuals, it seldom causes infection. However, in immunocompromised individuals, *Listeria* can cause serious disease as it grows as an intracellular pathogen and invades the central nervous system. Listeriosis is uncommon but shows high mortality.

Q Identify the food sources of *Listeria monocytogenes* infections. How does *Listeria* evade the immune system?

33.14 Viruses, especially norovirus, cause the highest incidence of foodborne illness, whereas the bacteria *Bacillus cereus, Shigella* spp., and *Yersinia enterocolitica* are only occasionally linked to foodborne disease outbreaks. Hepatitis A virus is also a serious foodborne pathogen. Some protists and prions also cause foodborne illness but are far less common foodborne pathogens than are bacteria and viruses.

Q What agent is the number one cause of gastrointestinal illness? What is the causative agent of vCJD? How does the structure of this agent differ from that of norovirus?

APPLICATION QUESTIONS

1. As a visitor to a country in which cholera is an endemic disease, what specific steps would you take to reduce your risk of cholera exposure? Will these precautions also prevent you from contracting other waterborne diseases? If so, which ones? Identify waterborne diseases for which your precautions may not prevent infection.
2. Argue a case for why perfringens foodborne illness can be considered both a food poisoning and a food infection.
3. Improperly prepared or handled potato salads are often the source of both staphylococcal food poisoning and salmonellosis. List some reasons why this might be the case. How do these differ from foodborne disease caused by *Clostridium botulinum*?
4. Both enterotoxigenic *Escherichia coli* and *Clostridium botulinum* cause foodborne illnesses. However, cells of one of these pathogens are rarely detected in the feces of a patient suffering from the disease, whereas cells of the other can be readily detected. Explain.

CHAPTER GLOSSARY

Botulism food poisoning due to ingestion of food containing botulinum toxin produced by *Clostridium botulinum*

Coliforms gram-negative, nonsporulating, facultatively aerobic rods that ferment lactose with gas formation within 48 hours at 35°C

Food infection a microbial infection resulting from the ingestion of pathogen-contaminated food followed by growth of the pathogen in the host

Food poisoning (food intoxication) a disease caused by the ingestion of food that contains preformed microbial toxins

Food spoilage a change in the appearance, smell, or taste of a food that makes it unacceptable to the consumer

Listeriosis a gastrointestinal food infection caused by *Listeria monocytogenes* that may lead to bacteremia and meningitis

Nonperishable foods foods of low water activity that have an extended shelf life and are resistant to spoilage by microorganisms

Pasteurization the use of controlled heat to reduce the microbial load, including both pathogens and spoilage organisms, in heat-sensitive liquids

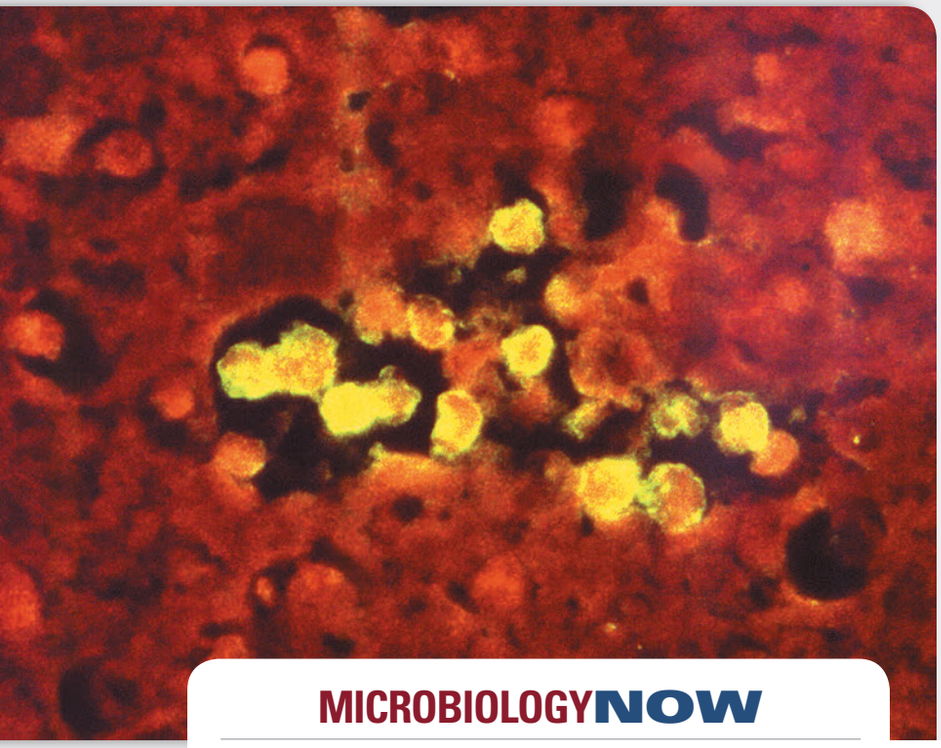
Perishable foods fresh foods generally of high water activity that have a very short shelf life because of spoilage by microbial growth

Potable in water purification, drinkable; safe for human consumption

Salmonellosis enterocolitis or other gastrointestinal disease caused by any of several subspecies of the bacterium *Salmonella*

Semiperishable foods foods of intermediate water activity that have a limited shelf life because of their potential for spoilage by growth of microorganisms

Eukaryotic Pathogens: Fungi, Protozoa, and Helminths 34



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III Blood and Tissue Parasitic Infections 1067

MICROBIOLOGYNOW

A Silver Bullet to Kill Brain-Eating Amoebae?

One of the most difficult-to-treat infectious diseases is primary amebic meningoencephalitis (PAM), caused by the "brain-eating" parasitic amoeba *Naegleria fowleri*. Although fewer than five cases are reported annually in the United States, *N. fowleri* infections are nearly always fatal and are linked to swimming in warm, stagnant, natural waters. If such waters are accidentally inhaled, the highly invasive amoebae (yellow cells in the photomicrograph) can attach to nasal epithelial cells and burrow to the central nervous system (CNS); there they begin to ingest brain cells (red in photo) and multiply quickly. The disease progresses rapidly, and death usually occurs within a few days.

PAM is difficult to treat for two key reasons. First, because it is so rare, diagnosis is often delayed, with the unfortunate result being cerebral hemorrhaging and rapid loss of brain cells to the voracious amoebae. Second, the pathogen in this case is a eukaryote. Thus, significant similarities between cells of the amoeba and those of the host often produce unwanted side effects when antiparasitic drugs are administered, especially at the high concentrations required to treat CNS infections.

A current trend in modern clinical medicine is to accelerate drug development for serious diseases by repurposing pharmaceuticals already approved by the U.S. Food and Drug Administration. In this connection, one research group recently tested three anti-seizure drugs (diazepam, marketed as Valium®; phenobarbital, marketed as Luminal®; and phenytoin, marketed as Dilantin®), which readily cross the blood–brain barrier, for cytotoxic effects against *N. fowleri*. Although the drugs alone could kill cells of *N. fowleri*, cytotoxicity was greatly amplified when the drugs were attached to silver nanoparticles, which are known to have antimicrobial activity.

As demonstrated by this study, the modification and repurposing of existing drugs may prove to be a key strategy for the rapid development and implementation of new disease therapies moving forward.

Source: Anwar, A., et al. 2019. Clinically approved drugs against CNS diseases as potential therapeutic agents to target brain-eating amoebae. *ACS Chem. Neurosci. 10*: 658. doi: 10.1021/acschemneuro.8b00484.

In this chapter we focus on *eukaryotic* pathogenic microorganisms. These include fungi—both molds and yeasts—and various parasitic protists. Some small helminths (worms) also cause infectious diseases, and we consider the most significant of these in the final section.

A common problem in treating human diseases caused by eukaryotic pathogens is the fact that humans are also eukaryotes. This thwarts many therapeutic strategies and often results in these diseases being highly refractory and chronic infections. This is especially true of systemic fungal pathogens, a group we consider first in our coverage of eukaryotic pathogenic microbes.

I • Fungal Infections

Pathogenic fungi cause a variety of diseases ranging from superficial and subcutaneous skin infections that are relatively easy to treat to serious systemic mycoses. The latter are deep-seated, potentially life-threatening infections that are much more difficult to treat.

Fungi cause a variety of human diseases. Some are mild and self-limiting, whereas others can be firmly entrenched systemic diseases. We begin by considering some of the major fungal pathogens followed by a description of some major fungal diseases, the mycoses.

34.1 Pathogenic Fungi and Classes of Infection

The fungi include the *yeasts*, which normally grow as single cells, and *molds*, which form branching filaments called *hyphae* with or without septa (cross-walls); hyphae eventually intertwine to form visible masses called *mycelia*. The diversity of the molds and yeasts was discussed in Chapter 18.

Common Fungal Pathogens

Fortunately, most fungi are harmless to humans. Most fungi grow in nature as saprophytes on dead organic material, and in so doing, fungi are important catalysts in the carbon cycle, especially in oxic environments in soil. Fungi are also important in medicine both as agents of disease and in chemotherapy (as producers of antibiotics). Only about 50 species of fungi cause human diseases, and in healthy individuals, the incidence of serious fungal infections is low, although certain superficial fungal infections (for example, athlete's foot) are common. In those with compromised immune systems, however, fungal infections can be systemic, reaching even the deepest of internal tissues. Such infections can cause serious health problems and be life-threatening.

Common fungal pathogens include both yeasts and molds (Figure 34.1). However, many pathogenic fungi are *dimorphic*, meaning that they can exist as *either* a yeast *or* in filamentous form, a feature

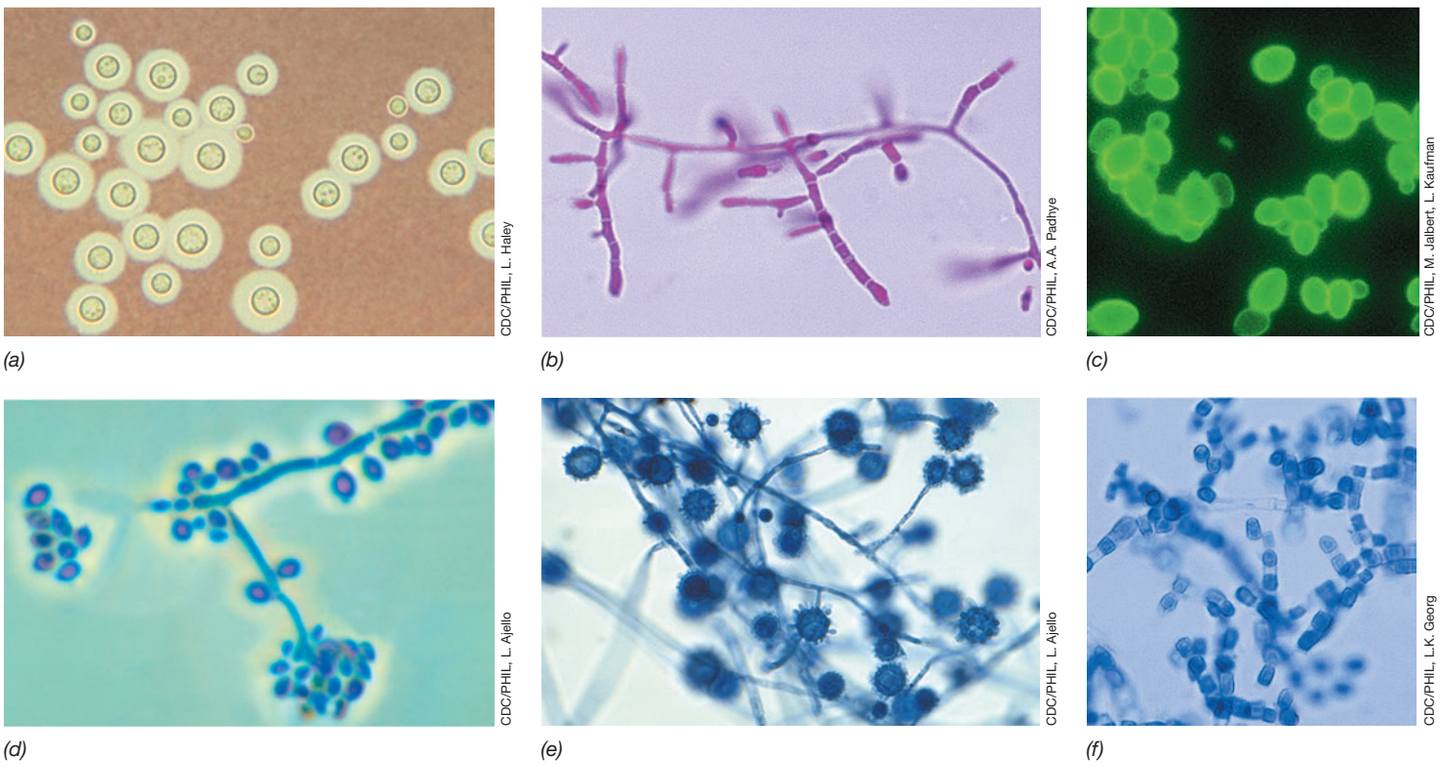


Figure 34.1 Pathogenic fungi. These organisms range from about 4 to 20 μm in diameter. *(a) Cryptococcus neoformans* yeast cells stained to reveal the capsule. *(b) Trichophyton mariatii* mycelia and hyphae. *(c) Candida albicans* yeast form stained with a fluorescent antibody. *(d) Sporothrix schenckii* mycelia and conidia. *(e) Histoplasma capsulatum* mycelia and large conidia. *(f) Coccidioides immitis* conidia. See fungal disease symptoms in Figure 34.5.

typically dictated by environmental factors, such as temperature and moisture levels. In *Histoplasma*, for example, cells in laboratory culture form hyphae and mycelia and thus exist in the mold form (Figure 34.1*e*). By contrast, when *Histoplasma* causes histoplasmosis, cells grow in the host in the yeast form (see Figure 34.5*a*). In the mold form, spores are produced, either asexual spores—*conidia*—or sexual spores (◀ Section 18.10). When filamentous fungi are cultured from an infection, the morphology of these spore-bearing structures is observed and is often a major clue in reaching a diagnosis. In addition to microscopy, a variety of clinically useful molecular and immunological tools (including fluorescent antibodies, Figure 34.1*c*) are also available to diagnose fungal infections. **Table 34.1** lists some major fungal pathogens and the types of infections they cause.

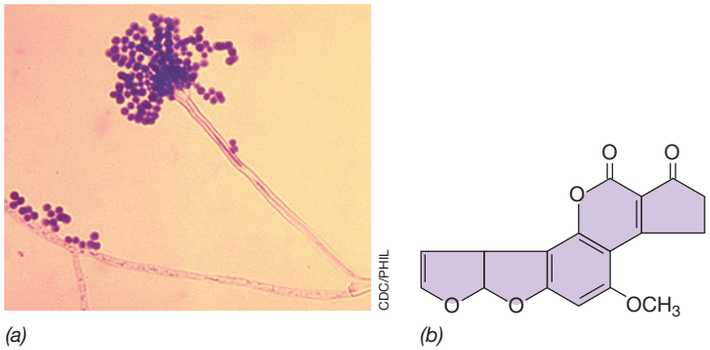
Fungal Disease Classes and Treatment

Fungi cause disease through three major mechanisms: inappropriate immune responses, toxin production, and mycosis. Some fungi trigger immune responses that result in allergic (hypersensitivity) reactions following exposure to specific fungal antigens. Reexposure to the same fungi, whether growing on the host or in the environment, may cause allergic symptoms. For example, *Aspergillus* spp. (**Figure 34.2*a***), common saprophytes often found in nature as a leaf mold, produce potent allergens, triggering asthma attacks or other hypersensitivity reactions in susceptible individuals.

TABLE 34.1 Major pathogenic fungal diseases[a]

Diseases by class	Causal organism	Site
Superficial mycoses		
Athlete's foot	*Epidermophyton*, *Trichophyton*	Between toes, skin
Jock itch	*Trichophyton*, *Epidermophyton*	Genital region
Ringworm	*Microsporum*, *Trichophyton*	Scalp, face
Subcutaneous mycoses		
Sporotrichosis	*Sporothrix schenckii*	Arms, hands
Chromoblastomycosis	*Phialophora verrucosa*, other fungi	Legs, feet, hands
Systemic mycoses		
Aspergillosis	*Aspergillus* spp.[b]	Lungs
Blastomycosis	*Blastomyces dermatitidis*	Lungs, skin
Candidiasis	*Candida albicans*[c]	Oral cavity, intestinal tract, vagina
Coccidioidomycosis	*Coccidioides immitis*[c]	Lungs
Paracoccidioidomycosis	*Paracoccidioides brasiliensis*	Skin
Cryptococcosis	*Cryptococcus neoformans*[c]	Lungs, meninges
Histoplasmosis	*Histoplasma capsulatum*[c]	Lungs
Pneumocystis pneumonia	*Pneumocystis jirovecii*[c]	Lungs

[a]Symptoms of many of these diseases are shown in Figures 34.3–34.5.
[b]*Aspergillus* can also cause allergies, toxemia, and limited infections.
[c]An opportunistic pathogen frequently infecting individuals with HIV/AIDS.



(a) (b)

Figure 34.2 *Aspergillus* and aflatoxin. *(a)* Mycelia and conidia of an *Aspergillus* species. *(b)* Structure of aflatoxin B1. This toxin is one of a group of related compounds produced by *Aspergillus flavus*.

Mastering Microbiology
Art Activity: Table 34.1 Major pathogenic fungal diseases

Fungal disease may occur from the production of *mycotoxins*, a large and diverse group of fungal exotoxins (◀ Section 25.6). The best-known examples of mycotoxins are the *aflatoxins* (Figure 34.2*b*) produced by *Aspergillus flavus*, a species that commonly grows on improperly stored dry foods, such as grain. Aflatoxins are highly toxic and are also carcinogenic, inducing tumors in some animals, especially in birds that feed on contaminated grain. Although aflatoxins are known to cause human liver damage including cirrhosis and even liver cancer, adults are not seriously affected by low-level aflatoxin exposure. However, chronic exposure in children can cause serious liver disease and stunt normal growth and development.

The final fungal disease-producing mechanism is through actual host infection. The growth of a fungus on or in the body is called a **mycosis** (plural, mycoses). Mycoses are fungal infections that range in severity from superficial to life-threatening. Mycoses fall into three classes (Table 34.1). **Superficial mycoses** are those in which the fungus infects only the surface layers of skin, hair, or nails (see Figure 34.3). **Subcutaneous mycoses** are infections of deeper layers of skin (see Figure 34.4) and are typically caused by different fungi than superficial infections (Table 34.1). The **systemic mycoses** are the most serious category of fungal infections. These are characterized by fungal growth in internal organs of the body (see Figure 34.5) and can be either primary or secondary infections. A *primary* infection occurs when an otherwise normal, healthy individual is infected with the fungal pathogen; these are rather uncommon. By contrast, a *secondary* infection occurs in a host that harbors a predisposing condition, such as antibiotic therapy or immunosuppression, that makes the individual more susceptible to infection.

Superficial and subcutaneous mycoses are for the most part easily treatable with topical drugs, including tolnaftate (applied topically), various azole drugs (applied either topically or orally), and griseofulvin, a relatively nontoxic drug that can be taken orally but passes through the bloodstream to the skin where it inhibits fungal growth. Chemotherapy against systemic fungal infections is more difficult because of issues with host toxicity (◀ Section 28.6). For example, one of the most effective antifungal agents, amphotericin B, is widely used to treat systemic fungal infections but can also reduce kidney function and have other unwanted side effects. Hence, effective treatment of the most serious of the mycoses is sometimes quite difficult.

Check Your Understanding

- Differentiate between superficial, subcutaneous, and systemic mycoses.
- What is a dimorphic fungus?
- Distinguish between a primary and a secondary fungal disease. Why do those suffering from HIV/AIDS often show secondary fungal infections of major internal organs?

34.2 Fungal Diseases: Mycoses

The two extremes of fungal infection are the superficial mycoses and the systemic mycoses. *Superficial mycoses* are quite common, and most individuals experience at least one in their lifetime. By contrast, *systemic mycoses* are far less common and primarily affect the elderly or otherwise immune compromised. As people age, cell-mediated immunity slowly declines as a result of surgeries, transplantations, immunosuppressive drug treatments for rheumatism and autoimmune diseases, and the onset of other conditions, such as pulmonary decline, diabetes, and cancer. Any of these can predispose the elderly to disease. Systemic mycoses also target those of any age whose immune systems have been impaired or destroyed, for example, by HIV/AIDS (◀ Figure 31.46). Systemic mycoses are thus diseases of *opportunistic pathogens*, microbes that cause disease only in those whose immune defenses can no longer fight them off (◀ Sections 25.4 and 31.15).

Superficial Mycoses

Table 34.1 listed some of the fungi that cause superficial mycoses; collectively, these pathogens are called *dermatophytes*. In general, superficial mycoses can be bothersome and often recurrent infections, but they are not serious health concerns. Fungi such as *Trichophyton* (Figure 34.1*b*) cause infections of the feet (athlete's foot) and other moist skin surfaces and are quite common (**Figure 34.3*a***). These infections cause flaking and itchy skin and are easily transmitted by cells or spores of the pathogen present in contaminated shower stalls, gymnasium and locker room floors, contaminated shared articles such as towels or bed linens, or from close person-to-person contact.

Mastering Microbiology
Art Activity: Figure 34.5 Systemic mycoses

Superficial mycoses can be treated with topical antifungal creams or liquid aerosols, although prophylactic application on a long-term basis may be necessary if constant exposure to the pathogen (for example, to *Trichophyton* on a locker room floor) is unavoidable.

Related surface mycoses include "jock itch," an itchy infection of the groin, skin folds, or anus, and *ringworm* (Table 34.1). Despite the name, ringworm is a fungal infection, typically localized to the scalp or the extremities; the infection causes hair loss and inflammatory reactions (Figure 34.3*b, c*). These more severe superficial mycoses are usually treated topically with either miconazole nitrate or griseofulvin.

Subcutaneous Mycoses

Subcutaneous mycoses are fungal infections of deeper layers of skin than those of the superficial mycoses (Table 34.1). One disease in this class is *sporotrichosis* (**Figure 34.4*a***), an occupational hazard of agricultural workers, miners, gardeners, and others who come into close and continual contact with the soil. The causative agent, *Sporothrix schenckii* (Figure 34.1*d*), is a ubiquitous soil saprophyte whose spores can enter through a cut or abrasion and infect subcutaneous tissues (Figure 34.4*a*). *Chromoblastomycosis* is due to pathogenic fungal growth in both surface (cutaneous) *and* subcutaneous skin layers, forming crusty, wartlike lesions on the hand (Figure 34.4*b*) or leg. The disease is primarily one of tropical and subtropical countries and occurs when the fungus becomes implanted under the skin from a puncture wound. Both sporotrichosis and chromoblastomycosis can be treated with oral administration of azoles.

Systemic Mycoses

Systemic fungal pathogens normally live in soil, and humans become infected by inhaling airborne spores that later germinate and grow in the lungs. From there the organism migrates throughout the body, causing deep-seated infections in the lungs and other organs and in the skin. In the United States, the three major systemic mycoses are, in order of decreasing incidence: histoplasmosis, coccidioidomycosis, and blastomycosis. Mortality from these is high, about 10%.

Gordon C. Sauer

(a)

CDC/PHIL

(b)

CDC/PHIL

(c)

Figure 34.3 Superficial mycoses caused by *Trichophyton* spp. *(a)* Athlete's foot. *(b)* Ringworm on a child's face and *(c)* on an adult index finger. "Jock itch" (ringworm of the groin) is another common *Trichophyton* infection and can occur in females as well as males. *Trichophyton* is a filamentous fungus (see Figure 34.1*b*).

(a) (b)

Figure 34.4 Subcutaneous mycoses. *(a)* Sporotrichosis, a subcutaneous infection caused by *Sporothrix schenckii*. *(b)* Chromoblastomycosis on the hand caused by the fungus *Phialophora verrucosa*. Chromoblastomycosis can also be caused by species of the fungal genera *Fonsecaea* and *Cladosporium*.

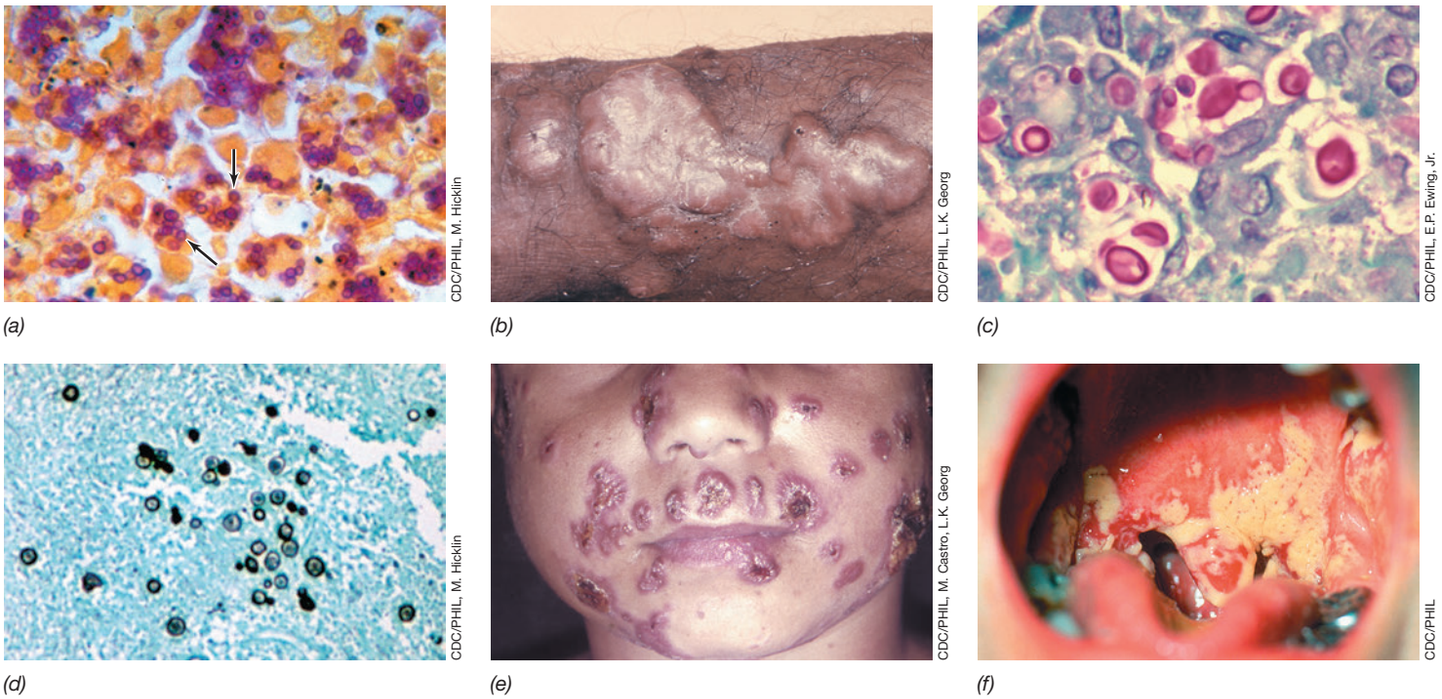
Histoplasmosis (**Figure 34.5*a***) is caused by *Histoplasma capsulatum* (Figure 34.1*e*), and *coccidioidomycosis* (San Joaquin Valley fever, Figure 34.5*d*) is caused by *Coccidioides immitis* (Figure 34.1*f*). Histoplasmosis is primarily a disease of rural areas in midwestern states of the United States, especially in the Ohio and Mississippi River valleys, whereas coccidioidomycosis is generally restricted to the desert regions of the southwestern United States. In more tropical climates *blastomycosis*, caused by *Blastomyces dermatitidis*, is prevalent (Figure 34.5*b*). *Paracoccidioidomycosis*, caused by the fungus *Paracoccidioides brasiliensis*, is primarily a subtropical disease with lesions forming on the face (Figure 34.5*e*) or other extremities.

Cryptococcosis (Figure 34.5*c*), caused by the dimorphic yeast *Cryptococcus neoformans* (Figure 34.1*a*), can occur in virtually any organ of the body and is the major mycosis seen in HIV/AIDS patients. The dimorphic yeast *Candida albicans* (Figure 34.1*c*) is often present as a minor component of the human microbiota. However, this fungus can cause a variety of diseases, including mild vaginal infections, more serious oral infections such as thrush (Figure 34.5*f*), and systemic infection of virtually any organ in those with HIV/AIDS. Like *Histoplasma* and *Coccidioides*, *Candida* and *Cryptococcus* are primarily opportunistic pathogens and rarely cause life-threatening infections except in immunocompromised individuals. An exception to this is *Candida auris*, a close relative of *Candida albicans* that has recently emerged as a deadly, multidrug-resistant threat in healthcare settings (see MicrobiologyNow in the Chapter 30 opener).

Our discussion transitions now from fungi to pathogenic parasites. Like fungi, parasites are eukaryotic microorganisms, but the pathogenic parasites typically attack quite different body tissues and organs than do the pathogenic fungi.

Check Your Understanding

- Give an example of a superficial, a subcutaneous, and a systemic mycosis.
- Why are systemic fungal pathogens called opportunistic?



(a) (b) (c)

(d) (e) (f)

Figure 34.5 Systemic mycoses. *(a)* Histoplasmosis; yeast-form cells of *Histoplasma* (arrows) in spleen tissue. *(b)* Cutaneous blastomycosis on the arm. *(c)* Cryptococcosis; yeast-form cells (stained red) in lung tissue. *(d)* Coccidioidomycosis; yeast-form cells (stained blue-black) in lung tissue. *(e)* Paracoccidioidomycosis lesions on the face. *(f)* Oral thrush. Masses of *Candida albicans* cells (yellow) line the back of the throat. See photomicrographs of cultures of the pathogens causing most of these mycoses in Figure 34.1.

II • Visceral Parasitic Infections

Visceral parasites include several pathogenic protozoa that cause diseases with symptoms ranging from mild gastroenteritis to lethal meningoencephalitis. These vicious pathogens are transmitted by various routes including contaminated food or water, environmental sources, or person-to-person contact.

Parasitism is a symbiotic relationship between two organisms, the parasite and the host (Chapters 23 and 24). The parasite derives essential nutrients from the host and may have little or no harmful effect on the host. However, in most cases, the parasite causes disease in the host. Many different phylogenetic groups of protists (Chapter 18) cause parasitic human diseases, and we examine some of the key ones here.

Parasitic infections can be either visceral—inducing vomiting, diarrhea, and other intestinal symptoms—or infections of blood and internal tissues. Some of the major diseases of human history, malaria for example, are parasitic diseases. We begin here with the visceral parasites and then consider blood and tissue parasites. **Table 34.2** summarizes some major parasitic human diseases.

Mastering Microbiology
Art Activity: Table 34.2 Major parasitic human diseases

34.3 Amoebae and Ciliates: *Entamoeba*, *Naegleria*, and *Balantidium*

The genera *Entamoeba* and *Naegleria* belong to a large group of protists, the *Amoebozoa*, that move by extending lobe-shaped pseudopodia (◀ Section 18.8). Both parasites can cause serious—even fatal—infections, although *Naegleria* infections are very rare. *Balantidium* is a ciliated species of the alveolate group (◀ Section 18.4) and is mainly a disease of tropical countries.

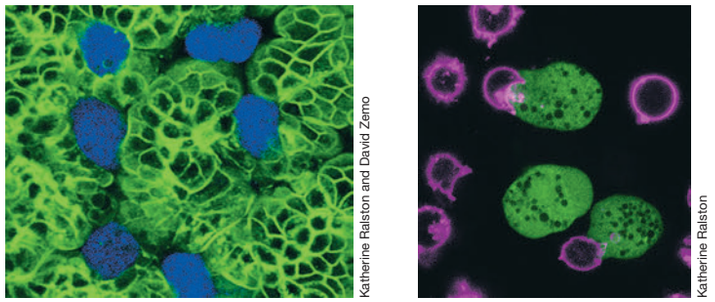
Amebic Dysentery

Entamoeba histolytica (**Figure 34.6**) is transmitted by contaminated water or occasionally through contaminated food. *E. histolytica* is an anaerobe, and the organism's *trophozoites* (the active, motile, feeding stage of the parasite) lack mitochondria. Like another common waterborne pathogen, *Giardia* (Section 34.4), the trophozoites of *E. histolytica* produce cysts, which are the means of transmission. Ingested cysts germinate to form amoebae that grow both on and in intestinal mucosa and can consume other available human cells (Figure 34.6*b*). This leads to breakdown of the intestinal epithelium, tissue invasion, and ulceration that triggers diarrhea and severe intestinal cramps.

TABLE 34.2 Major parasitic human diseases

Diseases by site	Causal organism[a]
Gastrointestinal	
Amebiasis	*Entamoeba histolytica*
Giardiasis	*Giardia intestinalis*
Cryptosporidiosis	*Cryptosporidium parvum*
Toxoplasmosis	*Toxoplasma gondii*
Blood and tissue	
Malaria	*Plasmodium* spp.
Leishmaniasis	*Leishmania* spp.
Trypanosomiasis (African sleeping sickness)	*Trypanosoma brucei*
Chagas disease	*Trypanosoma cruzi*
Schistosomiasis	*Schistosoma mansoni*

[a]All are protists (Chapter 18) except for *Schistosoma*, a helminth.



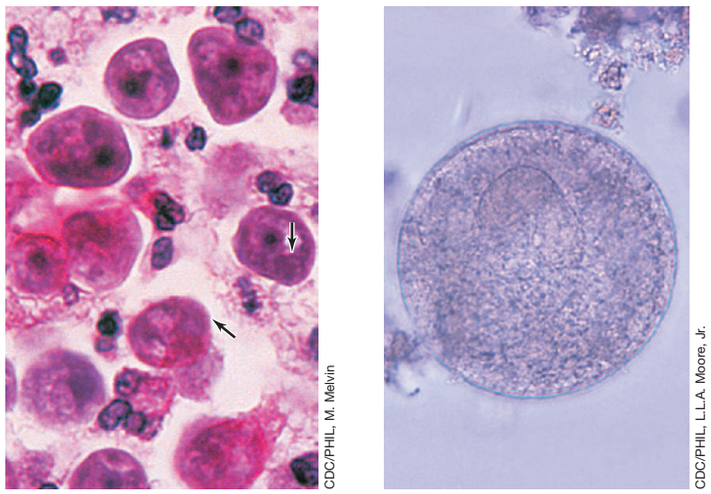
(a) (b)

Figure 34.6 *Entamoeba histolytica*, the causative agent of amebic dysentery. *(a)* Fluorescent photomicrograph of trophozoites of *E. histolytica* (blue) feeding on mouse intestinal cells (green); the trophozoites can be up to 60 μm in length. *(b)* Fluorescence photomicrograph of trophozoites of *E. histolytica* (green) ingesting human T cells (pink).

With further growth, the amoebae can invade the intestinal wall—a condition called **dysentery**, characterized by intestinal inflammation, fever, and the passage of intestinal blood and mucus, which often leads to anemia. If the infection is not treated, *E. histolytica* can invade the liver, the lungs, and even the brain. Growth in these tissues causes abscesses that can be fatal. Nearly 100,000 people, primarily from developing countries where untreated sewage is allowed to enter surface waters, die each year from invasive amebic dysentery. Although no vaccine is available, *E. histolytica* amebiasis can be treated with a variety of drugs, and the host immune system plays a significant role in recovery, as well. However, the immune response to a primary *E. histolytica* infection does not confer immune memory (◀ Section 27.1), and reinfection is common.

Naegleria and *Balantidium* Infections

Naegleria fowleri can cause amebiasis, but in a much different form from that of *E. histolytica*. *N. fowleri* is a free-living amoeba present in soil and in runoff waters (**Figure 34.7*a***). *N. fowleri* infections result from swimming or bathing in warm, soil-contaminated waters, such as warm springs or lakes and streams in summertime. *N. fowleri* enters the body through the nose and burrows directly into the brain. There the organism propagates, causing extensive hemorrhage and brain damage (Figure 34.7*a*), a condition called **primary amebic meningoencephalitis**. Diagnosis of an *N. fowleri* infection requires observation of the amoebae in cerebrospinal fluid. If a definitive diagnosis is made quickly, which is often not the case because the incidence of *Naegleria* meningoencephalitis is so low and the symptoms resemble those of meningitis, the drug amphotericin B can save the patient; untreated infections are almost always fatal (see also MicrobiologyNow on page 1059). Thus, although incidence of *Naegleria* meningoencephalitis is very low, mortality of untreated cases is near 100%, which makes this parasitic infection



(a) (b)

Figure 34.7 ***Naegleria fowleri* and *Balantidium coli*.** *(a)* Trophozoites (arrows) of *N. fowleri* in sectioned and stained brain tissue; the parasites are 10–25 μm in length. *(b) B. coli* cyst (about 50 μm wide) present in a fecal sample.

extremely dangerous. In the United States only 36 cases of *Naegleria* meningoencephalitis were reported in the 10-year period between 2008 and 2018, and virtually all were from recreational waters.

Balantidium coli is a ciliated intestinal human and swine parasite that alternates between the trophozoite and cyst (Figure 34.7*b*) stages; only the cysts are infective. *B. coli* is the only known ciliated parasite of humans. Cysts, typically transmitted in feces-contaminated water, germinate in the colon and infect mucosal tissues, leading to symptoms that resemble those of amebiasis, for which the disease is sometimes mistaken. An infected patient usually experiences a spontaneous recovery or may become an asymptomatic carrier, continuously shedding *B. coli* cysts in the feces. Compared with amebiasis, *B. coli* infections are uncommon, and cases are rarely fatal.

Check Your Understanding

- Contrast an *Entamoeba* and a *Naegleria* infection in terms of tissues infected and symptoms.
- Describe a scenario for contracting a *Naegleria* infection.

34.4 Other Visceral Parasites: *Giardia, Trichomonas, Cryptosporidium, Toxoplasma,* and *Cyclospora*

The protists *Giardia intestinalis* and *Trichomonas vaginalis* are flagellated anaerobic parasites that contain either mitosomes or hydrogenosomes in place of mitochondria (◀ Sections 18.1, 18.3); the parasites cause intestinal and sexually transmitted infections, respectively. The protist *Cryptosporidium* is transmitted primarily by contaminated water. *Cryptosporidium* is related to *Toxoplasma*, but *Toxoplasma* infection is primarily foodborne, as is infection by the pathogenic protist *Cyclospora*. We consider all five of these major human parasites here.

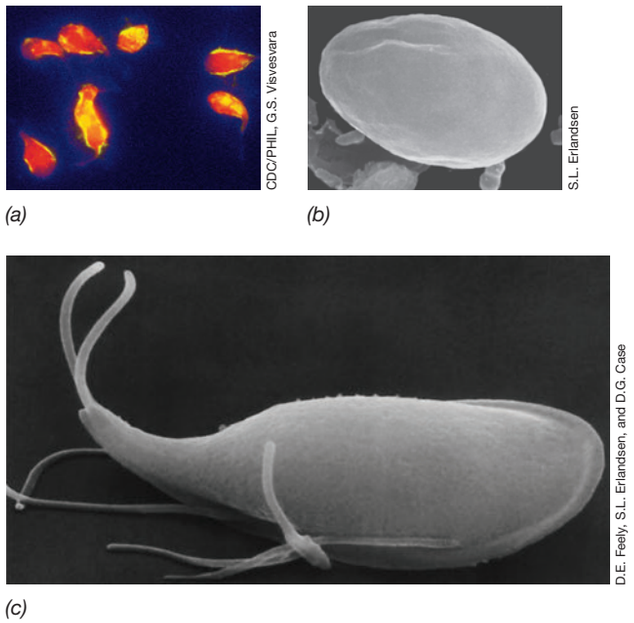
Giardiasis

Giardia intestinalis (also called *Giardia lamblia*) is typically transmitted to humans in fecally contaminated water and causes an acute gastroenteritis called *giardiasis*. The trophozoites of *Giardia* (**Figure 34.8*a, c***) produce highly resistant cysts (Figure 34.8*b*) that function in transmission. Ingested cysts germinate in the small intestine to form trophozoites, and these travel to the large intestine where they attach to the intestinal wall and cause the symptoms of giardiasis: an explosive, foul-smelling, watery diarrhea; intestinal cramps; flatulence; nausea; weight loss; and malaise. The distinct odor and absence of fecal blood distinguish giardiasis from diarrheas due to bacterial or viral intestinal pathogens.

G. intestinalis causes a significant number of infectious disease outbreaks linked to contaminated drinking water in the United States (◀ Section 33.1). The thick-walled cysts are resistant to chlorine, and most outbreaks have been associated with water systems that used only chlorination as a means of water purification. Water subjected to proper clarification and filtration followed by chlorination or other disinfection (◀ Section 22.9) should be free of *Giardia* cysts. Most surface water sources (lakes, ponds, and streams) contain *Giardia* cysts, as beavers and muskrats are carriers of this pathogen. This is why untreated surface waters should never be directly ingested but instead should be filtered and disinfected with iodine or chlorine or, alternatively, filtered and boiled. The drugs quinacrine, furazolidone, and metronidazole are useful for treating acute giardiasis.

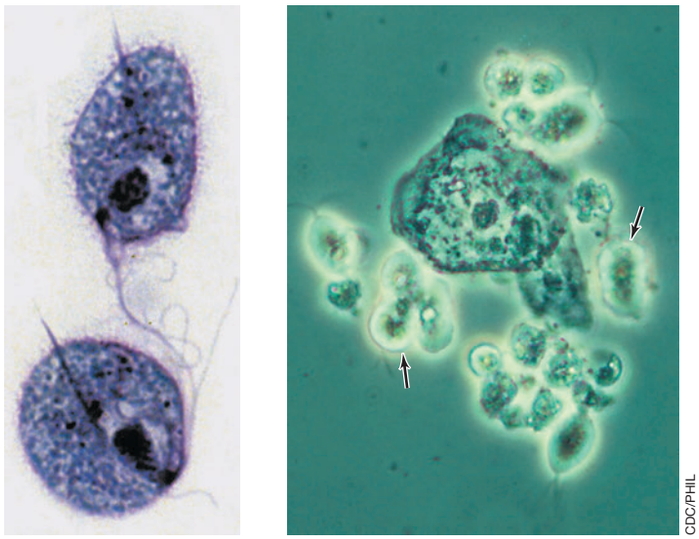
Trichomoniasis

Trichomonas vaginalis (**Figure 34.9**) causes a sexually transmitted infection, *trichomoniasis*. *T. vaginalis* does not produce resting cells or cysts, and as a result, *Trichomonas* transmission is typically from



(a) (b) (c)

Figure 34.8 ***Giardia.*** *(a)* Fluorescently stained cells of *Giardia intestinalis*. *(b, c)* Scanning electron micrographs of *(b)* a giardial cyst and *(c)* a motile *G. intestinalis* trophozoite. The trophozoite is 15 μm long and the cyst about 11 μm wide.



(a) (b)

Figure 34.9 ***Trichomonas vaginalis.*** *(a)* Light micrograph of stained cells; cells vary from 10 to 20 μm in diameter. *(b)* Light micrograph of vaginal discharge from a female with trichomoniasis. *T. vaginalis* cells (arrows) are present along with vaginal secretions and epithelial cells.

person to person through sexual intercourse. However, unlike most sexually transmitted *bacterial* pathogens, cells of *T. vaginalis* can survive for several hours on moist surfaces and up to a day in urine or semen. Hence, in addition to disease transmission by intimate contact, trichomoniasis can be transmitted by contaminated fomites, such as toilet seats, sauna benches, and towels.

T. vaginalis infects the vagina in women, the prostate and seminal vesicles of men, and the urethra of both males and females. Trichomoniasis is often asymptomatic in males. By contrast, trichomoniasis in females is characterized by a yellowish vaginal discharge that causes a persistent vaginal itching and burning. The infection is more common in females; surveys have shown that up to 25% of sexually active women are infected with *T. vaginalis*, whereas only about 5% of sexually active males are infected (conditions in the male are less conducive to colonization by the protist). Trichomoniasis is diagnosed by observation of the motile protists in a wet mount of fluid discharged from the patient (Figure 34.9*b*). The antiprotozoal drug metronidazole is effective for treating trichomoniasis.

Cryptosporidiosis, Toxoplasmosis, and Cyclosporiasis

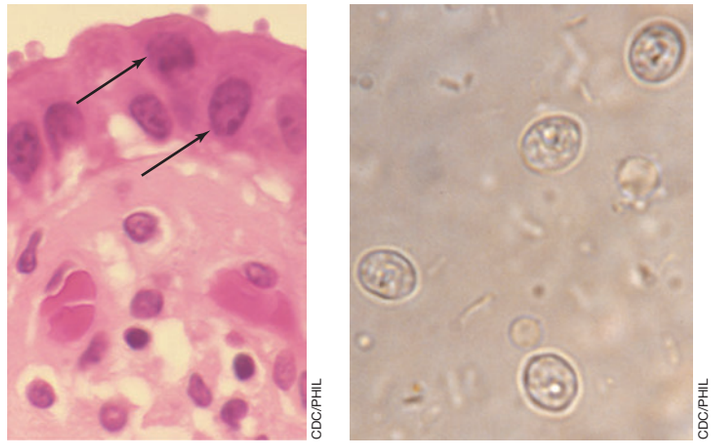
Cryptosporidium, *Toxoplasma*, and *Cyclospora* are genera of parasitic coccidia, which group among the alveolates (◀ Section 18.4). These parasites are transmitted to humans in feces-contaminated food or water and can trigger serious bouts of diarrhea, or in the case of *Toxoplasma*, serious internal organ damage.

Cryptosporidium parvum infects many warm-blooded animals, in particular cattle. The organism forms small, coccoid cells that invade and grow intracellularly in mucosal epithelial cells of the stomach and intestine (**Figure 34.10*a***), resulting in the gastrointestinal illness *cryptosporidiosis*. *C. parvum* produces thick-walled, highly resistant cysts called *oocysts* (Figure 34.10*b*), which enter water from the feces of infected animals. The infection is then transmitted to other animals and humans when they consume the feces-contaminated water.

Cryptosporidium oocysts are highly resistant to chlorine, and because of this, sedimentation followed by filtration is the only reliable way to remove them from water supplies. In an average year, *Cryptosporidium* is responsible for the majority of recreational waterborne disease outbreaks in the United States (Chapter 33) but is only occasionally associated with drinking water outbreaks. Nevertheless, *C. parvum* was responsible for the largest single outbreak of disease associated with drinking water ever recorded in the United States. In the spring of 1993, one-quarter of the population of Milwaukee, Wisconsin (USA), developed cryptosporidiosis from consuming water from the municipal water supply. Heavy spring rains and runoff from cattle manure on farmlands had drained into Lake Michigan, the water supply for the city, and overburdened the water purification system, leading to contamination by *C. parvum*.

In otherwise healthy individuals, cryptosporidiosis typically causes only a mild, self-limiting diarrhea, making treatment unnecessary. However, individuals with impaired immunity, such as that caused by HIV/AIDS, or the very young or old can develop serious complications from a *C. parvum* infection. For example, cryptosporidiosis is the leading cause of pediatric diarrhea worldwide, and it is responsible for nearly 9% of fatalities due to infectious disease in children. The primary laboratory diagnostic method for cryptosporidiosis is the demonstration of oocysts in feces (Figure 34.10*b*). Immunological and molecular tools are also available for more precise identification of strains of the pathogen when such tracking is necessary.

Like *C. parvum*, the parasite *Cyclospora cayetanensis* forms oocysts and causes a mild to occasionally severe gastroenteritis called *cyclosporiasis*. However, unlike *C. parvum*, *C. cayetanensis* is primarily transmitted by feces-contaminated, fresh foods rather than by contaminated water. Most cases of cyclosporiasis have been linked to



(a) (b)

Figure 34.10 ***Cryptosporidium parvum.*** *(a)* Light micrograph of intracellular trophozoites of *C. parvum* (arrows) embedded in human gastrointestinal epithelium. The trophozoites are about 5 μm in diameter. *(b)* Thick-walled *C. parvum* oocysts are about 3 μm in diameter in this fecal sample.

contaminated fruits or vegetables. A major outbreak in the United States in the summer of 2013 was linked to packaged lettuce (◀ Section 33.7). Another major *C. cayetanensis* outbreak in the United States occurred from May to August 2018, but in this incident, the outbreak was linked to multiple food items. Prepackaged vegetable trays containing broccoli, cauliflower, and carrots sold at a chain of convenience stores, and salads containing carrots, romaine lettuce, spinach, and kale sold at a fast food chain, were among the products that resulted in 2299 cases of cyclosporiasis.

Toxoplasmosis is caused by *Toxoplasma gondii* (**Figure 34.11**). This parasite infects many warm-blooded animals, and roughly half of all adults in the United States are infected but asymptomatic because their immune system keeps the organism in check. *T. gondii* is typically transmitted to humans in the form of cysts present in undercooked beef, pork, or lamb; by direct infection from cats, which are major carriers of *T. gondii*; and occasionally from contaminated water. A key step in the *T. gondii* life cycle is completed in felines, and thus they are obligate hosts; humans and other animals are only accidental hosts. Most transmission to humans is thus probably from cats.

Toxoplasmosis can be associated with mild to severe symptoms. When cysts of *T. gondii* are ingested, they germinate in and penetrate the wall of the small intestine. From this initial infection, symptoms can be inapparent or apparent but indistinguishable from those of a mild case of influenza (headache, muscle ache, general malaise). However, in some infected persons, *T. gondii* cysts migrate from the small intestine and circulate throughout the body. Subsequently, the parasite can penetrate nerve cells and infect tissues of the brain and eyes, a pathology that has been linked to altered behaviors, such as increased risk taking, obsessive–compulsive disorder, and schizophrenia. Although disease symptoms in healthy adults are uncommon, in immunocompromised individuals, damage to the eyes, brain, and other internal organ systems occurs with higher frequency. In addition, a first-time infection with *T. gondii* in expectant mothers can lead to congenital infection and birth defects in newborns. Thus, pregnant women who have not been in contact with cats should avoid cats until after giving birth.

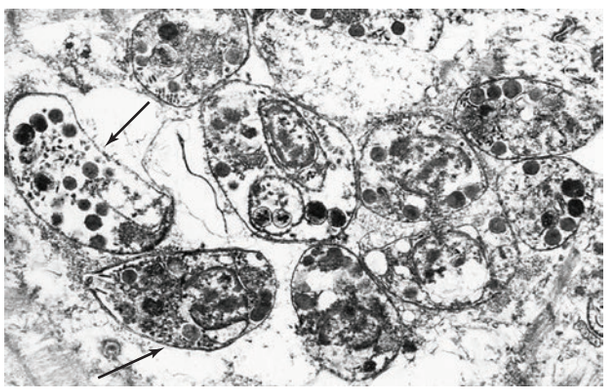


Figure 34.11 ***Toxoplasma.*** Tachyzoites (rapidly growing cells) of *Toxoplasma gondii*, an intracellular parasite. In this transmission electron micrograph, the tachyzoites (arrows) form a cystlike structure in a host cardiac cell. The *Toxoplasma* tachyzoites are 4–7 μm long. For a photo of *T. gondii* sporozoites (the infective phase of the parasite's life cycle), see Figure 18.11*b*.

Check Your Understanding

- What symptoms of giardiasis would suggest that your gastroenteritis was not due to a bacterial pathogen?
- How does one contract a case of trichomoniasis? A case of toxoplasmosis?
- What is unusual about the oocysts of *Cryptosporidium* that facilitates its transmission by a water route?

III • Blood and Tissue Parasitic Infections

Parasitic protozoa and helminths that infect the blood and other tissues cause some of the most prevalent and devastating diseases in the world, many of which are endemic in tropical and subtropical regions. Malaria is the most prevalent parasitic disease worldwide.

Several human parasites infect organs and tissues other than the gastrointestinal tract and are typically transmitted by insect vectors. We begin our consideration of these with malaria, the most devastating and widespread of parasitic diseases and one that remains a major global health problem today.

34.5 *Plasmodium* and Malaria

Malaria is caused by protists of the alveolate group (◀ Section 18.4). Several species of the protozoal genus *Plasmodium* cause malarial diseases in warm-blooded hosts; up to 250 million people worldwide contract malaria annually and about 600,000 die from the disease. Malaria is thus one of the most common causes of death worldwide from infectious disease and certainly the most prevalent of protozoal diseases.

In malaria, the complex parasite life cycle requires a mosquito vector. Four species of *Plasmodium*—*P. vivax, P. falciparum, P. ovale*, and *P. malariae*—cause most human malaria. The most widespread disease is caused by *P. vivax*, whereas the most serious disease is caused by *P. falciparum*. Humans are the only reservoirs for these four species. The protists carry out part of their life cycle in the human and part in the female *Anopheles* mosquito, the only vector that transmits *Plasmodium* spp. The vector spreads the protist from person to person.

Mastering Microbiology
Art Activity: Figure 34.12 The life cycle of *Plasmodium*

Malarial Life Cycle

The life cycle of *Plasmodium* is complex and involves a number of stages (**Figure 34.12**). First, the human host is infected by plasmodial *sporozoites*, small, elongated cells produced in the mosquito that localize in the salivary gland of the insect. The mosquito (Figure 34.12 inset) injects saliva containing the sporozoites into the human when obtaining a blood meal. The sporozoites travel to the liver where they infect liver cells. Here, some *Plasmodium* species, including *P. vivax*, may assume a dormant *hypnozoite* form for weeks to years, but eventually all species of the parasite replicate and become enlarged in a stage called the *schizont* (see Figure 34.13*b*). The schizonts then segment into a number of small cells called

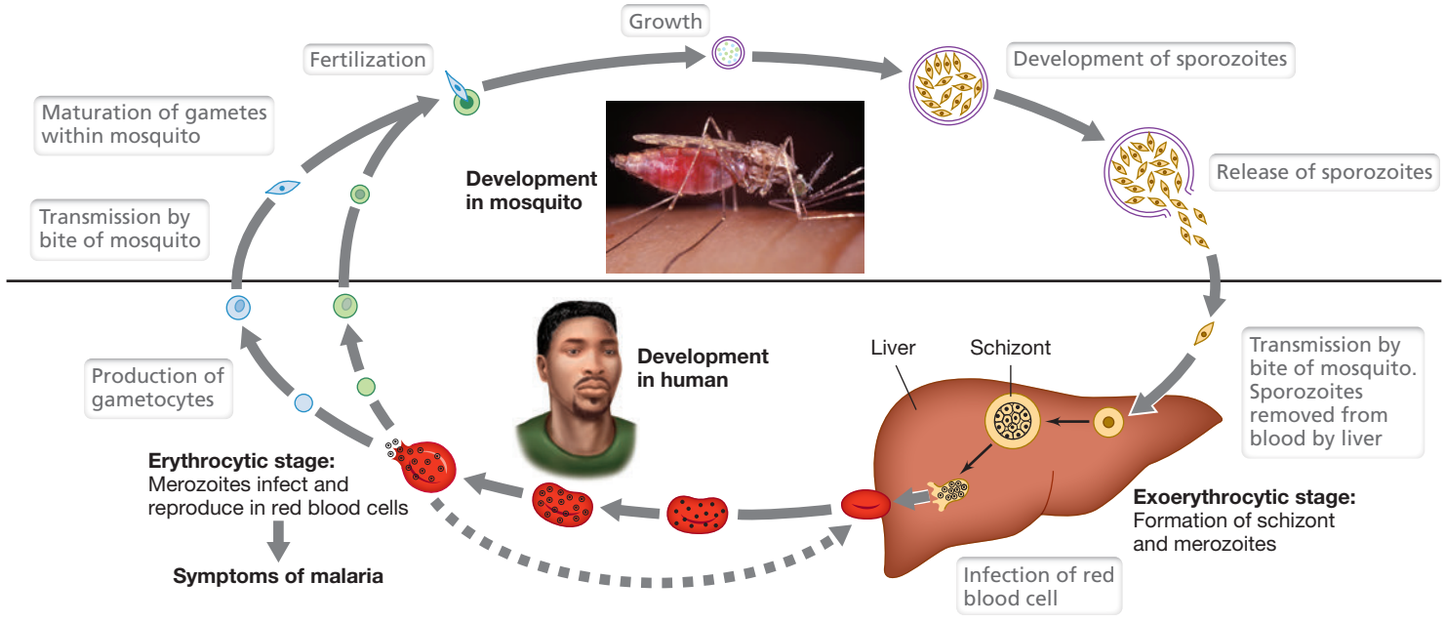


Figure 34.12 The life cycle of *Plasmodium*. The life cycle of *Plasmodium* requires both an endothermic (warm-blooded) host—in this case, a human—and the mosquito vector. Transmission of the protist to and from the human host is done by the bite of an *Anopheles gambiae* mosquito (inset) or certain other *Anopheles* species. Mosquito photo courtesy of CDC/PHIL, J. Gathany.

merozoites, which exit the liver into the bloodstream. Some of the merozoites then infect erythrocytes (red blood cells).

Mastering Microbiology
Art Activity: Figure 34.13 *Plasmodium* and malaria

The plasmodial life cycle in erythrocytes proceeds with repeated division, growth, and release of merozoites (**Figure 34.13**), resulting in destruction of the host red blood cells. Plasmodial growth in erythrocytes typically repeats at synchronized intervals of 48 h. During this period, the host experiences the defining clinical symptoms of malaria: chills followed by fever of up to 40°C (104°F). The chill–fever pattern coincides with the release of merozoites from the erythrocytes during the synchronized reproduction cycle. Vomiting and severe headache may accompany the chill–fever cycles, and over the longer term, characteristic symptomatic malaria can alternate with asymptomatic periods. Because of the destruction of red blood cells and consumption of hemoglobin by the parasite, malaria typically causes anemia, enlargement of the spleen, and fatigue.

Plasmodial merozoites eventually develop into *gametocytes*, cells that infect only mosquitoes. The gametocytes are ingested when an *Anopheles* mosquito takes a blood meal from an infected person, and they mature within the mosquito into *gametes*. Two gametes fuse to form a zygote, and the zygote migrates by amoeboid movement (◀ Section 18.8) to the outer wall of the insect's intestine where it enlarges and forms several sporozoites. These are released and reach the salivary gland of the mosquito, from where they can be introduced into another person during a subsequent bite, and the cycle begins anew (Figure 34.12).

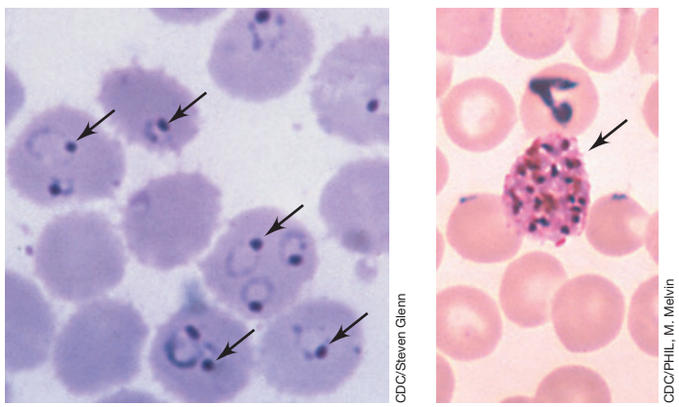


Figure 34.13 *Plasmodium* and malaria. *(a)* Merozoites of *Plasmodium falciparum* (arrows) growing within human red blood cells. *(b)* A schizont of *P. vivax* (arrow) along with red blood cells. When released from the schizont, the merozoites infect erythrocytes (Figure 34.12). Red blood cells about 8 μm in diameter; merozoites are 2–3 μm in diameter.

Epidemiology, Diagnosis, Treatment, and Control

Anopheles mosquitoes (Figure 34.12 inset) live predominantly in the tropics and subtropics and are the vector for malaria. Diagnosis of malaria requires the identification of *Plasmodium*-infected erythrocytes in blood smears (Figure 34.13*a*). Fluorescent nucleic acid stains, nucleic acid probes, PCR assays, and various antigen-detection methods (Chapter 29) are also used to verify *Plasmodium* infections or to differentiate between infections with various *Plasmodium* species.

Treatment of malaria is traditionally accomplished with quinine-based therapeutics, such as *chloroquine*. Chloroquine kills merozoites within red blood cells but does not kill sporozoites. The related drug *primaquine* eliminates sporozoites of *P. vivax* and *P. ovale* that may remain in liver cells. Thus treatment with both chloroquine and primaquine effectively cures malaria in some cases. More recently, the antimalarial drug *artemisinin*, derived from the wormwood plant originally but now made by genetically engineered yeast strains (◀ Section 12.11 and Figure 12.37), has provided more

reliable elimination of the parasite, and its use has become widespread. However, in some individuals, malaria recurs years after the primary infection when a few sporozoites not eliminated from the liver release a new generation of merozoites. Quinine- and artemisinin-resistant strains of *Plasmodium* are now widespread, especially in Southeast Asia where the prevalence of malaria is extremely high. As a result, *combination therapy*, in which the malaria patient is treated with several antimalarial drugs at once, is now a common form of treatment.

Malaria can be controlled by either draining swamps and other mosquito breeding areas or by eliminating the vector with insecticides. Together, these measures have all but eliminated malaria in the United States, with most cases being imported. Several malaria vaccines are also in development, including synthetic peptide vaccines, recombinant particle vaccines, and DNA vaccines (◀ Sections 12.8 and 28.3), but thus far no highly effective and reliable malaria vaccine has emerged for use in mass vaccination programs.

Check Your Understanding

- Which stages of the *Plasmodium* life cycle occur in humans, and which in the mosquito?
- What are the natural reservoirs and vectors for *Plasmodium* species? How can malaria be prevented or eradicated?
- What drugs are used to treat malaria?

34.6 Leishmaniasis, Trypanosomiasis, and Chagas Disease

Parasites of the genera *Leishmania* and *Trypanosoma* are transmitted by bloodsucking insect vectors. These parasites are *hemoflagellates*, organisms that reside in blood or related tissues such as the liver and spleen, and they cause major human diseases, primarily in tropical and subtropical countries.

Leishmaniasis

Leishmaniasis is a parasitic disease of various forms caused by species of the genus *Leishmania*, a flagellated protozoan related to *Trypanosoma*. The disease is transmitted to humans by a bite from the sand fly (**Figure 34.14*a***). *Cutaneous leishmaniasis*, caused by either *L. tropica* or *L. mexicana*, is the most common form of leishmaniasis. Following transmission of the parasite in a blood meal (Figure 34.14*a*, *b*), the parasite infects and grows within human macrophage cells (◀ Section 26.4), leading eventually (weeks or months later) to the formation of a small nodule on the skin. The nodule then enlarges to form an ulcerous skin lesion (Figure 34.14*c*) that contains active parasites. In the absence of secondary bacterial infections, which are common if the ulcerated tissue is left open, the lesions heal spontaneously over a period of several months but can leave a permanent scar.

Leishmaniasis has historically been treated with injections of pentavalent antimony (Sb^{5+}) compounds. Although the mode of action of these compounds is unknown, it is thought that Sb^{5+} in some way stimulates or activates the immune response to better attack the *Leishmania* parasites. At present, however, many *Leishmania* species are resistant to antimony compounds, but a variety of other drugs

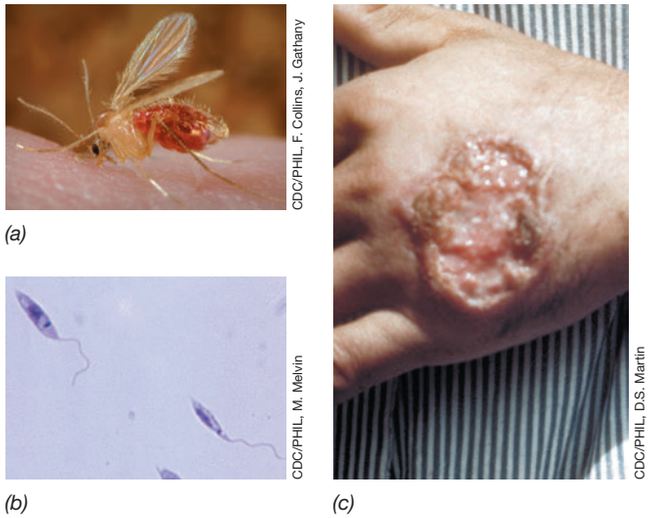


Figure 34.14 Leishmaniasis. *(a)* The sand fly (genus *Phlebotomus*) transmits leishmaniasis in a blood meal. *(b) Leishmania* spp. are flagellated protozoans and the cause of leishmaniasis. *(c)* Cutaneous leishmaniasis showing an open ulcer on the hand.

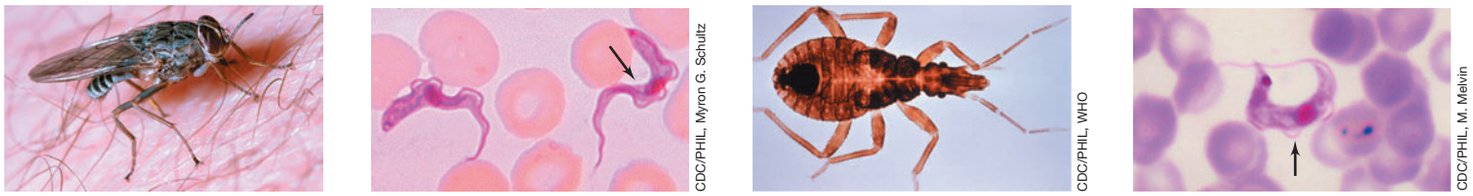
are available for treating resistant cutaneous forms of the disease. Cutaneous leishmaniasis exists in over 88 tropical and subtropical countries, with an estimated prevalence of about 1 million cases worldwide. Occasional reports of the disease are documented in the United States, most often in the state of Texas.

Visceral leishmaniasis is caused by *Leishmania donovani* and is the most severe form of the disease. In visceral leishmaniasis, the parasite travels from the site of infection to internal organs, in particular the liver, spleen, and bone marrow. If left untreated, the visceral disease is almost always fatal. Common symptoms of visceral leishmaniasis include a cycling of fever and chills, a slow reduction in both red and white blood cell numbers, and significant enlargement of the spleen and liver that can lead to pronounced distention of the abdomen. Treatment includes injections of antimony (as for the cutaneous disease), long periods of bed rest, and blood transfusions in acute cases if blood cell counts become dangerously low. Estimates of visceral leishmaniasis prevalence worldwide are about 300,000, causing about 20,000 deaths annually. In addition, because the range of the sand fly is already broad and is increasing with climate change and tropical and subtropical deforestation, it is estimated that worldwide nearly 400 million people (over 5% of the world's humans) could be at risk of some form of leishmaniasis.

Trypanosomiasis and Chagas Disease

Flagellated protozoans of the genus *Trypanosoma* (◀ Section 18.3) cause two related forms of **trypanosomiasis**. Two subspecies of *Trypanosoma brucei* native to Africa, *T. brucei gambiense* and *T. brucei rhodesiense*, cause *African trypanosomiasis*, better known as *African sleeping sickness*. The species *T. cruzi* causes *Chagas disease*, also known as *American trypanosomiasis*. As discussed below, both forms of trypanosomiasis are transmitted by the bite of specific insect vectors.

Sleeping sickness is transmitted by the tsetse fly (genus *Glossina*; **Figure 34.15*a***), an insect similar in dimensions to a housefly and native only to tropical regions of Africa; sleeping sickness is therefore endemic only in countries of sub-Saharan Africa. The disease begins



(a) (b) (c) (d)

Figure 34.15 African trypanosomiasis and Chagas disease. *(a)* The tsetse fly (genus *Glossina*), the vector of African trypanosomiasis. The insect is about 1 cm in length. *(b)* Two cells of *Trypanosoma brucei* (arrow), the causative agent of African sleeping sickness (African trypanosomiasis), in a blood smear. *(c)* The reduviid ("kissing") bug (*Triatoma infestans*), the vector for Chagas disease (American trypanosomiasis). The insect is about 2 cm in length. *(d)* A cell of *Trypanosoma cruzi* (arrow), the causative agent of Chagas disease, in a blood smear.

with intermittent fever, headache, and malaise. The trypanosome parasite multiplies in the blood (Figure 34.15*b*) and later infects the central nervous system and grows in cerebrospinal fluid. Neurological symptoms soon begin, with the primary symptom being disrupted sleep patterns marked by random periods of extreme lethargy and drowsiness. This is due to the parasite producing the aromatic alcohol *tryptophol*, a derivative of the amino acid tryptophan, which triggers a sleep response. Without treatment, the infection gradually progresses to a coma, multiple organ failure, and eventually death after months or years. A variety of anti-trypanosomal drugs are available for treating sleeping sickness; some are used primarily for treating the blood infection while others are used if the disease has progressed to the neurological stage. About 10,000 new cases of sleeping sickness are reported annually, but most cases are thought to go unreported.

Chagas disease, named for its discoverer, is caused by *T. cruzi*, a close relative of *T. brucei*, and is transmitted by the bite of the reduviid bug, or "kissing bug" (Figure 34.15*c, d*). Chagas disease mainly occurs in Latin American countries. The parasite affects several organs including the heart, gastrointestinal tract, and central nervous system, causing inflammatory reactions and tissue destruction. The acute illness is usually self-limiting, but if chronic illness develops, heart damage is significant and is the eventual cause of premature death. About 20,000 deaths due to Chagas disease occur annually in endemic Latin American countries. The disease is treated with anti-trypanosomal drugs, such as benznidazole, but currently no vaccines are available for prevention of African or American trypanosomiases.

Mastering Microbiology
Art Activity: Figure 34.16 Schistosomiasis

Check Your Understanding

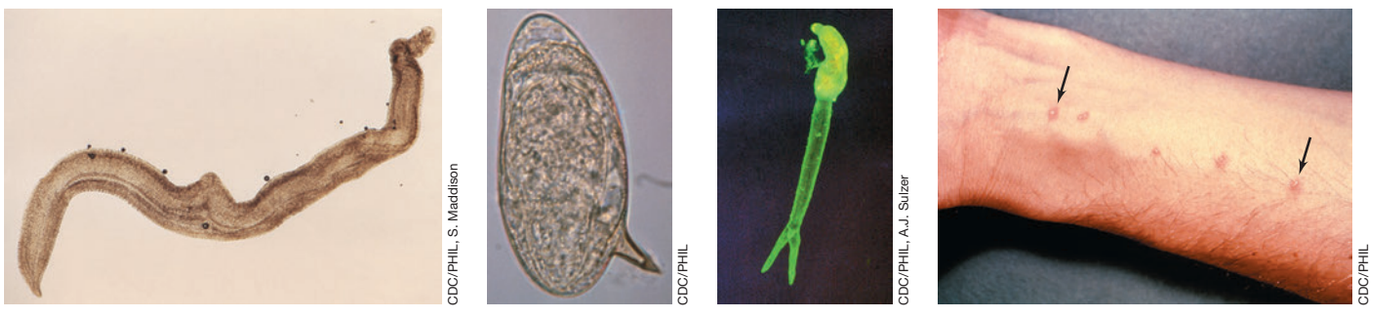
- How are trypanosome diseases similar to malaria and how do they differ?
- How do the symptoms of cutaneous and visceral leishmaniasis differ?
- How are sleep patterns altered in cases of African trypanosomiasis?

34.7 Parasitic Helminths: Schistosomiasis and Filariases

Some parasitic diseases are caused by helminths, tiny worms that burrow into the human host and cause debilitating diseases and death. We consider the most widespread of these, schistosomiasis, along with brief coverage of other, less common helminth infections.

Schistosomiasis

Schistosomiasis, also called *snail fever*, is a chronic parasitic disease caused by species of trematodes (flatworms) of the genus *Schistosoma*; the major species is *S. mansoni* and adult worms can be up to a centimeter in length (**Figure 34.16*a***). The life cycle of the parasite requires both snails and humans (or other mammals) as hosts. Schistosome eggs (Figure 34.16*b*) released into a freshwater aquatic environment hatch to generate *miracidia*, the form of the worm that



(a) (b) (c) (d)

Figure 34.16 Schistosomiasis. *(a)* Adult worm of *Schistosoma mansoni*; the worm is about 1 cm in length. *(b)* An *S. mansoni* egg, about 0.15 mm long. The lateral spur (bottom right) is characteristic of the eggs of this species. *(c)* Fluorescently stained cercaria, the infective form of *S. mansoni*. From the head (top) to the bifurcated tail is about 1 mm. *(d)* Cercarial infection of the forearm. Five infection sites (arrows) are apparent.

infects snails. In the snail, miracidia are transformed into *cercariae* (Figure 34.16*c*), the motile stage of the parasite that is released and infects humans.

A cercaria burrows into the skin, leaving a small surface lesion (Figure 34.16*d*), and then migrates to the lungs and liver; in the process, the worm establishes a long-term infection in the blood vessels. From the liver, the parasite infects the bladder, kidneys, and urethra, and the female worm produces large numbers of eggs. The eggs are shed in the urine and also pass through the intestinal wall and are shed in the feces. Large egg masses also become trapped along with fluids in the bladder, liver, and other organs, triggering an inflammatory response and severe distention of the abdomen, a condition commonly seen in infected children (**Figure 34.17*a***). Other symptoms include bloody urine, diarrhea, and abdominal pain. Eggs as well as adult worms can live in the body for years, causing chronic symptoms that can last from youth into adulthood.

Schistosomiasis is a disease of tropical countries, primarily those in Africa, but some cases also occur in subtropical countries, including those of Latin America and the Caribbean region. Poverty, poor sanitation, and land use changes are typically associated with widespread infection. Although there is no vaccine, schistosomiasis is treated with the drug praziquantel, and the diagnosis is made relatively easily by assessing symptoms and observing parasite eggs in the urine and feces. Mortality from schistosomiasis is low, about 0.1%, but schistosomiasis is second only to malaria in terms of total parasitic infections worldwide. In 2017, over 220 million cases of the disease were treated, and many others probably went untreated.

Filariases

Several other parasitic helminth infections are known, and chief among these are the *filariases*, infections by parasitic nematodes (roundworms). Unlike the schistosomiasis parasite, these worms are clearly macroscopic in the adult stage (several centimeters in length, depending on the filariasis).

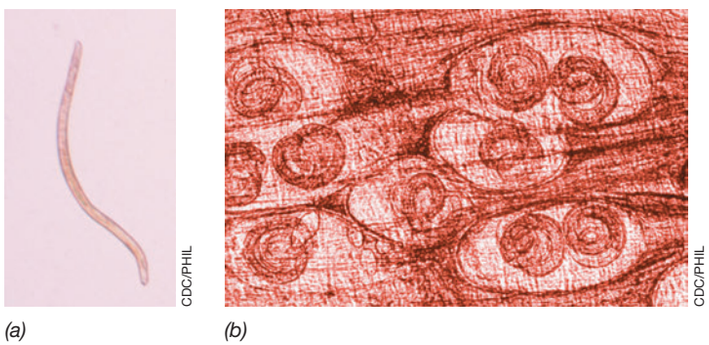
Bancroft's filariasis (also called elephantiasis) is a chronic infection of the lymphatic system by the roundworm *Wuchereria bancrofti*. The worm is transmitted to humans as tiny *microfilariae* in a mosquito bite. Once in the host, microfilariae develop into adult worms and these interrupt lymph flow, leading to massive accumulation of fluids (edema), especially in the lower extremities. Fluid accumulation in lower regions of the body can cause severe edema of the legs (Figure 34.17*b*). Over 120 million people in the tropics suffer from *W. bancrofti* infection, but the microfilarial stage of the disease is readily treatable with antihelminthic drugs (◀ Section 28.6) or the drug diethylcarbamazine, which kills both microfilariae and adult worms. Even simpler treatment may be available by administering antibacterial antibiotics. Although the worm itself is not sensitive to these drugs, the worm harbors an endosymbiotic bacterium, *Wolbachia* (*Alphaproteobacteria*, ◀ Sections 16.1, 32.5), which is killed by various antibiotics. If *Wolbachia* is eliminated by antibiotic treatment, the worms die, and so antihelminthic treatments often include antibiotics such as doxycycline (◀ Section 28.5) to accelerate the removal of worms from the patient.

Onchocerciasis (also called *river blindness*) is due to a chronic infection by the large parasitic roundworm *Onchocerca volvulus* (**Figure 34.18*a***). Humans are the only known host for this parasite, but biting flies are vectors when they become infected with microfilariae in a blood meal and transmit them to uninfected humans. Blackflies of the genus *Simulium* are the major means of transmission. The microfilariae invade the cornea, and from there the iris and retina, triggering an inflammatory response that causes scarring and partial to total loss of vision. *O. volvulus* infection is second only to trachoma (◀ Section 31.13) as a cause of infectious blindness. It is estimated that about 20 million people are infected with this parasite, primarily in equatorial Africa.

The disease *trichinosis* (also called *trichinellosis*) is caused by species of the parasitic roundworm *Trichinella* (Figure 34.18*b*). This worm commonly infects the muscle tissues of wild mammals and can occasionally infect domestic animals, especially swine; about 20 cases of human trichinosis are reported in the United States each year, usually from the consumption of undercooked wild game or undercooked pork. Human infection with *Trichinella* begins when worm larvae enter intestinal mucosal cells, leading to either an asymptomatic condition or mild gastroenteritis. As the larvae mature and reproduce, new larvae circulate throughout the body and lead to systemic inflammatory reactions such as malaise,

(a) (b)

Figure 34.17 Symptoms of parasitic helminth infections. *(a)* Schistosomiasis in a small child. The swollen abdomen from the accumulation of fluids and worm eggs is characteristic of the infection. *(b)* Bancroft's filariasis (elephantiasis). The swollen legs are the result of edema from infection of lymph tissues by the roundworm *Wuchereria bancrofti*.



(a) (b)

Figure 34.18 The roundworms of river blindness and trichinosis. *(a)* A worm larva of *Onchocerca volvulus*, the causative agent of river blindness. The microfilarial worm is about 0.3 mm long, but adult worms can be several centimeters in length. *(b)* Cysts of *Trichinella spiralis* containing worm larvae in muscle tissue. Unlike the much larger *O. volvulus*, adult worms of *T. spiralis* are just a few millimeters long.

facial swelling, and fever. Untreated cases of trichinosis can progress to more severe organ-specific symptoms including heart damage, encephalitis, and even death. However, if properly diagnosed (usually by immunological assays on biopsied muscle tissue), trichinosis is treatable with a variety of drugs, in particular the benzimidazole class of antihelminthics.

Check Your Understanding

- How does the pathogen that causes schistosomiasis differ from all other pathogens considered in this chapter?
- From what source are most cases of human trichinosis contracted?

Go to **Mastering Microbiology** for videos, animations, practice tests, and more.

CHAPTER REVIEW

I • Fungal Infections

34.1 Fungi include the molds and yeasts, and some fungi are dimorphic, meaning that both mycelial and yeast phases can occur. Superficial, subcutaneous, and systemic mycoses refer to fungal infections of the skin surface, skin subsurface, and internal organs, respectively. Fungal infections can be mild or serious, depending on the health and immune status of those infected.

Q What are the major mechanisms through which fungi cause disease?

34.2 Superficial mycoses such as athlete's foot or jock itch are mild and easily treatable, whereas subcutaneous mycoses, such as sporotrichosis, or especially systemic mycoses, such as histoplasmosis, are more difficult to treat effectively. The ability of fungi that cause systemic mycoses to infect internal organs makes these pathogens particularly dangerous to the elderly or those otherwise immune compromised.

Q How are systemic mycoses characterized? What is the difference between a primary and a secondary infection caused by systemic mycoses?

II • Visceral Parasitic Infections

34.3 The genera *Entamoeba* and *Naegleria* are amoebic human parasites that cause gastrointestinal and brain infections, respectively. *Entamoeba* is transmitted in feces-contaminated waters, whereas *Naegleria* inhabits warm, soil-contaminated waters. *Balantidium* is a ciliated intestinal parasite transmitted by feces-contaminated water.

Q If you were to contract one or the other, which would you rather have, an *Entamoeba* infection or a *Naegleria* infection, and why?

34.4 The protists *Giardia intestinalis* and *Cryptosporidium parvum* are major waterborne pathogenic parasites, whereas *Toxoplasma gondii* is primarily a foodborne or cat-transmitted parasite, and *Trichomonas vaginalis* is a sexually transmitted parasite. The pathogenic parasite *Cyclospora* is primarily transmitted by fresh vegetables such as lettuce and spinach contaminated with animal feces. None of these parasites causes life-threatening diseases in otherwise healthy individuals, although *T. gondii* can trigger severe and even fatal infections in immunocompromised hosts and can cross the placenta to cause serious congenital infections.

Q In contrast to disease caused by *Trichomonas*, what do giardiasis and cryptosporidiosis have in common?

III • Blood and Tissue Parasitic Infections

34.5 Infections with *Plasmodium* spp. cause malaria, a widespread, mosquito-transmitted disease of the blood that causes significant morbidity and mortality in tropical and subtropical regions of the world. Malaria is treatable with quinine-based drugs and artemisinin but is not yet preventable by vaccination.

Q Malaria symptoms include chills followed by fever. These symptoms are related to activities of the pathogen. Describe the growth stages of *Plasmodium* spp. in the human host and relate them to the chill–fever pattern.

34.6 Leishmaniasis is a parasitic disease caused by *Leishmania* species; the cutaneous form of the disease is most common. *Trypanosoma brucei* causes African trypanosomiasis (African sleeping sickness), while the related species *Trypanosoma cruzi* causes Chagas disease (American trypanosomiasis). All of these diseases are transmitted by the bite of specific insect vectors.

Q Contrast leishmaniasis with the two types of trypanosomiasis in terms of causative agents, symptoms, and transmission vectors.

34.7 Schistosomiasis is a prevalent parasitic disease caused by a microscopic worm, *Schistosoma mansoni*. The life cycle of the parasite requires both snails and mammals. The worm infects the liver and kidneys and produces large egg masses that accumulate in the body, leading to systemic inflammation and abdominal distention. Other parasitic worm diseases, such as elephantiasis and river blindness, also leave readily visible signs of infection. Trichinosis is caused by a roundworm that infects the intestine and muscle tissues and is a threat from the consumption of undercooked pork or wild game.

Q In what unusual way does the parasite *Schistosoma mansoni* enter the body to cause infection? Are there any physical signs of infection when it occurs?

APPLICATION QUESTIONS

1. Malaria eradication has been a goal of public health programs for at least 100 years. What factors preclude our ability to eradicate malaria? If an effective vaccine was developed, could malaria be eradicated?
2. In terms of public health, what is a common problem that unites many of the visceral parasitic infections covered in this chapter? How could this problem be attacked? Why are these diseases rare in developed countries?
3. Explain why the diseases malaria, leishmaniasis, and trypanosomiasis are primarily diseases of tropical regions. How could humans be affecting the future geographical ranges of these diseases?
4. Explain why systemic fungal infections are typically seen only in certain individuals even though many people have contact with the pathogen, whereas an outbreak of giardiasis affects virtually everyone that has come in contact with the pathogen.

CHAPTER GLOSSARY

Dysentery a disease characterized by intestinal ulceration and inflammation, fever, and the passage of diarrhea containing intestinal blood and mucus

Leishmaniasis a disease of the skin or viscera caused by infection with species of a parasitic flagellated protozoan, *Leishmania*

Malaria a disease characterized by recurrent episodes of fever and anemia; caused by species of the protist *Plasmodium*, usually transmitted between mammals through the bite of the *Anopheles* mosquito

Mycosis (plural, mycoses) any infection caused by a fungus

Primary amebic meningoencephalitis the invasion, inflammation, and destruction of brain tissue by the amoeba *Naegleria fowleri* or a variety of other amoebic parasites

Schistosomiasis a chronic disease caused by a parasitic worm that leads to internal organ damage and accumulation of fluids and worm egg masses

Subcutaneous mycoses fungal infections of deeper layers of skin

Superficial mycoses fungal infections of the surface layers of skin, hair, or nails

Systemic mycoses fungal growth in internal organs of the body

Trypanosomiasis any parasitic disease of the blood and internal tissues caused by species of the flagellated protozoan *Trypanosoma*; African sleeping sickness and Chagas disease are two major trypanosomiases

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binding of *Pseudomonas aeruginosa* to scratch-wounded human cystic fibrosis airway epithelial cells. *PLoS ONE* 11(3): e0150109; **7.16:** Dr. Ariane Briegel; **7.19:** Timothy C. Johnston, Murray State University; **7.29:** Jingyi Fei.

Chapter 8 Chapter Opener: Zarnowski, R. et al. 2018. Candida albicans biofilm-induced vesicles confer drug resistance through matrix biogenesis. *PLoS Biol*. 16(10): e2006872; **8.1a:** T. Doan, R. Losick, and D. Rudner; **8.1b:** Conrad L. Woldringh; **8.1c:** Fabai Wu and Cees Dekker Lab; **8.1d:** Pereira, A. R., Hsin, J., Krol, E., Tavares, A. C., Flores, P., Hoiczyk, E., Ng, N., Dajkovic, A., Brun, Y. V., VanNieuwenhze, M. S., Roemer, T., Carballido-Lopez, R., Scheffers, D. J., Huang, K. C., and Pinho, M. G. 2016. *MBio*: e00908-16; **8.2a:** S. Uphoff and A. Badrinarayanan; **8.2b:** Alexander von Diezmann, Andreas Gahlmann, and W.E. Moerner, Stanford University; **8.7:** R. Reyes-Lamothe; **8.8b:** T. den Blaauwen and Nanne Nanninga, University of Amsterdam, The Netherlands; **8.10b:** Alex Formstone; **8.10c:** Dr. Christine Jacobs-Wagner; **8.12:** Wanda Figueroa-Cuilan and Pamela Brown; **8.13b:** Akiko Umeda and K. Amako; **8.14:** Pereira, A. R., Hsin, J., Krol, E., Tavares, A. C., Flores, P., Hoiczyk, E., Ng, N., Dajkovic, A., Brun, Y. V., VanNieuwenhze, M. S., Roemer, T., Carballido-Lopez, R., Scheffers, D. J., Huang, K. C., and Pinho, M.G. 2016. *MBio*: e00908-16; **8.17:** Wang, C., Stanciu, C., Ehrhardt, C. J., Yadavalli, V. K. 2015. *Journal of Microscopy* 258: 49-58; **8.19:** Mutlu, A. et. al. 2018. *Nature Communications* 9 (1): 69; **8.20:** C. Fernandez-Fernandez and Justine Collier; **8.21a:** Alicia M. Muro-Pastor; **8.22b:** Rodney M. Donlan & Emerging Infectious Diseases; **8.24:** Turnbull, L. et al. 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications* 7: 11220; **8.25a:** Veysel Berk; **8.26a:** Koerdt, A., Godeke, J. Berger. K., Thormann, K.M. and Albers, S-V. 2010. Crenarchaeal biofilm formation under extreme conditions. PLoS ONE: e14104; **8.28:** Wang, Z., Fan, G., Hryc, C.F., Blaza, J.N., Serysheva, I.I., Schmid, M.F., Chiu, W., Luisi, B.F., and Du, D. RCSB Protein Databank: 5O66; **8.30:** Mutlu, A. et. al. 2018. *Nature Communications* 9 (1): 69.

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Chapter 10 Chapter Opener: Dong, Y.,et al. 2019. Physiology, metabolism, and fossilization of hot-springs filamentous microbial mats. *Astrobiology* DOI:10.1089/ast.2018.1965.; **10.1:** Steve Gschmeissner/Science Source; **10.3a:** Centers for Disease Control and Prevention; **10.3b:** Jose de la Torre and David Stahl, **inset:** Larry Staufer/CDC; **Explore the Microbial World Figure 2:** EMLab/Tommy Trenchard 2016; **10.11:** Yuuji Tsukii, Protist Information Server, (protist.i.hosei.ac.jp); **10.12a:** Don W. Fawcett/Science Source; **10.14a:** Dave Bunnell/Under Earth Images, **inset:** L. Ejim, C. Groves, G. Wright; **10.15b:** Gal Ofir, Sorek Lab, Weizmann Institute of Science; **10.16a:** Christen B. and Christen M.; **10.20:** ThermoFisher Scientific; **10.21:** Flaherty, B. L., Van Nieuwerburgh, F., Head, S. R., and Golden, J. W. 2011. BMC Genomics 12:332; **10.27:** Gary Siuzdak, Scripps Center for Metabolomics; **10.28:** Pieter Dorrestein; **10.29b:** Norbert Pfennig, University of Konstanz, Germany; **10.29c:** Courtesy of Jörg Overmann and Petra Henke; **10.32b:** CDC/PHIL; **10.34a:** Jennifer Li-Pook-Than and Michael P. Snyder, Stanford University.

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Chapter 12 Chapter Opener: Phillip Nadeau/MIT; **12.1 top:** Norbert Pfennig, University of Konstanz, Germany, **bottom:** Heidi Polumbo/U.S. Department of Energy; **12.4a:** Elizabeth Parker; **12.4b:** Jack Parker; **12.5a:** Dr. Laurie Ann Achenbach, Southern Illinois University at Carbondale; **12.5b:** Megan Kempher; **12.6:** Michael T. Madigan; **12.10:** Daniel L. Nickrent; **12.12 left:** Norbert Pfennig, University of Konstanz, Germany, **center:** Hans Hippe, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, **right:** Michael T. Madigan; **12.17a:** Martin Shields/Science Source; **12.17b:** Jason A. Kahana and Pamela A. Silver, Harvard Medical School; **12.22:** Stephen R. Padgette; **12.23:** Kevin McBride, Calgene; **12.24:** Aqua Bounty Technologies; **12.27:** Hwang, I. Y., Koh, E., Wong, A., March, J. C., Bentley, W. E., Lee, Y. S., and Chang, M. W. 2017. *Nature Communications* 8, 15028; **12.28:** Dinesh Chandra and Claudia Gravekamp; **12.30 top:** Klagyi/Shutterstock, **center:** Puchan/Shutterstock, **bottom:** Karen Lau/Shutterstock; **12.31:** Cinder Biological Inc.; **12.33a:** Chris Standlee and DOE/NREL; **12.33b:** Heidi Polumbo/U.S. Department of Energy; **12.33c:** Sara E. Blumer-Schuette, Jeffrey V. Zurawski, Jonathan M. Conway, and Robert M. Kelly; **12.34 top:** Todd Ciche, **bottom:** Marek Mis/Science Source; **12.35:** Rasala, B. A., Chao, S. S., Pier, M., Barrera, D. J., and Mayfield, S. P. 2014. *PLoS ONE* 9(4): e94028; **12.38c:** Aaron Chevalier and Matt Levy; **12.39b:** Images from M. Omar Din and Jeff Hasty; **12.41:** Image courtesy Clyde Hutchison and J. Craig Venter, JCVI and Thomas Deerinck and Mark Ellisman, NCMIR; **12.42c:** Hiroshi Nishimasu, Osama Nureki, and Feng Zhang; **12.43 left:** Joyce Van Eck and Zachary Lippman, **right:** Biao Ding & Yijun Qi; **12.44:** Guo, R., Wang, H., Cui, J., Wang, G., Li, W., and Hu, J. F. 2015. *PLoS ONE* 10(10): e0141335; **12.45a:** James Laboratory (UC Irvine) mosquitoes modified by Thai Binh Pham and image by Kiona M. Parker.

Chapter 13 Chapter Opener: Dr. Donald Hilvert, Dr. Angela Steinauer; **13.2:** Frances Westall, Lunar and Planetary Institute; **13.3:** Michael T. Madigan; **13.6a, b, e:** Malcolm R. Walter, Macquarie University, New South Wales, Australia; **13.6c:** Daniel H. Buckley; **13.6d:** Thomas D. Brock; **13.7:** J. William Schopf, University of California at Los Angeles; **13.8:** John M. Hayes; **13.11:** Rolf B. Pedersen; **13.14:** Daniel H. Buckley; **13.25 top:** Norbert Pfennig, University of Konstanz, Germany, **bottom:** Michael T. Madigan; **Table 13.1:** Norbert Pfennig, University of Konstanz, Germany.

Chapter 14 Chapter Opener: Dr. Clara S. Chan; **14.3:** Jessup M. Shively, Clemson University; **14.5a:** Norbert Pfennig, University of Konstanz, Germany, **14.5b:** Thomas D. Brock; **14.8b:** Simon Scheuring; **14.9 inset:** Yuuji Tsukii, Protist Information Server, (protist.i.hosei.ac.jp); **14.10:** Michael T. Madigan; **14.11a:** Niels-Ulrik Frigaard; **14.14a:** Susan Barns and Norman R. Pace, University of Colorado; **14.14c:** Kaori Ohki, Tokai University, Shimizu, Japan; **14.15a:** George Feher, University of California at San Diego; **14.15b:** The model is based on PDB ID: 3I4D (Fujii et al. (2010) Photosynthetic reaction center from rhodobacter sphaeroides 2.4.1) and was visualized in the RCSB PDB (Berman et al. (2000) Nucleic Acids Research, 28: 235-242) using the NGL Viewer (Rose et al. (2018) *Bioinformatics* 34: 3755–3758); **14.18:** Yehuda Cohen and Moshe Shilo; **14.19:** Thomas D. Brock; **14.21a:** William Strode; **14.21b:** Thomas D. Brock; **14.23:** Reproduced from Armin Ehrenreich and Friedrich Widdel, *Applied and Environmental Microbiology* 60:4517-4526 (1994), with permission of the American Society for Microbiology; **14.26a:** Marc Strous, University of Nijmegen, The Netherlands; **14.26b:** John A. Fuerst, University of Queensland, Australia; **14.32:** Dianne K. Newman and Stephen Tay, previously published in *Applied and Environmental Microbiology* 63:2022-2028 (1997); **14.37:** Thomas D. Brock; **14.41a:** Antje Boetius and Armin Gieseke, Max Planck Institute for Marine Microbiology, Bremen, Germany; **14.41c:** Viola Krukenberg, Katrin Knittel, and Gunter Wegener; **14.42:** Laura van Niftrik and Mingliang Wu; **14.52:** H. J. M. Harmsen.

Chapter 15 Chapter Opener: NASA Earth Observatory, Image by Joshua Stevens; **15.2a:** Susan Barns and Norman R. Pace, University of Colorado; **15.2b-e:** Daniel H. Buckley; **15.4:** M. R. Edwards; **15.5:** Daniel H. Buckley; **15.6:** Thomas D. Brock; **15.7a:** Rachel Foster; **15.7b, c:** Angel White; **15.8:** Daniel H. Buckley; **15.9a:** Thomas D. Brock; **15.9b, c:** Jörg Overmann, University of Munich, Germany; **15.10a-c:** Norbert Pfennig, University of Konstanz, Germany; **15.10d:** Johannes F. Imhoff, University of Kiel, Germany; **15.11a:** Charles C. Remsen, University of Wisconsin at Milwaukee; **15.11b:** Jeffrey C. Burnham and Samuel F. Conti; **15.12:** Norbert Pfennig, University of Konstanz, Germany; **15.13a-e:** Norbert Pfennig, University of Konstanz, Germany; **15.13f:** Peter Hirsch, University of Kiel, Germany; **15.14a, b:** Norbert Pfennig, University of Konstanz, Germany; **15.14c, d:** Courtesy of Alice C. Dohnalkova; **15.15:** Daniel H. Buckley; **15.16:** Daniel H. Buckley; **15.17a, d:** Douglas E. Caldwell, University of Saskatchewan; **15.17b, c:** Jörg Overmann, University of Munich, Germany; **15.18a:** Daniel H. Buckley; **15.18b:** Vladimir M. Gorlenko, Institute of Microbiology, Russian Academy of Sciences; **15.18c:** Charles A. Abella, University of Girona, Girona, Spain; **15.18d:** Deborah Jung; **15.20a:** F. Rudy Turner and Howard Gest, Indiana University; **15.20b, c:** Daniel H. Buckley; **15.21a:** Don Bryant; **15.21b:** Amaya Garcia Costas and Donald A. Bryant; **15.23:** J. H. Becking, Wageningen Agricultural University, Wageningen, Netherlands; **15.24:** Harold L. Sadoff, Michigan State University; **15.25:** Stanley W. Watson; **15.26a:** Holger Daims, Frank Maixner, Michael Wagner; **15.26b:** Holger Daims; **15.28a, b, f:** Norbert Pfennig, University of Konstanz, Germany; **15.28c-e:** Friedrich Widdel, Max Planck Institute for Marine Microbiology, Bremen, Germany; **15.28g:** Daniel H. Buckley; **15.29a:** Michael F. McGlannan, Florida International University; **15.29b:** Andreas Teske; **15.30a:** Jessup M. Shively, Clemson University; **15.30b:** Hans-Dietrich Babenzien, Institute of Freshwater Ecology and Inland Fisheries, Neuglobsow, Germany; **15.31a:** Verena Salman; **15.31b:** Michael F. McGlannan, Florida International University; **15.32a:** Michael Richard, Colorado State University; **15.32b:** Markus Huettel, Max Planck Institute for Marine Microbiology, Bremen, Germany; **15.33:** Verena Salman; **15.34a:** Daniel H. Buckley; **15.34b, d:** Tom Fenchel; **15.35:** Derek R. Lovley; **15.36:** William C. Ghiorse; **15.37:** D. W. Ribbons; **15.38:** Charles R. Fisher; **15.39:** Susan Koval and Ryan Chanyi; **15.40a:** Susan F. Koval, University of Western Ontario; **15.41:** Hans Reichenbach; **15.42:** Hans Reichenbach;

15.43: Patricia L. Grillione; **15.44:** David White, **inset:** Hans Reichenbach; **15.45:** E. Canale-Parola; **15.46a:** E. Canale-Parola; **15.47a:** Noel Krieg; **15.47b:** Stanley L. Erlandsen; **15.47c:** Harkisan D. Raj; **15.48:** Antionette Ryter; **15.49a:** B. J. Paster and E. Canale-Parola; **15.49b:** Susan Koval and George Chaconas; **15.51a:** Peter Hirsch; **15.51b:** S. F. Conti and P. Hirsch; **15.52a:** Einar Leifson; **15.52b, c:** Germaine Cohen-Bazire; **15.53a:** J. L. Pate; **15.53b:** James T. Staley; **15.53c:** Heinz Schlesner; **15.55:** William C. Ghiorse; **15.56a:** Thomas D. Brock; **15.56b, c:** J. F. M. Hoeniger; **15.57:** Richard Blakemore.

Chapter 16 Chapter Opener: J. Waters and R. Ley; **16.4:** Odile Berge; **16.5a:** Willy Burgdorfer; **16.5b:** G. Devauchelle; **16.6:** Michael T. Madigan; **16.8:** James A. Shapiro, University of Chicago; **16.9a**: CDC/PHIL; **16.9b:** Thomas D. Brock; **16.11:** Arthur Kelman, University of Wisconsin-Madison; **16.12:** Cheryl L. Broadie and John Vercillo; **16.13a:** Daniel E. Snyder; **16.13b:** James A. Shapiro, University of Chicago; **16.14:** Cheryl L. Broadie and John Vercillo, Southern Illinois University at Carbondale; **16.15:** Arthur Kelman, University of Wisconsin-Madison; **16.18a, b:** Otto Kandler; **16.18c:** V. Bottazi; **16.19a:** Bryan Larsen; **16.19b, c:** Thomas D. Brock; **16.20a:** Akiko Umeda, Kyushu University School of Medicine, Fukuoka, Japan; **16.20b:** Susan F. Koval, University of Western Ontario; **16.21:** Terry J. Beveridge, University of Guelph, Guelph, Ontario; **16.22:** Hans Hippe, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; **16.23:** James R. Norris; **16.24:** Dieter Claus, University of Gottingen, Germany; **16.25:** Alan Rodwell; **16.26:** Thomas D. Brock; **16.27:** David L. Williamson; **16.28:** Terry A. Krulwich, Mount Sinai School of Medicine; **16.29:** Terry A. Krulwich, Mount Sinai School of Medicine; **16.30:** Hans Veldkamp; **16.31:** CDC/PHIL; **16.32a:** N. Rist; **16.32b:** Victor Lorian; **16.32c:** CDC/PHIL; **16.33:** CDC/PHIL; **16.34:** Hubert and Mary P. Lechevalier; **16.35a:** Peter Hirsch, University of Kiel, Germany; **16.35b:** Hubert and Mary P. Lechevalier; **16.38a:** Michael T. Madigan; **16.38b:** David A. Hopwood, John Innes Centre, UK; **16.39a:** © Eli Lilly and Company. Used with permission; **16.39b:** David A. Hopwood, John Innes Centre, UK; **16.42:** Hans Reichenbach, Gesellschaft fur Biotechnologische Forschung mbH, Braunschweig, Germany; **16.44b:** Morris D. Cooper, Southern Illinois University School of Medicine; **16.45:** Robert R. Friis, Tiefenau Laboratory, Bern, Switzerland; **16.46:** John A. Fuerst, University of Queensland, Australia; **16.47:** Margarete Schüler and Harald Engelhardt; **16.48:** John Bauld, Australian Geological Survey Organisation; **16.49:** Heinz Schlesner, University of Kiel, Germany; **16.50:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **16.51a:** Friedrich Widdel, Max Planck Institute for Marine Microbiology, Bremen, Germany; **16.52a:** David M. Ward; **16.52b:** Daniel H. Buckley; **16.52c:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **16.53a, b:** Diane Moyles and R.G.E. Murray, University of Western Ontario; **16.53c:** Daniel H. Buckley; **16.54:** Courtesy of Svetlana N. Dedysh; **16.55:** Jessica Mark Welch and Gary Borisy.

Chapter 17 Chapter Opener: Marie Bulínová; **17.1:** Thomas D. Brock; **17.2a:** Thomas D. Brock; **17.2b:** NASA; **17.2c:** Daniel H. Buckley; **17.2d:** Francisco Rodriguez-Valera, Universidad Miguel Hernandez, San Juan de Alicante, Spain; **17.3:** Mary C. Reedy, Duke University Medical Center; **17.5:** Alexander Zehnder, Swiss Federal Institute for Environmental Science and Technology, Dubendorf, Switzerland; **17.6:** J. Gregory Zeikus and V.G. Bowen; **17.7a, c:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.7b:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.7d:** Stephen H. Zinder, Cornell University; **17.8a:** Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.9a:** Thomas D. Brock; **17.9b:** A. Segerer and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.10:** Thomas D. Brock; **17.12a:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.12b:** G. Fiala and Karl O. Stetter, University of Regensburg, Germany; **17.13a:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Germany; **17.13b:** Karl O. Stetter and Reinhard Rachel, University of Regensburg, Germany; **17.14:** Martin Könneke; **17.15:** Christa Schleper; **17.16:** Edward DeLong, Monterey Bay Aquarium Research Institute; **17.17:** Reinhard Rachel; **17.18:** Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.19a:** R. B. Pedersen, K. G. Jebsen Centre for Deep Sea Research, University of Bergen; **17.19b, c:** J. Beam and W. Inskeep; **17.20:** Thomas D. Brock; **17.21a:** Thomas D. Brock; **17.21b:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.22a:** Helmut Konig, University of Regensburg, Germany; **17.22b:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.22c:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.23a, b:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.23c:** Karl O. Stetter and Reinhard Rachel, University of Regensburg, Germany; **17.23d:** Kazem Kashefi; **17.24a:** Helmut Konig and Karl O. Stetter, University of Regensburg, Germany; **17.24b:** Reinhard Rachel and Karl O. Stetter, University of Regensburg, Germany; **17.25:** Helmut Konig and Karl O. Stetter, University of Regensburg, Regensburg, Germany; **17.26:** Anna-Louise Reysenbach and Woods Hole Oceanographic Institution; **17.28:** Gertraud Rieger, R. Hermann, Reinhard Rachel, and Karl O. Stetter, University of Regensburg, Germany; **17.29:** Suzette L. Pereira, Ohio State University.

Chapter 18 Chapter Opener: NASA's Earth Observatory, **inset:** Dr. Jeremy R. Young; **18.1:** Jian-ming Li, Nancy Martin/University of Louisville School of Medicine; **18.4a:** Michael Abbey/Science Source; **18.4b:** Steve J. Upton/Kansas State University; **18.5:** Blaine Mathison/CDC; **18.6a:** M. I. Walker/Science Source; **18.6b:** Biophoto Associates/Science Source; **18.7a:** Michael T. Madigan; **18.7b:** Sydney Tamm; **18.8:** Steve J. Upton/Kansas State University; **18.9:** Courtesy of Digital Microscopy Facility, Mount Allison University; **18.10a:** M.Arai/Getty Images; **18.10b:** Biophoto Associates/Science Source; **18.10c, d:** Joseph E. Kleinman/North Carolina State University Center for Applied Ecology; **18.11a:** Mae Melvin/CDC; **18.11b:** Silvia Botero Kleiven/The Swedish Institute for Infectious Disease Control; **18.12a:** Jörg Piper; **18.12b-d:** Courtesy of Digital Microscopy Facility, Mount Allison University; **18.13a:** Frank Mayer, University of Gottingen, Gottingen, Germany; **18.13b:** EpicStockMedia/Fotolia; **18.13c:** Michael Plewka; **18.14a:** Andrew Syred/Science Source; **18.14b:** Eye of Science/Science Source; **18.15:** Dr. Jeremy R. Young; **18.16:** M. Haberey; **18.17:** Stephen Sharnoff; **18.18:** Kenneth B. Raper; **18.20a:** Nano Creative/Science Source; **18.21a:** Cheryl L. Broadie/Southern Illinois University at Carbondale; **18.21b:** Michael T. Madigan; **18.22:** Michael T. Madigan; **18.23:** J. Forsdyke/SPL/Science Source; **18.25a:** London School of Hygiene & Tropical Medicine/Science Source; **18.25b:** Forrest Brem; **18.26a:** Premierlight Images/Alamy Stock Photo; **18.26b:** Alena Kubátová/Charles University in Prague; **18.27:** Thomas D. Brock; **18.29:** Samuel F. Conti and Thomas D. Brock; **18.31a:** Damian Palus/Shutterstock; **18.31b:** U.S. Department of Agriculture; **18.31c:** Ed Reschke/Photolibrary/Getty Images; **18.32:** Patrick J. Lynch/Science Source; **18.33a:** Christine Oesterhelt and Gerald Schonknecht; **18.33b:** Richard W. Castenholz/University of Oregon; **18.34a:** Arthur M. Nonomura; **18.34b:** Bob Gibbons/Alamy Stock Photo; **18.34c:** Thomas D. Brock; **18.34d:** Ralf Wagner; **18.34e:** Blickwinkel/NaturimBild/Alamy Stock Photo; **18.34f:** Dr. Aurora M. Nedelcu; **18.34g:** Arthur M. Nonomura; **18.35a:** Guillaume Dargaud; **18.35b:** Yuuji Tsukii/Hosei University, Japan.

Chapter 19 Chapter Opener: Steven Wilbert, Jessica Mark Welch, and Gary Borisy; **19.2b:** Norbert Pfennig, University of Konstanz, Germany; **19.3a:** James A. Shapiro, University of Chicago; **19.3b:** Marie Asao, Deborhah O. Jung, and Michael T. Madigan; **19.6a:** Adam Deutschbauer and Hans Carlson; **19.6b top:** Excellent backgrounds/Shutterstock, **bottom:** Steve Giovannoni; **19.7:** Rustem Ismagilov and Liang Ma; **19.8a, b:** Marc Mussman and Michael Wagner; **19.8c:** Willm Martens-Habbena; **19.9:** ThermoFisher Scientific; **19.10b, c:** Daniel Gage; **19.11:** Alex Toftgaard Nielsen; **19.12:** Norman R. Pace; **19.13a:** Michael Wagner and David A. Stahl; **19.13b:** Michael Wagner and Jiri Snaidr; **19.14:** Michael Wagner and Marc Mussman; **19.15c:** Rachel Spietz, George Schaible, Roland Hatzenpichler; **19.17ab:** Jennifer A. Fagg and Michael J. Ferris, Montana State University; **19.17c:** Gerard Muyzer and Abdelaziz Belila; **19.20a:** Jizhong (Joe) Zhou, Zhili He, and Joy Van Nostrand; **19.22:** Vaughn Iverson and Ginger Armbrust; **19.27:** Trent R. Northen, Mia Jones; **19.33:** Niels Peter Revsbech; **19.34a, b:** The figure data and components were generated by Dr. Klaus Koren (Department of Bioscience, University of Aarhus) and Dr. Michael Kühl (Department of Biology, University of Copenhagen) with technical assistance of Sofie Lindegaard Jakobsen (Department of Biology, University of Copenhagen); **19.34c:** Philippe Laissue; **19.38:** Colin Murrell; **19.39b, d:** Jennifer Pett-Ridge, Peter K. Weber; **19.39c:** Peter K. Weber; **19.40:** Eva Heinz and Matthias Horn; **19.41a:** Michael Wagner, Kilian Stöcker, and Holger Daims; **19.41b, c:** Michael Wagner, Per Nielsen, and Natuscka Lee.

Chapter 20 Chapter Opener: Belinda Ferrari; **20.1:** Hans Paerl, University of North Carolina at Chapel Hill; **20.4a:** Frank Dazzo; **20.4b:** Thomas D. Brock; **20.4c:** Cindy E. Morris, INRA, Centre de Recherche d'Avignon, France. Previously published in *Applied and Environmental Microbiology* 63:1570-1576; **20.4d:** C. T. Huang, Karen Xu, Gordon McFeters, and Philip S. Stewart; **20.5:** Courtesy of Marilyn B. Vogel and David J. Des Marais; **20.6:** Anna Depetris and Tom Ian Battin; **20.7:** The SEM picture was taken by Maria Arias-Andres andReingard Rossberg in the frame of the Leibniz SAW-project 'MikrOMIK' (SAW-2014-IOW-2); **20.8a:** Jesse Dillon and David A. Stahl; **20.8b:** David M. Ward; **20.9a:** J. M. Sanchez, J. Lidel Lope and Ricardo Amils; **20.9b:** W. Inskeep; **20.10:** Andreas Teske and Markus Huettel; **20.11b:** Michael T. Madigan; **20.13:** Jayne Belnap; **20.14:** Winai Tepsuttinun/Fotolia; **20.15a:** Terry C. Hazen; **20.15b:** Esta van Heerden; **20.17:** Luis R. Comolli; **20.19b:** Thomas D. Brock; **20.20:** Yuriy Brykaylo/Alamy Stock Photo; **20.21:** Otis Brown and Robert Evans, NASA; **20.23a:** US Coast Guard Photo/Alamy Stock Photo; **20.23b:** NASA; **20.24:** David L. Valentine; **20.25:** Sallie Chisholm, **inset:** Alexandra Z. Worden and Mya E. Breitbart, Scripps Institution of Oceanography, University of California at San Diego; **20.27a:** Hans W. Paerl, University of North Carolina at Chapel Hill; **20.27b:** Alexandra Z. Worden and Brian P. Palenik, Scripps Institution of Oceanography, University of California at San Diego; **20.28:** Vladimir Yurkov; **20.30:** Daniela Nicastro; **20.32:** Jed Fuhrman/Matt Sullivan, **inset:** Jennifer R. Brum/Matt Sullivan; **20.34:** Kathryn Kauffman, Nicki Watson, Martin Polz; **20.35:** Photographer: Nick Verola; **20.38:** Hideto Takami, Japan Marine Science and Technology Center, Kanagawa, Japan; **20.39a:** Doug Bartlett; **20.40a:** Andreas Teske; **20.42:** Sea Research Foundation (SRF) and the Ocean Exploration Trust (OET)/NOAA; **20.44:** Robert D. Ballard/Woods Hole Oceanographic Institution; **20.45:** Deborah Kelley, University of Washington; **20.46:** Christian Jeanthon; **20.47:** Carl Wirsen/Woods Hole Oceanographic Institution.

Chapter 21 Chapter Opener: Rebecca Albright; **21.3:** Evan Solomon; **21.5:** David L. Valentine; **21.8a:** John A. Breznak; **21.8b, c:** Monica Lee and Stephen H. Zinder; **21.13a:** Eye of Science/Science Source; **21.13b:** Lars Peter Nielsen; **21.17:** J. M. Sanchez, J. Lidel Lope and Ricardo Amils; **21.18a-g:** David Emerson; **21.19a:** Clara Chan and Deborah Powell, UD Biolmaging Center; **21.19b:** Shingo Kato, Clara Chan, and Deborah Powell, UD Biolmaging Center; **21.20a:** Jörg Bollmann; **21.20b:** M. L. Cros Miguel and J. M. Fortuño Alós; **21.21a:** Jörg Piper; **Explore the Microbial World Figure 1c:** David A. Stahl.

Chapter 22 Chapter Opener: Jason Flowers; **22.1:** Thomas D. Brock; **22.3:** Kodzo Gbewonyo; **22.4a:** Ravin Donald; **22.4b:** Thomas D. Brock; **22.5:** Thomas D. Brock; **22.6:** Kenneth Hurst Williams & Derek R. Lovley; **22.7a, b:** U.S. Environmental Protection Agency Headquarters; **22.7c:** Bassam Lahoud/Lebanese American University; **22.8:** Thomas D. Brock; **22.9:** Designelements/Fotolia; **22.14a:** Dr. Helmut Brandl/University of Zurich, Switzerland;

22.14d: Shutterstock/Mariyana Misaleva; **22.15a:** John M. Martinko; **22.15c:** Michael T. Madigan; **22.16:** Michael T. Madigan; **22.18:** Richard F. Unz/Penn State University Archives; **22.21b:** Royal Haskoning DHV, www.Nereda.net; **22.21c:** Mari Winkler; **22.22a:** Thomas D. Brock; **22.23a:** Louisville Water Company; **22.25:** Don Howard/Centers for Disease Control and Prevention; **22.26:** Norman R. Pace; **22.27c:** Shawna Johnston and Gerrit Voordouw.

Chapter 23 Chapter Opener: Shunichi Takahashi; **23.1a:** Pierre Philippe Laissue; **23.1b:** Thomas D. Brock; **23.1c:** Michael T. Madigan; **23.2a:** Pierre Philippe Laissue; **23.2b:** Thomas D. Brock; **23.4:** Michael T. Madigan; **23.5:** Michael T. Madigan; **23.6:** Viola Krukenberg, Katrin Knittel, and Gunter Wegener; **23.8:** Joe Burton; **23.9:** Ben B. Bohlool; **23.10:** Joe Burton; **23.12a:** M. D. Shakhawat Hossain; **23.12b, c:** Howard Berg, Donald Danforth Plant Science Center; **23.17:** B. Dreyfus; **23.18:** J. H. Becking/Wageningen Agricultural University, Wageningen, Netherlands; **23.20a:** Félix Fracchia; **23.20b-d:** Heike Bucking; **23.21:** Sergey Ivanov and Maria Harrison; **23.23:** S. A. Wilde, **inset:** D. J. Read/University of Sheffield, England; **23.24:** Jo Handelsman/University of Wisconsin at Madison; **23.27:** Amparo Latorre; **Explore the Microbial World Figure 1a, b:** Shee-Mei Lok and Guntur Fibriansah, **Figure 1c:** James Gathany/CDC; **23.28a:** blickwinkel/Teigler/Alamy Stock Photo; **23.29:** Michael Poulsen and Cameron Currie; **23.30:** Nancy Moran; **23.31c:** Michael Pettigrew/Shutterstock; **23.32:** Michael Pettigrew/Shutterstock; **23.33:** Kenneth H. Nealson, University of Wisconsin; **23.34:** Margaret McFall-Ngai; **23.35a, b, d:** Margaret McFall-Ngai; **23.36a:** Dudley Foster/Woods Hole Oceanographic Institution; **23.36b:** Carl Wirsen/Woods Hole Oceanographic Institution; **23.37:** Colleen M. Cavanaugh, Harvard University; **23.38a, b:** Heidi Goodrich-Blair, Mengyi Cao, Matthew Stilwell, and Elle Kielar Grevstad at the Biochemistry Optical Core, Biochemistry Dept. UW-Madison; **23.38c:** Sam Kyu-Kim and S. Patricia Stock; **23.39:** Kazuhiko Koike; **23.40:** Pierre Philippe Laissue; **23.41:** Michael T. Madigan; **23.44 sheep:** Gallas/Fotolia, **bird:** Bernard Swain, **horse:** Nancy L. Spear, **rabbit:** D Photo Sudbury/Shutterstock; **23.45b:** Sharisa D. Beck; **23.47:** Eric Isselee/Shutterstock.

Chapter 24 Chapter Opener: Dr. Andrey Shkoporov**; 24.4a:** Dwayne C. Savage and R. V. H. Blumershine; **24.4b:** Dwayne C. Savage; **24.8:** Ryan Hunter, Batbileg Bor, Xuesong He and Jeffrey McLean; **24.10:** Jessica Mark Welch and Gary Borisy; **24.13b:** John Durham/Science Source; **24.17:** Amina Bouslimani & Pieter Dorrestein; **24.18c:** Jeremy J. Barr; **24.26:** Tryggve Lie, School of Dentistry, University of Bergen; **24.29:** Deborah O. Jung and John Martinko.

Chapter 25 Chapter Opener: Joshua Jenkins; **25.2a:** USDA Agricultural Research Service; **25.3a:** Larry Stauffer/Oregon State Public Health Laboratory/CDC; **25.3b:** CDC/PHIL; **25.3c:** J. William Costerton/Montana State University; **25.4a:** M. Miller/CDC; **25.4b:** CDC/PHIL; **25.4c:** Richard Facklam/CDC; **25.5:** CDC/PHIL; **25.7a, b:** C. Lai, Max A. Listgarten, and B. Rosan; **25.7c:** Richard Facklam/CDC; **25.7d:** Isaac L. Schechmeister and John J. Bozzola/Southern Illinois University at Carbondale; **25.8:** Jessica Mark Welch and Gary Borisy; **25.9:** Janice Haney Carr/CDC; **25.13:** CDC/PHIL; **25.16:** Zang, R. G., Scott, D. L., Westbrok, M.L., Nance, S., Spangler, B.D., Shipley, G.G., Westbrook, E.M. Journal: (1995) *J.Mol. Biol.* 251: 563-573; **25.17a:** Thomas D. Brock; **25.17b:** Leon J. Le Beau/University of Illinois at Chicago; **25.18 left:** 2-methyl-2, 4-pentanediol induces spontaneous assembly of staphylococcal alpha-hemolysin into heptameric pore structure. Tanaka, Y. Hirano, N., Kaneko, J., Kamio, Y., Yao, M., Tanaka, I. (2011) *Protein Sci.* 20: 448-456, **right:** Janice Haney Carr/CDC; **25.19:** Janice Haney Carr/CDC; **25.20:** Arthur O. Tzianabos and R.D. Millham.

Chapter 26 Chapter Opener: Florian Ermini/Cortexyme; **26.3:** Dr. Deepak Kumar, Vijaya Kumar, and Dr. Robert Moir; **Table 26.2a:** John M. Martinko and Michael T. Madigan; **26.7b:** NIAID (CDC/PHIL); **Explore the Microbial World Figure 1:** U.S. National Oceanic and Atmospheric Administration; **26.10:** J. G. Hirsch; **26.12a:** Centers for Disease Control and Prevention; **26.12b:** James V. Little; **26.15b:** Michelle Dunstone and Bradley Spicer, Centre of Excellence for Advanced Moleular Imaging.

Chapter 27 Chapter Opener: NIAID/NIH; **27.5 left to right:** CDC/PHIL, US WPA/Library of Congress, D. Jordan, M. A./CDC, Edward J. Wozniak, D.V.M, PH.D/CDC; **27.8b:** Richard J. Feldmann, National Institutes of Health; **27.13d:** Don C. Wiley, Howard Hughes Medical Institute; **27.13e:** Aideen C. M. Young, Albert Einstein College of Medicine, Bronx, New York; **27.13f:** Jerry H. Brown; **27.16:** Boensch, B./Arco Images GmbH/Alamy Stock Photo; **27.22b:** Alex Ritter, Jennifer Lippincott Schwartz, and Gillian Griffiths, National Institutes of Health; **27.23b:** Lennart Nilsson, Albert Bonniers Forlag AB.

Chapter 28 Chapter Opener: Oliver Meckes/Science Source; **28.2:** Edward J. Wozniak/D.V.M/PH.D/CDC; **28.3:** CDC/PHIL; **28.4:** CDC/PHIL; **28.5:** CDC/PHIL; **28.7:** NIAID (CDC/PHIL); **28.8:** Amanda Mills/CDC; **28.15:** Biophoto Associates/Science Source; **28.16:** Dr. P. Marazzi/Science Source.

Chapter 29 Chapter Opener: National Institute of Allergy and Infectious Diseases (NIAID); **29.1:** CDC/PHIL; **29.2:** USAMRIID; **Table 29.3:** Janice Haney Carr/CDC, Peta Wardell/CDC, Erskine Palmer/CDC, A. Harrison/CDC, Frederick Murphy/CDC, Charles D. Humphrey/CDC; **Explore the Microbial World Figure 1a:** Alberto Lerner/CHROMagar, **Figure1b:** NIAID (CDC/PHIL); **29.6a:** Norman Jacobs/CDC; **29.6b:** CDC/PHIL; **29.7a, b:** Dr. Todd Parker/CDC; **29.7c:** John M. Martinko and Cheryl L. Broadie; **29.8:** Matthew Sattley; **29.9:** Matthew Sattley; **29.10:** Leon J. Le Beau; **29.11a:** Leon J. Le Beau; **29.11b, c:** Matthew Sattley; **29.11g:** Richard Facklam/CDC; **29.14:** C. Weibull, W. D. Bickel, W. T. Hashius, K. C. Milner, and E. Ribi; **29.15a:** Norman L. Morris; **29.16:** Matthew Sattley; **29.18a:** Wellcome Research Laboratories; **29.18b:** William B. Cherry/CDC; **29.19a:** P. M. Feorino/CDC; **29.19b:** William Kaplan/CDC; **29.19c:** Govinda S. Visvesvara/CDC; **29.21a:** Matthew Sattley; **29.22b:** Victor Tsang/CDC; **29.24b:** Matthew Sattley.

Chapter 30 Chapter Opener: Jeniel E. Nett; **30.5 left:** James Gathany/CDC, **right:** CDC/PHIL; **30.6 left:** Janice Haney Carr/CDC, **right:** NIAID (CDC/PHIL); **30.8 left:** CDC/PHIL, **right:** Jim Goodson/CDC; **30.12a:** James Gathany and CDC/Melissa Dankel; **30.12b:** Gilda Jones/CDC; **30.17:** CDC/PHIL; **30.18a, d:** CDC/PHIL; **30.18b:** Larry Stauffer, Oregon State Public Health Laboratory, and CDC/PHIL; **30.18c:** Archil Navdarashvili, Georgia (Republic)/CDC.

Chapter 31 Chapter Opener: Brinkmann, Max Planck Institute for Infection Biology; **31.1:** Thomas D. Brock; **31.3a:** Eye of Science/Science Source; **31.3b:** CDC/PHIL; **31.4:** Michael T. Madigan; **31.5:** CDC/PHIL; **31.6:** Franklin H. Top; **31.7:** Dr. Thomas F. Sellers/CDC; **31.8:** Michael T. Madigan; **31.9:** Franklin H. Top; **31.10:** Dr. M.A. Ansary/Science Source; **31.11:** Isaac L. Schechmeister; **31.12:** CDC/PHIL; **31.13a:** CDC/PHIL; **31.13b:** Franklin H. Top; **31.14:** J. H. Carr/CDC; **31.15a:** Dr. Edwin P. Ewing Jr./CDC; **31.15b:** CDC/PHIL; **31.16a:** R. W. Smithwick/CDC; **31.16b, c:** Aaron Friedman; **31.17a:** Jorge Adorno/Reuters; **31.17b:** CDC/PHIL; **31.18:** CDC/PHIL; **31.19a:** C. Goldsmith/CDC; **31.19b:** Centers for Disease Control and Prevention; **31.19c:** CDC/PHIL; **31.20:** CDC/PHIL; **31.21:** Centers for Disease Control and Prevention; **31.22:** A. D. Langmuir/CDC; **31.23a:** Biophoto Associates/Science Source; **31.23b:** David A. J. Tyrrell; **31.24:** E. L. Palmer, M. L. Martin, and F. Murphy/CDC; **31.25:** Irene T. Schulze; **31.28:** CDC/PHIL, M. J. Arduino and J. H. Carr/CDC; **31.29:** Gregory Moran/CDC; **31.30:** Michael T. Madigan; **31.31:** Juergen Berger/Science Source; **31.33a:** Eye of Science/Science Source; **31.33b:** CDC/PHIL; **31.34a:** Cynthia Goldsmith/CDC; **31.34b:** CDC/PHIL; **31.36a:** Joe Miller/CDC; **31.36b:** Morris D. Cooper; **31.37a:** H. Russell/CDC; **31.37b, c:** CDC/PHIL; **31.38a:** Centers for Disease Control and Prevention; **31.38b:** Sidney Olansky; **31.38c:** Robert Sumpter/CDC; **31.39:** Dr. Peter Perine/CDC; **31.40:** Morris D. Cooper; **31.41a:** Gordon A. Tuffli, MD; **31.41b:** K. L. Hermann/CDC; **31.42a:** N. J. Flumara and G. Hart/CDC; **31.42b:** Susan Lindsley/CDC; **31.46:** CDC/PHIL; **31.47a:** CDC/PHIL; **31.47b:** S. Krans/CDC **31.48a:** M. Metcalfe and T. Hodge/CDC.

Chapter 32 Chapter Opener: National Institute of Allergy and Infectious Diseases (NIAID); **32.2a:** Dr. Fred Murphy/CDC; **32.2b:** Mekonnen Fekadu/CDC; **32.3a:** Cynthia Goldsmith and Luanne Elliot/CDC; **32.3b:** Natalie Dolan/CDC; **32.4a:** James Gathany/CDC; **32.4b:** CDC/PHIL; **32.6a:** Willy Burgdorfer, M.D; **32.6b:** CDC/PHIL; **32.6c:** Willy Burgdorfer, MD; **32.6d:** Kenneth E. Greer/University of Virginia School of Medicine; **32.7a:** D. H. Walker/CDC; **32.7b:** National Institute of Allergy and Infectious Diseases (NIAID); **32.8a:** National Institute of Allergy and Infectious Diseases (NIAID); **32.8b:** Janice Haney Carr/CDC; **32.9a:** Pfizer Central Research; **32.9b:** James Gathany/CDC; **32.10:** Pfizer Central Research; **32.12a:** James Gathany/CDC; **32.12b:** CDC/PHIL; **32.12c:** Frederick Murphy/CDC; **32.13:** Cynthia Goldsmith/CDC; **32.14a:** W. Brogdon, J. Gathany/CDC; **32.14b:** Cynthia Goldsmith/CDC; **32.15:** CDC/PHIL; **32.17a:** Larry Stauffer/CDC; **32.17b:** T. Parker/CDC; **32.18a:** CDC/WHO; **32.18b:** Larry Staufer/CDC; **32.19a:** CDC/PHIL; **32.19b:** Christina Nelson, MD, MPH/CDC; **32.21a:** Larry Stauffer/CDC; **32.21b:** Janice Haney Carr/CDC; **32.22a:** Larry Stauffer/CDC; **32.22b-d:** CDC/PHIL; **32.23:** CDC/PHIL; **32.24:** CDC/PHIL; **32.24c:** Biophoto Associates/Science Source.

Chapter 33 Chapter Opener: Michael T. Madigan; **33.1a:** Thomas D. Brock; **33.1b:** U.S. Environmental Protection Agency Headquarters; **33.1c:** Jade ThaiCatwalk/Shutterstock; **33.2 clockwise from top:** Kimberley Seed, Tufts University School of Medicine, Centers for Disease Control and Prevention, James Cavallini/Science Source, Centers for Disease Control and Prevention, Mark L. Tamplin, Juergen Berger/Science Source; **33.3a, b:** CDC/PHIL; **33.3c:** Janice Haney Carr/CDC; **33.3d:** Jim Feeley/CDC; **33.4:** CDC/PHIL; **33.5:** Thomas D. Brock; **33.6:** John M. Martinko and Cheryl Broadie; **33.7:** Seward Laboratory Systems Inc.; **33.8a:** CDC/PHIL; **33.8b:** Janice Haney Carr/CDC; **33.9:** CDC/PHIL; **33.10a:** Eric Grafman/CDC; **33.10b, c:** James Gathany/CDC; **33.10d:** Michael T. Madigan; **33.11:** CDC/PHIL; **33.12a:** CDC/PHIL; **33.12b:** Janice Haney Carr/CDC; **33.12c, d:** CDC/PHIL; **33.13a, b:** CDC/PHIL; **33.13c:** Mediscan/Alamy Stock Photo; **33.14a:** Michael T. Madigan; **33.14b:** Elizabeth White/CDC; **33.15b:** Dr. Pascale Cossart, Institut Pasteur, Paris; **33.16:** CDC/PHIL; **33.17:** CDC/PHIL.

Chapter 34 Chapter Opener: Dr. Visvesvara/CDC; **34.1a:** L. Haley/CDC; **34.1b:** A. A. Padhye/CDC; **34.1c:** M. Jalbert/L. Kaufman/CDC; **34.1d, e:** L. Ajello/CDC; **34.1f:** L. K. Georg/CDC; **34.2a:** CDC/PHIL; **34.3a:** Gordon C. Sauer, MD; **34.3b, c:** CDC/PHIL; **34.4a:** Gordon C. Sauer, MD; **34.4b:** L. K. Georg/CDC; **34.5a:** M. Hicklin/CDC; **34.5b:** L. K. Georg/CDC; **34.5c:** E. P. Ewing, Jr./CDC; **34.5d:** M. Hicklin/CDC; **34.5e:** M. Castro/L.K. Georg/CDC; **34.5f:** CDC/PHIL; **34.6:** Katherine Ralston and David Zemo; **34.7a:** M. Melvin/CDC; **34.7b:** L. L. A. Moore, Jr./CDC; **34.8a:** G.S. Visvesvara/CDC; **34.8b:** Stanley L. Erlandsen; **34.8c:** Dennis E. Feely; **34.9a:** Steve J. Upton; **34.9b:** CDC/PHIL; **34.10:** CDC/PHIL; **34.11:** Edwin P. Ewing/CDC; **34.12:** Jim Gathany/CDC; **34.13a:** Steven Glenn/CDC; **34.13b:** M. Melvin/CDC; **34.14a:** F. Collins/J. Gathany/CDC; **34.14b:** M. Melvin/CDC; **34.14c:** D. S. Martin/CDC; **34.15a:** Scott Camazine/Alamy Stock Photo; **34.15b:** Myron G. Schultz/CDC; **34.15c:** WHO (CDC/PHIL); **34.15d:** M. Melvin/CDC; **34.16a:** S. Maddison/CDC; **34.16b:** CDC/PHIL; **34.16c:** A. J. Sulzer/CDC; **34.16d:** CDC/PHIL; **34.17:** CDC/PHIL; **34.18:** CDC/PHIL.

Glossary Terms

NOTE: Page numbers indicate where the term is defined in a Chapter Glossary. A few entries with more than one page number are used in more than one sense. Comprehensive glossary available at Mastering Microbiology®.

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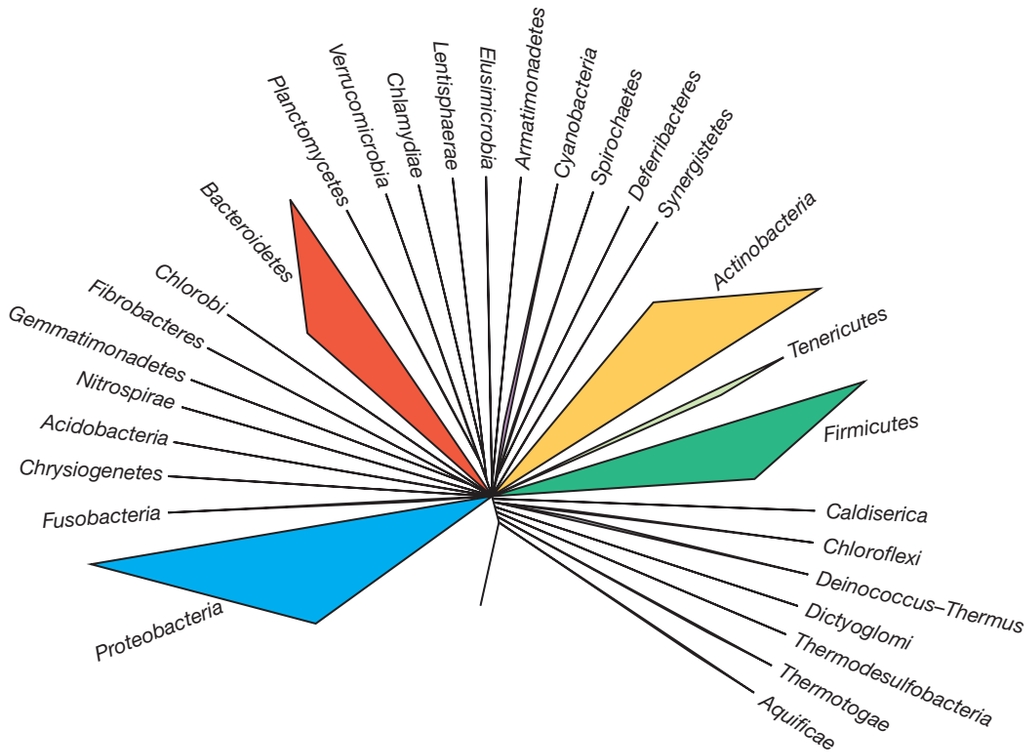
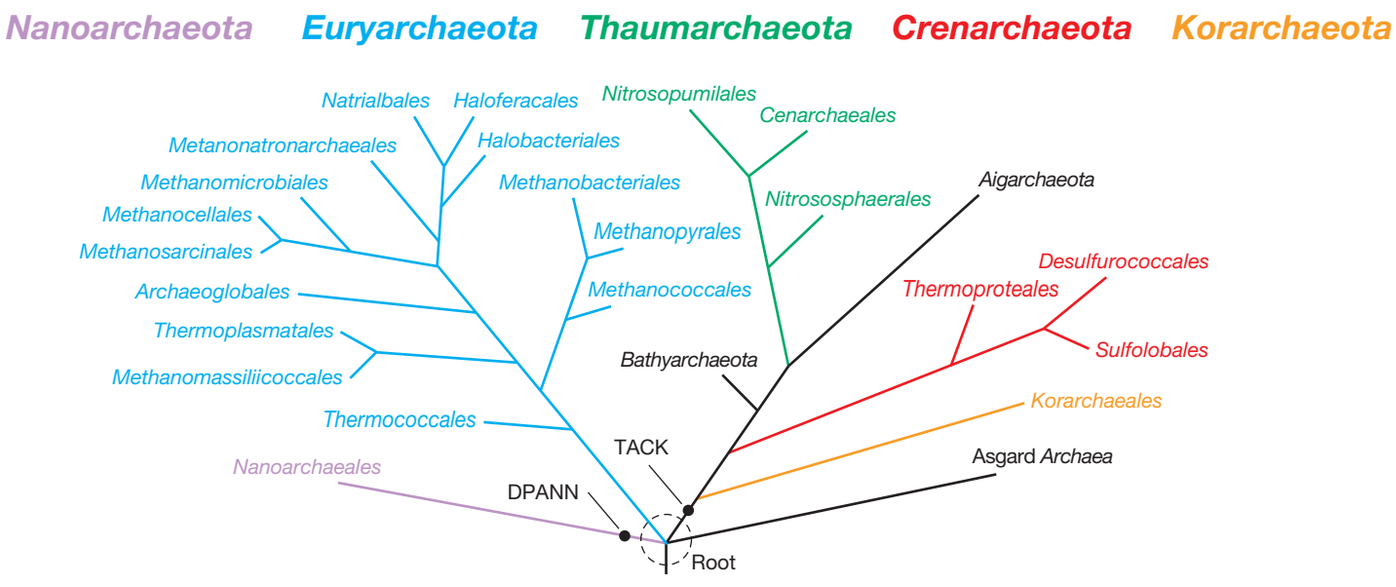
Phylogeny of *Bacteria*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