second edition

Prescott's Principles of Microbiology

Joanne M. Willey
HOFSTRA UNIVERSITY

Kathleen M. Sandman

PRESCOTT'S PRINCIPLES OF MICROBIOLOGY, SECOND EDITION

This book is printed on acid-free paper.

1 2 3 4 5 6 7 8 9 LWI 24 23 22 21 20 19

ISBN 978-1-260-25903-2 (bound edition)
MHID 1-260-25903-X (bound edition)
ISBN 978-1-260-80563-5 (loose-leaf edition)
MHID 1-260-80563-8 (loose-leaf edition)

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Cover Image: *Kateryna Kon/Shutterstock*
Compositor: *MPS Limited*

Library of Congress Cataloging-in-Publication Data

Willey, Joanne M., author. | Sandman, Kathleen M., author. |
Prescott's principles of microbiology / Joanne M. Willey (Hofstra University), Kathleen M. Sandman
Principles of microbiology
Second edition. | New York, NY : McGraw-Hill Education, [2021] |
Includes index.
LCCN 2019020105 | ISBN 9781260259032 (student edition : alk. paper)
LCSH: Microbiology—Textbooks.
LCC QR41.2 .W545 2021 | DDC 579—dc23
LC record available at https://lccn.loc.gov/2019020105

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Brief Contents

About the Authors

Courtesy of Jeffrey Shelkey

Joanne M. Willey has been a professor at Hofstra University on Long Island, New York, since 1993, where she is the Leo A. Guthart Professor of Biomedical Science and Chair of the Department of Science Education at the Donald and Barbara Zucker School of Medicine at Hofstra/Northwell. Dr. Willey received her B.A. in Biology from the University of Pennsylvania, where her interest in microbiology began with work on cyanobacterial growth in eutrophic streams. She earned her Ph.D. in biological oceanography (specializing in marine microbiology) from the Massachusetts Institute of Technology–Woods Hole Oceanographic Institution Joint Program in 1987. She then went to Harvard University, where she spent her postdoctoral fellowship studying the filamentous soil bacterium *Streptomyces coelicolor.* Dr. Willey has coauthored a number of publications that focus on its complex developmental cycle. She is an active member of the American Society for Microbiology (ASM), and served on the editorial board of the journal *Applied and Environmental Microbiology* for nine years and as Chair of the Division of General Microbiology. Dr. Willey taught microbiology to biology majors for 20 years and now teaches microbiology and infectious disease to medical students. She has taught courses in cell biology, marine microbiology, and laboratory techniques in molecular genetics. Dr. Willey lives on the north shore of Long Island and has two grown sons. She is an avid runner and enjoys skiing, hiking, sailing, and reading. She can be reached at joanne.m.willey@hofstra.edu.

Courtesy of Adele Anderson

Kathleen M. Sandman received her B.A. in Biology from La Salle University and her Ph.D. in Cellular and Developmental Biology from Harvard University. She was inspired to a career in science by her older brother's experience as an organic chemist and by the developing technology in recombinant DNA in the 1970s. Her graduate work used a transposable element as a mutagen in *Bacillus subtilis* to study gene expression during endospore formation. She continued in the genetics of Gram-positive bacteria with a postdoctoral year studying *Bacillus thuringiensis* at the University of Cambridge in the United Kingdom. Another postdoctoral opportunity at The Ohio State University provided an introduction to the emerging field of archaeal molecular biology, where Dr. Sandman discovered archaeal histones and continued research in the structural biology of archaeal chromatin for about 20 years. She served the National Science Foundation as a research grant reviewer and panelist for the Life in Extreme Environments program, and has organized conference sessions on archaeal molecular biology and proteins from extremophiles. Dr. Sandman has taught microbiology to hundreds of students, at both the introductory level and in an advanced molecular microbiology laboratory. Dr. Sandman has worked as a consultant in a variety of industries, including industrial microbiology, environmental geomicrobiology, and technical publishing. She lives with her husband in Columbus, Ohio, and has two grown daughters. She enjoys biking, fabric arts, reading, and genealogy, and can be reached at kathleenmsandman@gmail.com.

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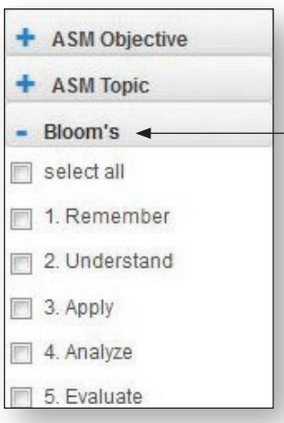

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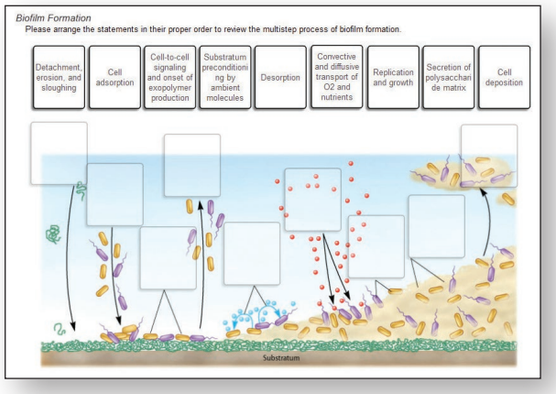

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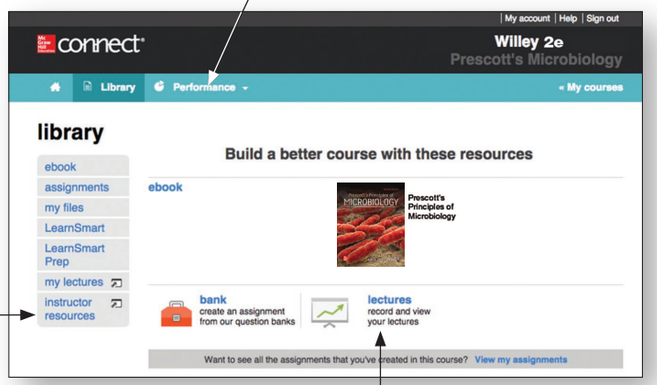

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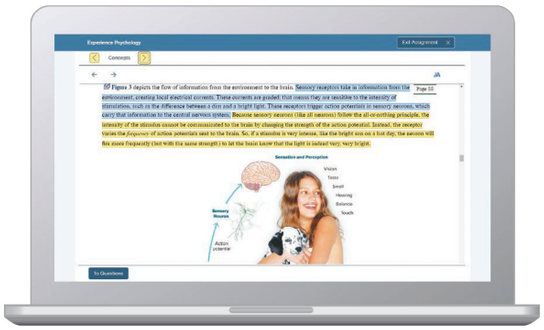

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A Modern Approach to Microbiology

Evolution as a Framework

Introduced immediately in chapter 1 and used as an overarching theme throughout, evolution helps unite microbiological concepts and provides a framework upon which students can build their knowledge.

An Introduction to Microbial Structure and Growth

Chapters 3 and 4 introduce the structure and function of bacteria, archaea, and eukaryotic cells. Chapter 5 introduces students to the dynamics of microbial growth. This provides the foundation for the chapters that follow on metabolism (6–8) and molecular biology and genetics (9–12).

Integration of Microbial Diversity and Pathogenesis

Microbial diversity is presented in the context of metabolic adaptations that enable students to relate the physiological adaptations that make an organism successful in its environment, whether that is the natural environment or the human body.

Broad Coverage of Microbial Ecology

The importance and multidisciplinary nature of microbial ecology are demonstrated by content that ranges from global climate change to the human microbiome.

Brief Contents

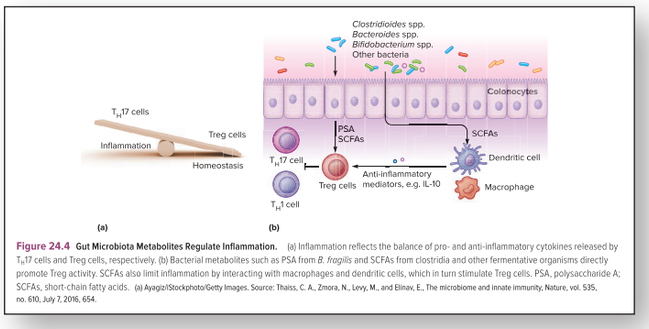
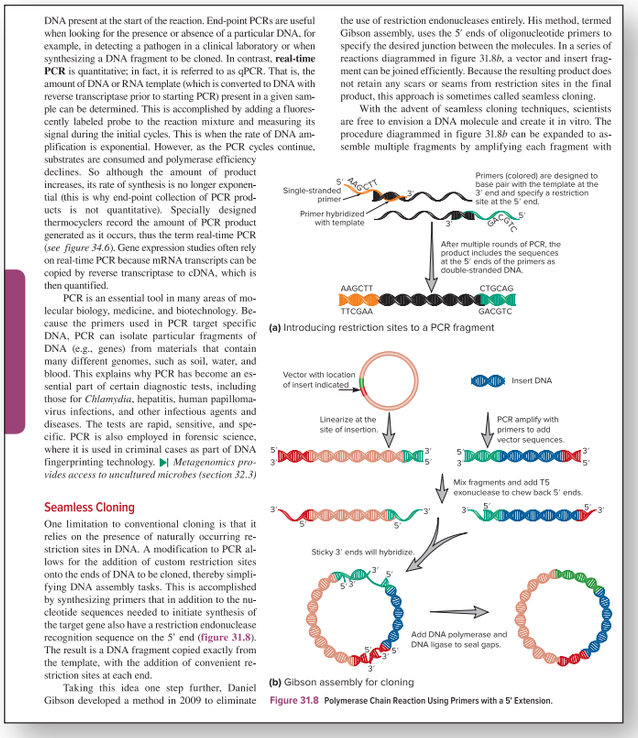


Figure 24.4 Gut Microbiota Metabolites Regulate Inflammation. (a) Inflammation reflects the balance of pro- and anti-inflammatory cytokines released by T_H17 cells and Treg cells, respectively. (b) Bacterial metabolites such as PSA from *B. fragilis* and SCFAs from clostridia and other fermentative organisms directly promote Treg activity. SCFAs also limit inflammation by interacting with macrophages and dendritic cells, which in turn stimulate Treg cells. PSA, polysaccharide A; SCFAs, short-chain fatty acids. (a) Ayagizi/Stockphoto/Getty Images. Source: Thaiss, C. A., Zmora, N., Levy, M., and Elinav, E., The microbiome and innate immunity, Nature, vol. 535, no. 610, July 7, 2016, 654.

Molecular Microbiology and Immunology

The second edition includes comprehensive coverage of genetics, biotechnology, genomics and metagenomics, immunology, and the human microbiome. A streamlined discussion of immunity, with enhanced coverage of innate and adaptive linkages, helps students grasp the complexity and specificity of immune responses. A new chapter, The Human Microbiome and Host Interactions, introduces students to the development and impact of the human microbiome.

A Modern Approach to Microbiology



DNA present at the start of the reaction. End-point PCRs are useful when looking for the presence or absence of a particular DNA, for example, in detecting a pathogen in a clinical laboratory or when synthesizing a DNA fragment to be cloned. In contrast, **real-time PCR** is quantitative; in fact, it is referred to as qPCR. That is, the amount of DNA or RNA template (which is converted to DNA with reverse transcriptase prior to starting PCR) present in a given sample can be determined. This is accomplished by adding a fluorescently labeled probe to the reaction mixture and measuring its signal during the initial cycles. This is when the rate of DNA amplification is exponential. However, as the PCR cycles continue, substrates are consumed and polymerase efficiency declines. So although the amount of product increases, its rate of synthesis is no longer exponential (this is why end-point collection of PCR products is not quantitative). Specially designed thermocyclers record the amount of PCR product generated as it occurs, thus the term real-time PCR (*see figure 34.6*). Gene expression studies often rely on real-time PCR because mRNA transcripts can be copied by reverse transcriptase to cDNA, which is then quantified.

PCR is an essential tool in many areas of molecular biology, medicine, and biotechnology. Because the primers used in PCR target specific DNA, PCR can isolate particular fragments of DNA (e.g., genes) from materials that contain many different genomes, such as soil, water, and blood. This explains why PCR has become an essential part of certain diagnostic tests, including those for *Chlamydia*, hepatitis, human papillomavirus infections, and other infectious agents and diseases. The tests are rapid, sensitive, and specific. PCR is also employed in forensic science, where it is used in criminal cases as part of DNA fingerprinting technology. ▶| *Metagenomics provides access to uncultured microbes (section 32.3)*

Seamless Cloning

One limitation to conventional cloning is that it relies on the presence of naturally occurring restriction sites in DNA. A modification to PCR allows for the addition of custom restriction sites onto the ends of DNA to be cloned, thereby simplifying DNA assembly tasks. This is accomplished by synthesizing primers that in addition to the nucleotide sequences needed to initiate synthesis of the target gene also have a restriction endonuclease recognition sequence on the 5′ end (**figure 31.8**). The result is a DNA fragment copied exactly from the template, with the addition of convenient restriction sites at each end.

Taking this idea one step further, Daniel Gibson developed a method in 2009 to eliminate the use of restriction endonucleases entirely. His method, termed Gibson assembly, uses the 5′ ends of oligonucleotide primers to specify the desired junction between the molecules. In a series of reactions diagrammed in figure 31.8*b*, a vector and insert fragment can be joined efficiently. Because the resulting product does not retain any scars or seams from restriction sites in the final product, this approach is sometimes called seamless cloning.

With the advent of seamless cloning techniques, scientists are free to envision a DNA molecule and create it in vitro. The procedure diagrammed in figure 31.8*b* can be expanded to assemble multiple fragments by amplifying each fragment with

Figure 31.8 Polymerase Chain Reaction Using Primers with a 5′ Extension.

21st-Century Microbiology

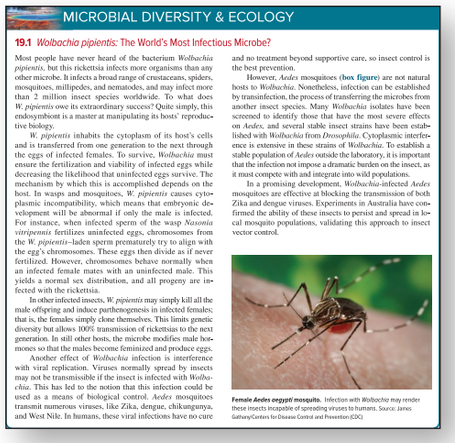
Prescott's Principles of Microbiology leads the way with text devoted to genome editing, global climate change and microbial fuel cells. For more, see chapters 20, 30, and 31.

Metagenomics and the Human Microbiome

The importance of metagenomics in understanding the role of microbes in all environments and in exploring symbionts of invertebrates is threaded throughout the text. Chapter 24 is new and focuses exclusively on the human microbiome and its interaction with the host.

Special Interest Essays

Organized into four themes—Microbial Diversity & Ecology, Techniques & Applications, Historical Highlights, and Disease—these focused and interesting essays provide additional insight into relevant topics.



MICROBIAL DIVERSITY & ECOLOGY

19.1 *Wolbachia pipientis:* The World's Most Infectious Microbe?

Most people have never heard of the bacterium *Wolbachia pipientis*, but this rickettsia infects more organisms than any other microbe. It infects a broad range of crustaceans, spiders, mosquitoes, millipedes, and nematodes, and may infect more than 2 million insect species worldwide. To what does *W. pipientis* owe its extraordinary success? Quite simply, this endosymbiont is a master at manipulating its hosts' reproductive biology.

W. pipientis inhabits the cytoplasm of its host's cells and is transferred from one generation to the next through the eggs of infected females. To survive, *Wolbachia* must ensure the fertilization and viability of infected eggs while decreasing the likelihood that uninfected eggs survive. The mechanism by which this is accomplished depends on the host. In wasps and mosquitoes, *W. pipientis* causes cytoplasmic incompatibility, which means that embryonic development will be abnormal if only the male is infected. For instance, when infected sperm of the wasp *Nasonia vitripennis* fertilizes uninfected eggs, chromosomes from the *W. pipientis*–laden sperm prematurely try to align with the egg's chromosomes. These eggs then divide as if never fertilized. However, chromosomes behave normally when an infected female mates with an uninfected male. This yields a normal sex distribution, and all progeny are infected with the rickettsia.

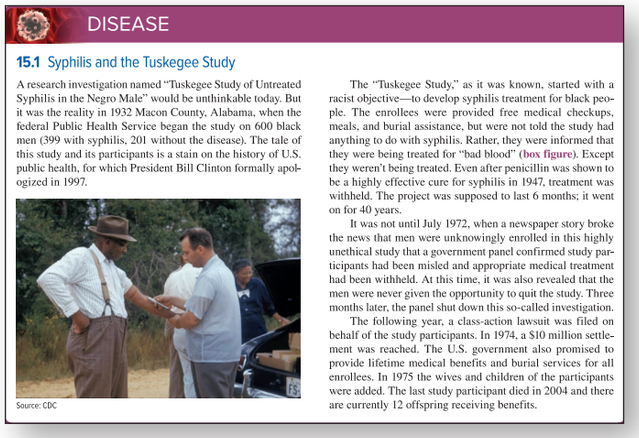
In other infected insects, *W. pipientis* may simply kill all the male offspring and induce parthenogenesis in infected females; that is, the females simply clone themselves. This limits genetic diversity but allows 100% transmission of rickettsias to the next generation. In still other hosts, the microbe modifies male hormones so that the males become feminized and produce eggs.

Another effect of *Wolbachia* infection is interference with viral replication. Viruses normally spread by insects may not be transmissible if the insect is infected with *Wolbachia*. This has led to the notion that this infection could be used as a means of biological control. *Aedes* mosquitoes transmit numerous viruses, like Zika, dengue, chikungunya, and West Nile. In humans, these viral infections have no cure and no treatment beyond supportive care, so insect control is the best prevention.

However, *Aedes* mosquitoes (**box figure**) are not natural hosts to *Wolbachia*. Nonetheless, infection can be established by transinfection, the process of transferring the microbes from another insect species. Many *Wolbachia* isolates have been screened to identify those that have the most severe effects on *Aedes*, and several stable insect strains have been established with *Wolbachia* from *Drosophila*. Cytoplasmic interference is extensive in these strains of *Wolbachia*. To establish a stable population of *Aedes* outside the laboratory, it is important that the infection not impose a dramatic burden on the insect, as it must compete with and integrate into wild populations.

In a promising development, *Wolbachia*-infected *Aedes* mosquitoes are effective at blocking the transmission of both Zika and dengue viruses. Experiments in Australia have confirmed the ability of these insects to persist and spread in local mosquito populations, validating this approach to insect vector control.

Female *Aedes aegypti* mosquito. Infection with *Wolbachia* may render these insects incapable of spreading viruses to humans. Source: James Gathany/Centers for Disease Control and Prevention (CDC)



DISEASE

15.1 Syphilis and the Tuskegee Study

A research investigation named "Tuskegee Study of Untreated Syphilis in the Negro Male" would be unthinkable today. But it was the reality in 1932 Macon County, Alabama, when the federal Public Health Service began the study on 600 black men (399 with syphilis, 201 without the disease). The tale of this study and its participants is a stain on the history of U.S. public health, for which President Bill Clinton formally apologized in 1997.

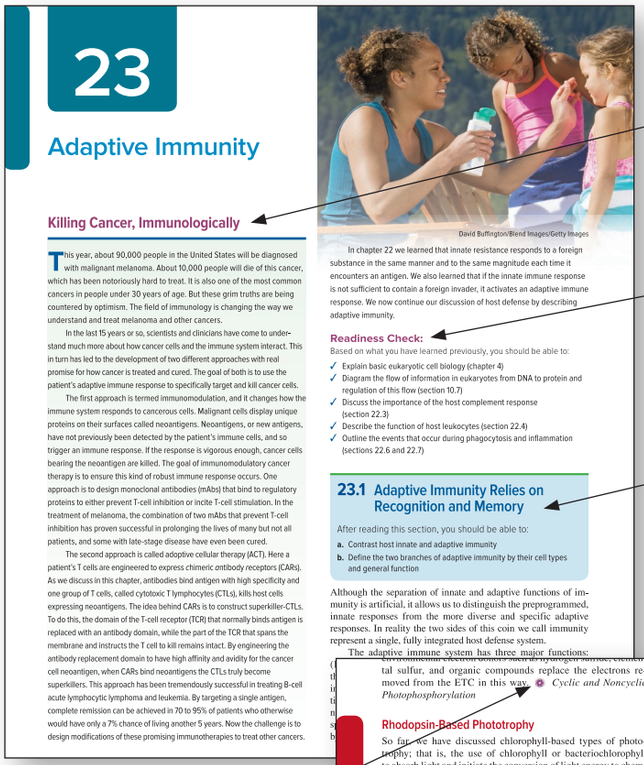
Source: CDC

The "Tuskegee Study," as it was known, started with a racist objective—to develop syphilis treatment for black people. The enrollees were provided free medical checkups, meals, and burial assistance, but were not told the study had anything to do with syphilis. Rather, they were informed that they were being treated for "bad blood" (**box figure**). Except they weren't being treated. Even after penicillin was shown to be a highly effective cure for syphilis in 1947, treatment was withheld. The project was supposed to last 6 months; it went on for 40 years.

It was not until July 1972, when a newspaper story broke the news that men were unknowingly enrolled in this highly unethical study that a government panel confirmed study participants had been misled and appropriate medical treatment had been withheld. At this time, it was also revealed that the men were never given the opportunity to quit the study. Three months later, the panel shut down this so-called investigation.

The following year, a class-action lawsuit was filed on behalf of the study participants. In 1974, a $10 million settlement was reached. The U.S. government also promised to provide lifetime medical benefits and burial services for all enrollees. In 1975 the wives and children of the participants were added. The last study participant died in 2004 and there are currently 12 offspring receiving benefits.

Student-Friendly Organization



23

Adaptive Immunity

Killing Cancer, Immunologically

This year, about 90,000 people in the United States will be diagnosed with malignant melanoma. About 10,000 people will die of this cancer, which has been notoriously hard to treat. It is also one of the most common cancers in people under 30 years of age. But these grim truths are being countered by optimism. The field of immunology is changing the way we understand and treat melanoma and other cancers.

In the last 15 years or so, scientists and clinicians have come to understand much more about how cancer cells and the immune system interact. This in turn has led to the development of two different approaches with real promise for how cancer is treated and cured. The goal of both is to use the patient's adaptive immune response to specifically target and kill cancer cells.

The first approach is termed immunomodulation, and it changes how the immune system responds to cancerous cells. Malignant cells display unique proteins on their surfaces called neoantigens. Neoantigens, or new antigens, have not previously been detected by the patient's immune cells, and so trigger an immune response. If the response is vigorous enough, cancer cells bearing the neoantigen are killed. The goal of immunomodulatory cancer therapy is to ensure this kind of robust immune response occurs. One approach is to design monoclonal antibodies (mAbs) that bind to regulatory proteins to either prevent T-cell inhibition or incite T-cell stimulation. In the treatment of melanoma, the combination of two mAbs that prevent T-cell inhibition has proven successful in prolonging the lives of many but not all patients, and some with late-stage disease have even been cured.

The second approach is called adoptive cellular therapy (ACT). Here a patient's T cells are engineered to express chimeric antibody receptors (CARs). As we discuss in this chapter, antibodies bind antigen with high specificity and one group of T cells, called cytotoxic T lymphocytes (CTLs), kills host cells expressing neoantigens. The idea behind CARs is to construct superkiller-CTLs. To do this, the domain of the T-cell receptor (TCR) that normally binds antigen is replaced with an antibody domain, while the part of the TCR that spans the membrane and instructs the T cell to kill remains intact. By engineering the antibody replacement domain to have high affinity and avidity for the cancer cell neoantigen, when CARs bind neoantigens the CTLs truly become superkillers. This approach has been tremendously successful in treating B-cell acute lymphocytic lymphoma and leukemia. By targeting a single antigen, complete remission can be achieved in 70 to 95% of patients who otherwise would have only a 7% chance of living another 5 years. Now the challenge is to design modifications of these promising immunotherapies to treat other cancers.

David Buffington/Blend Images/Getty Images

In chapter 22 we learned that innate resistance responds to a foreign substance in the same manner and to the same magnitude each time it encounters an antigen. We also learned that if the innate immune response is not sufficient to contain a foreign invader, it activates an adaptive immune response. We now continue our discussion of host defense by describing adaptive immunity.

Readiness Check:

Based on what you have learned previously, you should be able to:

- ✓ Explain basic eukaryotic cell biology (chapter 4)
- ✓ Diagram the flow of information in eukaryotes from DNA to protein and regulation of this flow (section 10.7)
- ✓ Discuss the importance of the host complement response (section 22.3)
- ✓ Describe the function of host leukocytes (section 22.4)
- ✓ Outline the events that occur during phagocytosis and inflammation (sections 22.6 and 22.7)

23.1 Adaptive Immunity Relies on Recognition and Memory

After reading this section, you should be able to:

a. Contrast host innate and adaptive immunity
b. Define the two branches of adaptive immunity by their cell types and general function

Although the separation of innate and adaptive functions of immunity is artificial, it allows us to distinguish the preprogrammed, innate responses from the more diverse and specific adaptive responses. In reality the two sides of this coin we call immunity represent a single, fully integrated host defense system.

The adaptive immune system has three major functions:

tal sulfur, and organic compounds replace the electrons removed from the ETC in this way. *Cyclic and Noncyclic Photophosphorylation*

Rhodopsin-Based Phototrophy

So far, we have discussed chlorophyll-based types of phototrophy; that is, the use of chlorophyll or bacteriochlorophyll to absorb light and initiate the conversion of light energy to chemical energy. Within the last decade, it has become apparent that many bacteria and archaea are capable of chlorophyll-independent phototrophy. These microbes rely on microbial rhodopsin, a molecule similar to that found in the eyes of many multicellular organisms. The first microbial rhodopsin was discovered in certain archaea. It was first described so long ago that it was called bacteriorhodopsin; it is now more correctly called **archaerhodopsin.** Since that time, a variety of rhodopsins have been found in members of Proteobacteria (and thus called proteorhodospin), Flavobacteria, extremely halophilic bacteria (xanthorhodopsin), and some eukaryotes. The archaerhodopsin of the halophilic archaeon *Halobacterium salinarum* remains the best studied and is the focus of our discussion here. ▶| *Haloarchaea (section 17.3)*

H. salinarum normally depends on aerobic respiration for the release of energy from its organic energy source. It cannot grow anaerobically by anaerobic respiration or fermentation. However, under conditions of low oxygen and high light intensity, it synthesizes archaerhodopsin, a deep-purple pigment *(see figure 16.15).* Archaerhodopsin's color comes from retinal, a type of carotenoid. Retinal is covalently attached to a protein embedded in the plasma membrane such that retinal is in the center of the membrane.

Archaerhodopsin functions as a light-driven proton

come temporarily anoxic, the archaeon uses light energy to synthesize sufficient ATP to survive until oxygen levels rise again.

The discovery that microbial rhodopsins are common in marine ecosystems blurs the line between metabolic strategies. To date, all rhodopsin-containing microbes are chemoorganoheterotrophs, using organic carbon as their source of carbon, electrons, and energy. Note that rhodopsin-based proton pumping generates a PMF without having to oxidize NADH via an ETC. What does this mean for these microorganisms? Many of these microbes live in nutrient-depleted environments where organic carbon is limited. With each organic molecule the organism takes up, it must "decide" to either oxidize it to make NADH and ATP or reduce it at the expense of NADPH and ATP to make more macromolecules for growth. The use of rhodopsin-based proton pumping enables the synthesis of ATP without having to catabolize as much precious organic carbon. That is, by performing rhodopsin-mediated phototrophy, these chemoorganoheterotrophs can shunt more organic carbon to growth rather than oxidizing it for energy. ▶| *Microorganisms in the open ocean are adapted to nutrient limitation (section 21.2)*

Comprehension Check

1. Define the following terms: *light reactions, dark reactions, chlorophyll, carotenoid, phycobiliprotein, antenna,* and *photosystems I* and *II.*
2. What happens to a reaction center chlorophyll pair, such as P700, when it absorbs light?
3. What is the function of accessory pigments?
4. What is photophosphorylation? What is the difference between cyclic and noncyclic photophosphorylation?

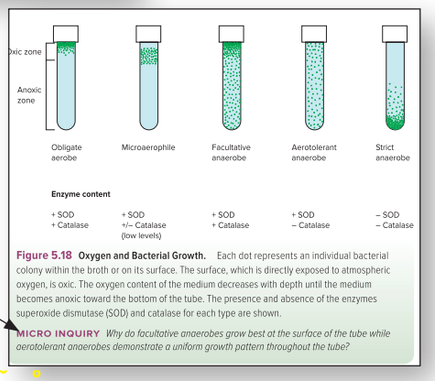


Figure 5.18 **Oxygen and Bacterial Growth.** Each dot represents an individual bacterial colony within the broth or on its surface. The surface, which is directly exposed to atmospheric oxygen, is oxic. The oxygen content of the medium decreases with depth until the medium becomes anoxic toward the bottom of the tube. The presence and absence of the enzymes superoxide dismutase (SOD) and catalase for each type are shown.

MICRO INQUIRY *Why do facultative anaerobes grow best at the surface of the tube while aerotolerant anaerobes demonstrate a uniform growth pattern throughout the tube?*

Micro Focus—Each chapter begins with a real-life story illustrating the relevance of content covered in the upcoming text.

Readiness Check—The introduction to each chapter includes a skills checklist that defines the prior knowledge students need to understand the material that follows.

Learning Outcomes—Every section in each chapter begins with a list of content-based activities students should be able to perform after reading.

Comprehension Check—Questions within the narrative of each chapter help students master section concepts before moving on to other topics.

Animation Icon—This symbol indicates that material presented in the text is accompanied by an animation within Instructor Resources in Connect. Create a file attachment assignment in Connect to have your students view the animation, or post it to your Learning Management System for students.

Cross-Referenced Notes—In-text references refer students to other parts of the book to review.

Micro Inquiry—Selected figures in every chapter contain probing questions, adding another assessment opportunity for students.

Student-Friendly Organization

Vivid Instructional Art—Three-dimensional renditions and bright, attractive colors enhance learning.

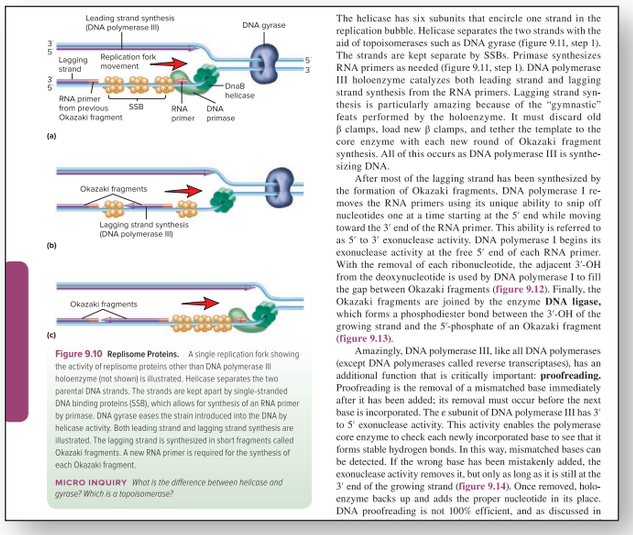


Figure 9.10 Replisome Proteins. A single replication fork showing the activity of replisome proteins other than DNA polymerase III holoenzyme (not shown) is illustrated. Helicase separates the two parental DNA strands. The strands are kept apart by single-stranded DNA binding proteins (SSB), which allows for synthesis of an RNA primer by primase. DNA gyrase eases the strain introduced into the DNA by helicase activity. Both leading strand and lagging strand synthesis are illustrated. The lagging strand is synthesized in short fragments called Okazaki fragments. A new RNA primer is required for the synthesis of each Okazaki fragment.

MICRO INQUIRY *What is the difference between helicase and gyrase? Which is a topoisomerase?*

The helicase has six subunits that encircle one strand in the replication bubble. Helicase separates the two strands with the aid of topoisomerases such as DNA gyrase (figure 9.11, step 1). The strands are kept separate by SSBs. Primase synthesizes RNA primers as needed (figure 9.11, step 1). DNA polymerase III holoenzyme catalyzes both leading strand and lagging strand synthesis from the RNA primers. Lagging strand synthesis is particularly amazing because of the "gymnastic" feats performed by the holoenzyme. It must discard old β clamps, load new β clamps, and tether the template to the core enzyme with each new round of Okazaki fragment synthesis. All of this occurs as DNA polymerase III is synthesizing DNA.

After most of the lagging strand has been synthesized by the formation of Okazaki fragments, DNA polymerase I removes the RNA primers using its unique ability to snip off nucleotides one at a time starting at the 5′ end while moving toward the 3′ end of the RNA primer. This ability is referred to as 5′ to 3′ exonuclease activity. DNA polymerase I begins its exonuclease activity at the free 5′ end of each RNA primer. With the removal of each ribonucleotide, the adjacent 3′-OH from the deoxynucleotide is used by DNA polymerase I to fill the gap between Okazaki fragments (figure 9.12). Finally, the Okazaki fragments are joined by the enzyme **DNA ligase,** which forms a phosphodiester bond between the 3′-OH of the growing strand and the 5′-phosphate of an Okazaki fragment (figure 9.13).

Amazingly, DNA polymerase III, like all DNA polymerases (except DNA polymerases called reverse transcriptases), has an additional function that is critically important: **proofreading.** Proofreading is the removal of a mismatched base immediately after it has been added; its removal must occur before the next base is incorporated. The ε subunit of DNA polymerase III has 3′ to 5′ exonuclease activity. This activity enables the polymerase core enzyme to check each newly incorporated base to see that it forms stable hydrogen bonds. In this way, mismatched bases can be detected. If the wrong base has been mistakenly added, the exonuclease activity removes it, but only as long as it is still at the 3′ end of the growing strand (figure 9.14). Once removed, holoenzyme backs up and adds the proper nucleotide in its place. DNA proofreading is not 100% efficient, and as discussed in

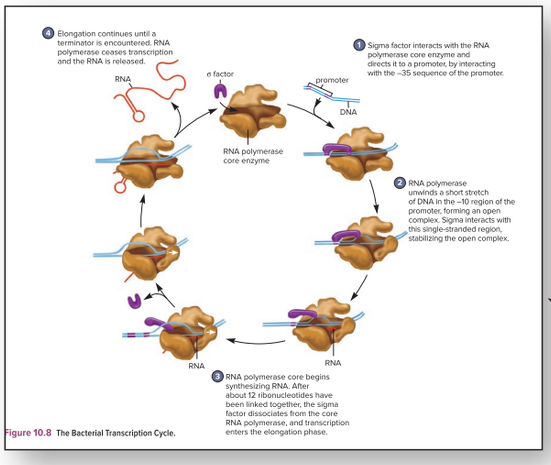


Figure 10.8 The Bacterial Transcription Cycle.

Annotated Figures—All key metabolic pathways and molecular processes are annotated, so each step is clearly illustrated and explained.

Key Concepts—At the end of each chapter, organized by numbered headings, this feature distills the content to its essential components with cross-references to figures and tables.

Key Concepts

20.1 Biogeochemical Cycling Sustains Life on Earth

- The redox potential of the environment has a major impact on the type of metabolic processes that can occur **(figure 20.1)**.
- The cycling between inorganic (CO_2) and organic carbon occurs aerobically and anaerobically by all types of microbes **(figure 20.2)**. Only methanogenic archaea can produce methane, an anaerobic process. Methane can be oxidized aerobically or anaerobically **(figure 20.3)**.
- Assimilatory processes involve incorporation of nutrients into the organism's biomass during metabolism; dissimilatory processes involve the release of nutrients to the environment after metabolism. Dissimilatory reduction involves the use of the oxidized compound (e.g., NO_3^- or SO_4^{2-}) as the terminal electron acceptor during anaerobic respiration **(figures 20.4** and **20.6)**.
- Chemolithotrophs use reduced elements as electron donors and generate the oxidized form, which can then be used for assimilatory or dissimilatory processes **(figures 20.4** and **20.6)**.
- Phosphorus is chiefly cycled in its oxidized state, phosphate. In marine systems, phosphite and phosphonates are also cycled **(figure 20.5)**.
- Iron cycles between its oxidized and reduced forms without the accumulation of intermediates **(figure 20.7)**.
- Although it is convenient to consider a single element cycling, in nature there are extensive and complex interactions between nutrient cycles **(figure 20.8)**.

20.2 Global Climate Change: Biogeochemical Cycling Out of Balance

- Microbes have evolved slowly over time; this has given rise to the biogeochemical cycles that sustain life on Earth. However, since the beginning of the twentieth century, the rate at which CO_2, CH_4, and nitrogen oxides have been released into the atmosphere has outpaced the rate at which they can be recycled. Thus these greenhouse gases are accumulating in Earth's atmosphere **(figures 20.9** and **20.10)**.
- The increase in greenhouse gases is correlated with an increase in the global annual-mean surface air temperature **(figure 20.11)**.

Active Learning—Includes questions based on examples from the current literature; designed to stimulate analytical problem-solving skills.

Active Learning

1. Examine figure 20.10. Farmers in Argentina use a crop/cattle rotation system in which cattle graze on pastures for about 5 years. Cattle are then moved to a different area and crops are planted where they had grazed. Crops are grown for 3 years, and cattle are rotated back and the field reverts to a pasture. This dramatically reduces the amount of nitrogen fertilizer that must be added when crops are grown. Discuss why this practice minimizes fossil fuel emissions.
2. A bacterium isolated from sewage sludge was recently found to be capable of anoxygenic photosynthesis using nitrite as the electron donor, converting it to nitrate. Compare this form of nitrification to that which is well characterized. By removing nitrite from the environment, what other processes within the nitrogen cycle might this bacterium be influencing?
3. It has been shown that the addition of phosphorus to lake ecosystems promotes excess nitrogen removal. This has real-world implications, as many communities have implemented management plans to reduce phosphorus influx to natural waters. Explain how an increase in one element, phosphorus, could lead to the removal of another, nitrogen. Think about the carbon cycle. How could an increase in another element in a given ecosystem alter the loss of carbon as CO_2?
4. Microbial methane oxidation is an important process because it drastically limits the release of this dangerous greenhouse gas. A group of European microbiologists used enrichment cultures of archaea from Dutch canal sediments to obtain two isolates. One oxidized CH_4 to CO_2 using NO_3^- as an electron acceptor to generate N_2. The other isolate reduced Fe(III) to Fe(II) during methane oxidation. Refer to figures 20.2, 20.3, 20.4, and 20.7 to review the parts of the carbon, nitrogen, and iron cycles involved. If you mixed the two isolates with equal amounts of nitrate and Fe(III), which methane-oxidizing archaeon do you think would grow fastest? Why?

Read the original paper: Etwing, K. F. 2016. Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc. Nat. Acad. Sci. USA.* 113:12792–6. doi:10.1073/pnas.1609534113.

Chapter Highlights

Part One Introduction to Microbiology

Chapter 1 The Evolution of Microorganisms and Microbiology—Evolution is the driving force of all biological systems; this is made clear by introducing essential concepts of microbial evolution first. Advances in the discipline of microbiology and the increasing contributions of genomics and metagenomics are discussed.

Chapter 2 Microscopy—This chapter introduces students to the foundations of microscopy as well as the principles of organism preparation for microscopic visualization.

Chapter 3 Bacterial and Archaeal Cell Structure—In addition to the basics of bacterial and archaeal cellular structure and function, this chapter compares the cellular organization in these two domains to offer insight into common challenges to cellular life.

Chapter 4 Eukaryotic Cell Structure—Eukaryotic cell structure and function is reviewed, with emphasis on eukaryotic microbes. The current understanding of the evolution of mitochondria and mitochondria-like organelles is considered. Key comparisons between bacteria, archaea, and eukaryotes are included throughout the chapter.

Chapter 5 Bacterial and Archaeal Growth—This chapter introduces microbial growth styles and the environmental conditions that affect them. Current understanding of growth arrested and persister cells is summarized. Strategies for laboratory culture of microbes are also described.

Part Two Microbial Metabolism

Chapter 6 Introduction to Metabolism—This chapter reviews the thermodynamic principles that govern energy capture and includes a section outlining the nature of biochemical pathways. The concept of metabolic flux is presented by discussing the interconnected biochemical pathways used by cells.

Chapter 7 Catabolism: Energy Release and Conservation—This introduction to metabolic diversity and nutritional types starts with aerobic respiration and is followed by an exploration of the energy-conserving process of each nutritional type, including recently discovered flavin-based electron bifurcation.

Chapter 8 Anabolism: The Use of Energy in Biosynthesis—This chapter presents the anabolic reactions that fix carbon and nitrogen, as well as the biosynthetic pathways to generate macromolecular precursors. Formation of the components of the complete bacterial cell wall is also described in detail.

Part Three Microbial Molecular Biology and Genetics

Chapter 9 Genome Replication—DNA replication in all three domains is covered. Differences among the organisms are related to overall chromosome structure.

Chapter 10 Gene Expression—Transcription, translation, and secretion are described and compared among the three domains. Membrane vesicles are introduced.

Chapter 11 Regulation of Cellular Processes—This chapter surveys the regulation of bacterial cellular processes, with examples of both protein-based and RNA-based mechanisms. Global regulatory mechanisms are introduced, followed by specific examples of complex control in chemotaxis, quorum sensing, endospore formation, and responses to viral infection.

Chapter 12 Mechanisms of Genetic Variation—This chapter covers mutation, repair, and recombination in the context of processes that introduce genetic variation into populations. Recent progress in the study of integrative conjugative elements and mobilizable genomic islands is summarized.

Part Four The Diversity of the Microbial World

Chapter 13 Gram-Positive Bacteria—This overview of Gram-positive bacteria includes firmicutes and actinobacteria. In addition to morphology and physiology, the role of these organisms in both disease and the production of important natural products is discussed.

Chapter 14 Proteobacteria—This chapter is organized by taxonomic class. The content ranges from the adaptations seen in α-proteobacteria that enable survival in oligotrophic environments to the pathogenesis of some of the γ-proteobacteria.

Chapter 15 Nonproteobacterial Gram-Negative Bacteria—In addition to a description of Deinoccoci and Mollicutes, the ecology and physiology of photosynthetic bacteria and the recently described Planctomycetes, Verrucomicrobia, Chlamydiae (PVC) superphylum is introduced. The role of mycoplasma, chlamydia, and spirochetes in human disease is discussed.

Chapter 16 Archaea—The discussion of archaeal taxonomy includes the new diversity uncovered by metagenomics. The methanogenesis discussion includes the mechanism of flavin-based electron bifurcation, first introduced in chapter 7.

Chapter 17 Eukaryotic Microbes—This chapter introduces protist and fungal morphology and diversity, with an emphasis on morphology, physiological adaptation, and ecology. The global importance of protozoan infections and burden of fungal infections in immunocompromised hosts are stressed.

Chapter Highlights

Chapter 18 Viruses and Other Acellular Agents—This chapter surveys essential morphological, physiological, and genetic elements of viruses as well as viroids, satellites, and prions. Pathogenic viruses and the human disease they cause are discussed.

Part Five Ecology and Symbiosis

Chapter 19 Microbial Interactions—Important model systems for the exploration of microbial symbioses are presented.

Chapter 20 Biogeochemical Cycling and Global Climate Change—The description of each major nutrient cycle is accompanied by a student-friendly figure that distinguishes between reductive and oxidative reactions. Coverage of the role of biogeochemical cycling in global climate change lends real-world relevance to nutrient cycling.

Chapter 21 Microorganisms in Natural Ecosystems—This chapter views microbes in their natural environments through the lens of the evolutionary adaptations that enable them to survive conditions that seem uninhabitable. An updated discussion of the role of marine microbes in the global carbon budget is included. The concepts of the phyllosphere and rhizoplane are introduced along with discussion of mycorrhizae and rhizobia.

Part Six Host Defense and Pathogenicity

Chapter 22 Innate Host Resistance—This chapter begins with the physical and chemical components of the nonspecific host response, followed by an overview of cells, tissues, and organs of the immune system. The chapter concludes with an overview of the molecular mechanisms that drive phagocytosis and inflammation.

Chapter 23 Adaptive Immunity—The development and activation of cell and antibody mediated immune responses is introduced. Discussions integrate concepts of cell biology, physiology, and genetics to present the immune system as a unified response having multiple components. Implications of dysfunctional immune actions are also discussed.

Chapter 24 The Human Microbiome and Host Interactions—This chapter introduces the establishment of the human microbiome as a developmental process from infancy through adulthood. The importance of the microbiome to host homeostasis is emphasized by discussion of its role in metabolism, immune function, and the gut-brain axis as well as an introduction to the consequences of dysbiosis.

Chapter 25 Infection and Pathogenicity—This chapter delineates the development of disease from microbial transmission to host cell damage. Emphasis is placed on the overlap between microbial molecules that facilitate survival and those that act as virulence factors. Its placement after the immunology chapters enables discussion of host-parasite relationships.

Part Seven Applied Microbiology

Chapter 26 Epidemiology and Public Health Microbiology—This chapter presents the development of modern epidemiology as an investigative science, emphasizing its role in preventative medicine. The latest epidemiological data from the Centers for Disease Control and Prevention are reported.

Chapter 27 Control of Microorganisms in the Environment—This chapter surveys the many ways to control microbial growth. Major control mechanisms are described in detail, with examples of uses in real-world scenarios.

Chapter 28 Antimicrobial Chemotherapy—This chapter starts with a brief history of the development of antibiotics. This is followed by sections on each type of antimicrobial agent (antibacterial, antiviral, etc.). Finally, the concept of prudent antibiotic use is discussed in the context of the growing threat of resistance.

Chapter 29 Microbiology of Food—The essentials of both food safety and microbial processes involved in food production are discussed.

Chapter 30 Industrial and Environmental Microbiology—This chapter complements our 21st-century approach to microbiology by emphasizing the importance of clean water and the power of microbial environmental remediation.

Part Eight Methods in Microbiology

Chapter 31 Microbial DNA Technologies—This chapter describes the steps in gene cloning, PCR, and heterologous gene expression. Cas9 genome engineering methodologies are described.

Chapter 32 Microbial Genomics—This chapter walks students through genomics from Sanger and next-generation nucleotide sequencing to the process of genome annotation, transcriptomics and proteomics. Methods employed in single-cell genomics and metagenomics are also briefly described and their impact on microbial biology is stressed.

Chapter 33 Methods in Microbial Biology—This chapter emphasizes culture-based techniques as the gold standard and reviews culture-independent approaches such as mass spectrometry in the identification of microbial taxa as well as metatranscriptomics, metaproteomics, and stable isotope probing in the study of community activity.

Chapter 34 Clinical Microbiology and Immunology—Culture-based and common molecular techniques used in the modern clinical laboratory are introduced. Laboratory safety is also stressed.

Lab Tools for Your Success

Prep for Microbiology is an adaptive learning tool that prepares students for college-level work in Microbiology. Prep for Microbiology individually identifies concepts the student does not fully understand and provides learning resources to teach essential concepts so he or she enters the classroom prepared. Data-driven reports highlight areas where students are struggling, helping to accurately identify weak areas.

Acknowledgements

In the preparation of each edition, we are guided by the collective wisdom of reviewers who are expert microbiologists and excellent teachers. They represent experience in community colleges, liberal arts colleges, comprehensive institutions, and research universities. We have followed their recommendations, while remaining true to our overriding goal of writing readable, student-centered content. Each feature incorporated into this edition has been carefully considered in terms of how it may be used to support student learning in both the traditional and the flipped learning environment.

Also in this edition, we are very excited to incorporate real student data points and input, derived from thousands of our LearnSmart users, to help guide our revision. With this information, we were able to hone both book and digital content.

The authors wish to extend their gratitude to our team at McGraw-Hill Education, including Lauren Vondra, Darlene Schueller, Jim Connely, Kristine Rellihan, Sandy Wille, David Hash, Tammy Juran, and Beth Cray. Finally, we thank our spouses and children, who provided support and tolerated our absences (mental, if not physical) while we completed this demanding project.

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The Evolution of Microorganisms and Microbiology

Source: NASA, ESA, and The Hubble Heritage Team (STScI/AURA)-ESA/Hubble Collaboration

The Microbial Universe

If you have ever gazed at the night sky on a cloudless evening in a region far from light pollution, you have probably been amazed and perhaps a little humbled by the vast number of stars. It's hard to estimate just how many stars are out there; best estimates start with our own Milky Way Galaxy, which has roughly 100 billion (1×10^{11}) stars. Astronomers figure there is something like 10 trillion (1×10^{13}) galaxies in the universe. Assuming all galaxies are roughly the size of the Milky Way, you wind up with a number around 1×10^{24} stars in the universe—not the most accurate number, but nonetheless daunting.

If you turn your gaze back to Earth, you will find even more awesome abundance. For example, if you stacked all of the 1×10^{31} viruses on Earth one on top of the other, they would stretch about 100 million light-years. That's about 43 times farther away than the Andromeda Galaxy. And next time you take a dip in the sea, consider that there are at least a hundred thousand (1×10^{5}) times more bacteria in the ocean (about 1.3×10^{29}) than stars in the universe. Or perhaps you prefer staying on land where a teaspoon of soil has about a billion (1×10^{9}) microorganisms.

What are all these microorganisms doing? The short answer is, making life for the rest of us possible. How? Starting about 2.4 billion years ago, bacteria called cyanobacteria started releasing oxygen in abundance into the atmosphere. This has been dubbed the "great oxidation event," and it set the stage for oxygen-consuming organisms (like us) to evolve. Microorganisms have another starring role in the evolution of life because only bacteria can fix nitrogen—that is, take gaseous nitrogen and convert it to organic nitrogen used by plants, animals, and other microbes. Finally, can you imagine what life on Earth would look like if dead organic material were not degraded? Probably best not to. Much better to think of microbial products like beer, wine, chocolate, cheese, and yogurt.

But of course not all microorganisms make life possible, or even easier. Each year about 16 million (1.6×10^{7}) people die from infectious disease; and many of these deaths are preventable by either vaccination or antibiotic treatments. Ironically most vaccines and antibiotics are also microbial products. Although we know the most about disease-causing microorganisms, because less than 1% of all microorganisms cause disease, there is a lot left to learn about microbes. In fact, like the number of stars, the number of microbial species (including bacteria, viruses, fungi, and protists) is debated. What is certain is that microbes are important for all other life on Earth.

Our goal in this chapter is to introduce you to this amazing world of microorganisms and to outline the history of their evolution and discovery. Microbiology is a biological science, so much of what you will learn in this text is similar to what you have learned in high school and college biology classes that focus on large organisms. But microbes have unique properties, and microbiology has unique approaches to understanding them. These too will be introduced. But before you delve into this chapter, check to see if you have the background needed to get the most from it.

Readiness Check:

Based on what you have learned previously, you should be able to:

- ✓ List the features of eukaryotic cells that distinguish them from other cell types
- ✓ Understand the basic structure of the macromolecules, nucleic acids, proteins, carbohydrates, and lipids (*see appendix I*)
- ✓ Explain the terms *genome, genotype,* and *mutation*

1.1 Members of the Microbial World

After reading this section, you should be able to:

a. Define the term *microbiology*
b. Explain Carl Woese's contributions in establishing the three-domain system for classifying cellular life
c. Determine the type of microbe (bacterium, fungus, etc.) when given a description of a newly discovered microorganism
d. Provide an example of the importance to humans of each of the major types of microbes

Microorganisms are defined as those organisms too small to be seen clearly by the unaided eye (**figure 1.1**). They are generally 1 millimeter or less in diameter. Although small size is an important characteristic of microbes, it is not sufficient to define them. Most unicellular microbes are microscopic, but some, such as bread molds, are visible without microscopes. Some macroscopic microorganisms are multicellular. They are

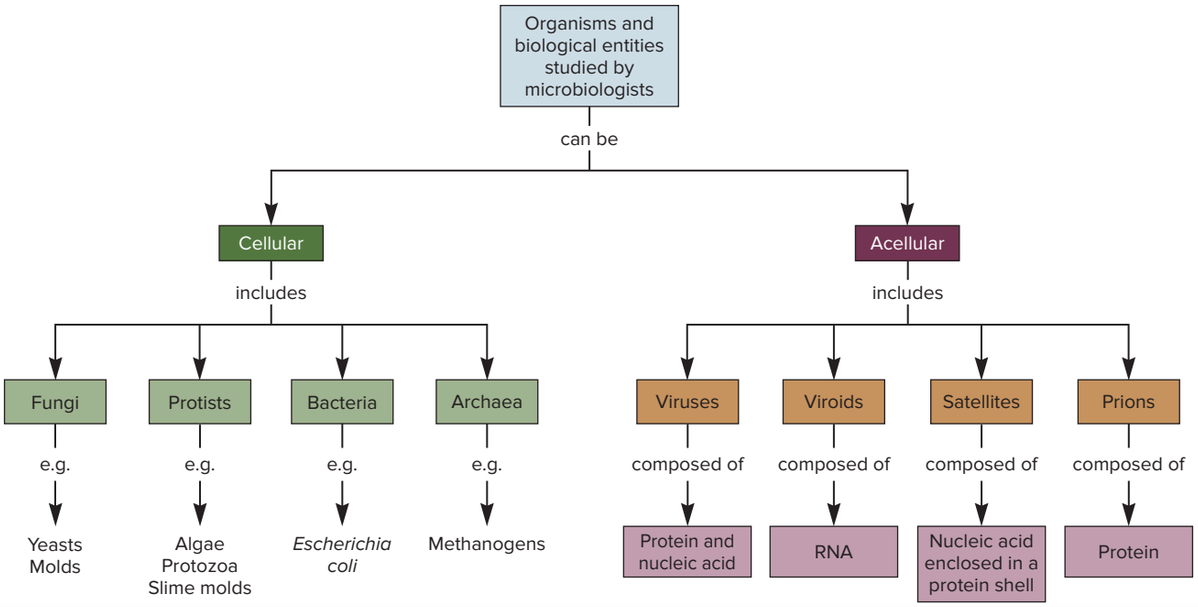


Figure 1.1 **Concept Map Showing the Types of Biological Entities Studied by Microbiologists.**

MICRO INQUIRY *How would you alter this concept map so that cellular organisms are differentiated by their key features?*

distinguished from other multicellular life forms such as plants and animals by their lack of highly differentiated tissues. In summary, cellular microbes are usually smaller than 1 millimeter in diameter, often unicellular and, if multicellular, lack differentiated tissues.

In addition to microorganisms, microbiologists study a variety of acellular biological entities (figure 1.1). These include viruses and subviral agents. The terms "microorganism" and "microbe" are sometimes applied to these acellular agents as well.

The diversity of microorganisms has always presented a challenge to microbial taxonomists. Early descriptions of cellular microbes as either plants or animals were too simple. For instance, some microbes are motile like animals but also have cell walls and are photosynthetic like plants. An important breakthrough in microbial taxonomy arose from studies of their cellular architecture, when it was discovered that cells exhibited one of two possible "floor plans." Cells that came to be called **prokaryotic cells** (Greek *pro,* before; *karyon,* nut or kernel) have an open floor plan. That is, their contents are not divided into compartments ("rooms") by membranes. Only **eukaryotic cells** (Greek *eu,* true) have a nucleus and other membrane-bound organelles (e.g., mitochondria, chloroplasts) that separate some cellular materials and processes from others.

These observations eventually led to the development of a classification scheme that divided organisms into five kingdoms: Monera, Protista, Fungi, Animalia, and Plantae. Microorganisms (except for viruses and other acellular infectious agents) were placed in the first three kingdoms. In this scheme, all organisms with prokaryotic cell structure were placed in Monera. However, the five-kingdom system is no longer accepted by microbiologists. This is because "prokaryotes" are too diverse to be grouped together in a single kingdom. ▶| *Use of the term "prokaryote" is controversial (section 3.1)*

Great progress has been made in three areas that profoundly affect microbial classification. First, much has been learned about the detailed structure of microbial cells from the use of electron microscopy. Second, microbiologists have determined the biochemical and physiological characteristics of many different microorganisms. Third, the sequences of nucleic acids and proteins from a wide variety of organisms have been compared. The comparison of ribosomal RNA (rRNA) nucleic acid sequences, begun by Carl Woese (1928–2012) in the 1970s, was instrumental in demonstrating that there are two very different groups of organisms with prokaryotic cell architecture: Bacteria and Archaea. Later studies based on rRNA comparisons showed that Protista is not a cohesive taxonomic unit (i.e., taxon) and that it should be divided into three or more kingdoms. These studies and others led many taxonomists to reject the five-kingdom system in favor of one that divides cellular organisms into three domains: Bacteria, Archaea, and Eukarya (all eukaryotic organisms) (**figure 1.2**). ▶| *Nucleic acids (appendix I); Proteins (appendix I)*

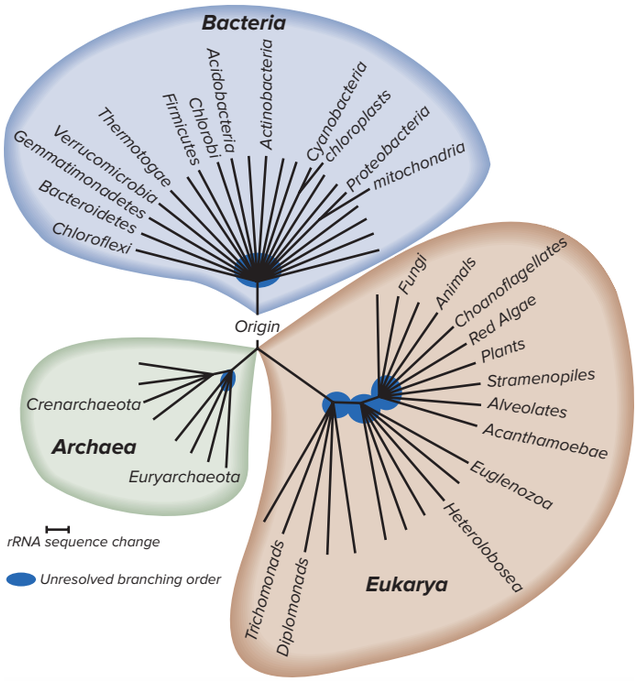


Figure 1.2 Universal Phylogenetic Tree. These evolutionary relationships are based on rRNA sequence comparisons. Only representative lineages have been identified.

MICRO INQUIRY *How many of the taxa listed in the figure include microbes?*

Members of domain **Bacteria** are usually single-celled organisms.[1] Most have cell walls that contain the structural molecule peptidoglycan. Bacteria are abundant in soil, water, and air, including sites that have extreme temperatures, pH, or salinity. Bacteria are also major inhabitants of our bodies, forming the human **microbiome.** Indeed, more microbial cells are found in and on the human body than there are human cells. These microbes begin to colonize humans shortly after birth. As the microbes establish themselves, they contribute to the development of the body's immune system. Those microbes that inhabit the large intestine help the body digest food and produce vitamins. In these and many other ways, the human microbiome helps maintain our health and well-being. ▶| *Overview of bacterial cell wall structure (section 3.4); Human microbiome and host interactions (chapter 24)*

Unfortunately some bacteria cause disease, and some of these diseases have had a huge impact on human history. In 1347 the plague (Black Death), a disease carried by bacteria living in fleas, struck Europe with brutal force, killing one-third of the population within 4 years. Over the next 80 years, the disease struck repeatedly, eventually wiping out roughly half of the European population. The plague's effect was so great that most historians believe it changed European culture and prepared the way for the Renaissance.

Members of domain **Archaea** are distinguished from bacteria by many features, most notably their distinctive rRNA sequences, lack of peptidoglycan in their cell walls, and unique membrane lipids. Some have unusual metabolic characteristics, such as the ability to generate methane (natural) gas. Many archaea are found in extreme environments, including those with high temperatures (thermophiles) and high concentrations of salt (extreme halophiles). Archaea do not appear to directly cause disease in humans.

Domain **Eukarya** includes plants, animals, and microorganisms classified as protists or fungi. **Protists** are generally unicellular but larger than most bacteria and archaea. They have traditionally been divided into **protozoa,** which have an animal-like metabolism, and **algae,** which are photosynthetic. However, these terms lack taxonomic value because protists, algae, and protozoa do not form three groups, each with a single evolutionary history. Nonetheless, for convenience, we use these terms here. ▶| *Protist diversity reflects broad phylogeny (section 17.1)*

Fungi are a diverse group of microorganisms that range from unicellular forms (yeasts) to molds and mushrooms. Molds and mushrooms are multicellular fungi that form thin, threadlike structures called hyphae. Because of their metabolic capabilities, many fungi play beneficial roles, including making bread dough rise, producing antibiotics, and decomposing dead organisms. Some fungi associate with plant roots to form mycorrhizae. Mycorrhizal fungi transfer nutrients to the roots, improving growth of the plants, especially in poor soils. Other fungi cause plant diseases (e.g., rusts, powdery mildews, and smuts) and diseases in humans and other animals. ▶| *Fungal biology reflects vast diversity (section 17.6)*

The microbial world also includes numerous acellular infectious agents. **Viruses** are acellular entities that must invade a host cell to multiply. The simplest virus particles (also called virions) are composed only of proteins and a nucleic acid, and can be extremely small (the smallest is 10,000 times smaller than a typical bacterium). However, their small size belies their power. They cause many animal and plant diseases and have caused epidemics that have shaped human history. Viral diseases include smallpox, rabies, influenza, AIDS, the common cold, and some cancers. Viruses are also important in aquatic environments, where they play a critical role in shaping microbial communities. **Viroids** are infectious agents composed only of ribonucleic acid (RNA). They cause numerous plant diseases. **Satellites** are composed of a nucleic acid enclosed in a protein shell. They must coinfect a host cell with virus, called a helper virus, to complete their life cycle. Satellites and their helper viruses cause both plant and animal diseases. Finally, **prions,**

1 In this text, the term *bacteria* (s., *bacterium*) is used to refer to those microbes belonging to domain Bacteria, and the term *archaea* (s., *archaeon*) is used to refer to those that belong to domain Archaea. In some publications, the term *bacteria* is used to refer to all cells having prokaryotic cell structure. That is not the case in this text.

infectious agents composed only of protein, are responsible for causing a variety of spongiform encephalopathies such as scrapie and "mad cow disease." ▶| *Viruses and other acellular infectious agents (chapter 18)*

Comprehension Check

1. How did the methods used to classify microbes change, particularly in the last half of the twentieth century? What was the result of these technological advances?
2. Identify one characteristic for each of these types of microbes that distinguishes it from the other types: bacteria, archaea, protists, fungi, viruses, viroids, satellites, and prions.

1.2 Microbes Have Evolved and Diversified for Billions of Years

After reading this section, you should be able to:

a. Explain the RNA world hypothesis and the evidence that supports it
b. Design a set of experiments that could be used to place a newly discovered cellular microbe on a phylogenetic tree based on small subunit (SSU) rRNA sequences
c. Compare and contrast the evolution of mitochondria and chloroplasts

A review of figure 1.2 reminds us that in terms of the number of taxa, microbes are the dominant organisms on Earth. How has microbial life been able to radiate to such an astonishing level of diversity? To answer this question, we must consider microbial evolution. The field of microbial evolution, like any other scientific endeavor, is based on the formulation of hypotheses, the gathering and analysis of data, and the reformation of hypotheses based on newly acquired evidence. That is to say, the study of microbial evolution is based on the scientific method. To be sure, it is difficult to amass evidence when considering events that occurred millions, and often billions, of years ago, but the application of molecular methods has revealed a living record of life's ancient history. This section describes the outcome of this scientific research.

Theories of the Origin of Life Depend Primarily on Indirect Evidence

Dating meteorites through the use of radioisotopes places our planet at an estimated 4.5 to 4.6 billion years old. However, conditions on Earth for the first 100 million years or so were far too harsh to sustain any type of life. Eventually bombardment by meteorites decreased, water appeared on the planet in liquid form, and gases were released by geological activity to form Earth's atmosphere. These conditions were amenable to the origin of the first life forms. But how did this occur, and what did these life forms look like?

To find evidence of life and to develop hypotheses about its origin and subsequent evolution, scientists must be able to define life. Although even very young children can examine an object and correctly determine whether it is living or not, defining life succinctly has proven elusive for scientists. Thus most definitions of life consist of a set of attributes. The attributes of particular importance to paleobiologists are an orderly structure, the ability to obtain and use energy (i.e., metabolism), and the ability to reproduce. Just as NASA scientists are using the characteristics of microbes on Earth today to search for life elsewhere, so too are scientists examining **extant organisms,** those organisms present today, to explore the origin of life. Some extant organisms have structures and molecules that represent relics of ancient life forms. These can provide scientists with ideas about the type of evidence to seek when testing hypotheses.

The best direct evidence for the nature of primitive life would be a fossil record. There have been reports of microbial fossil discoveries since 1977 (**figure 1.3**). These have always met with skepticism because finding them involves preparing thin slices of ancient rocks and examining the slices for objects that look like cells. Unfortunately some things that look like cells can be formed by geological forces that occurred as the rock was formed. The result is that the fossil record for microbes is sparse and always open to reinterpretation. Despite these problems most scientists agree that life was present on Earth about 3.5 to 3.7 billion years ago (**figure 1.4**). To reach this conclusion, biologists rely primarily on indirect evidence. Among the indirect evidence used are molecular fossils. These are chemicals found in rock or sediment that are chemically related to molecules found in cells. For instance, the presence of molecules called hopanes in a rock indicates that bacteria were present when the rock was formed. This conclusion is reached because hopanes are formed from hopanoids, which are found in the plasma membranes of extant bacteria. As you can see, no single piece of evidence can stand alone. Instead many pieces of evidence are

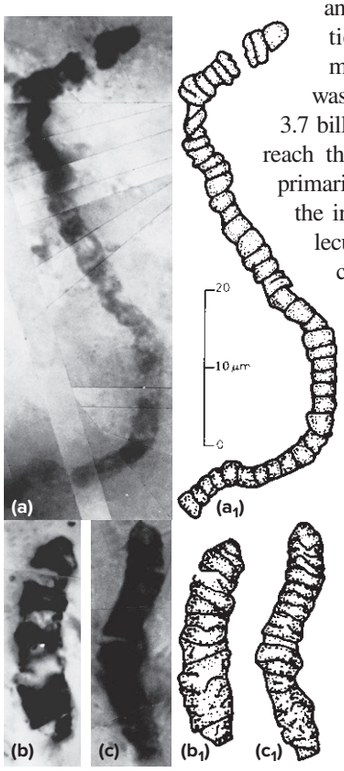


Figure 1.3 Possible Microfossils Found in the Archaeon Apex Chert of Australia. Chert is a type of granular sedimentary rock rich in silica. These structures were discovered in 1977. Because of their similarity to filamentous cyanobacteria they were proposed to be microfossils. In 2011 scientists reported that similar structures from the same chert were not biological in origin. They used spectrometry and microscopy techniques not available in 1977 to show that the structures were fractures in the rock filled with quartz and hematite. Scientists are still debating whether or not these truly are microfossils. (a-c) J. William Schopf

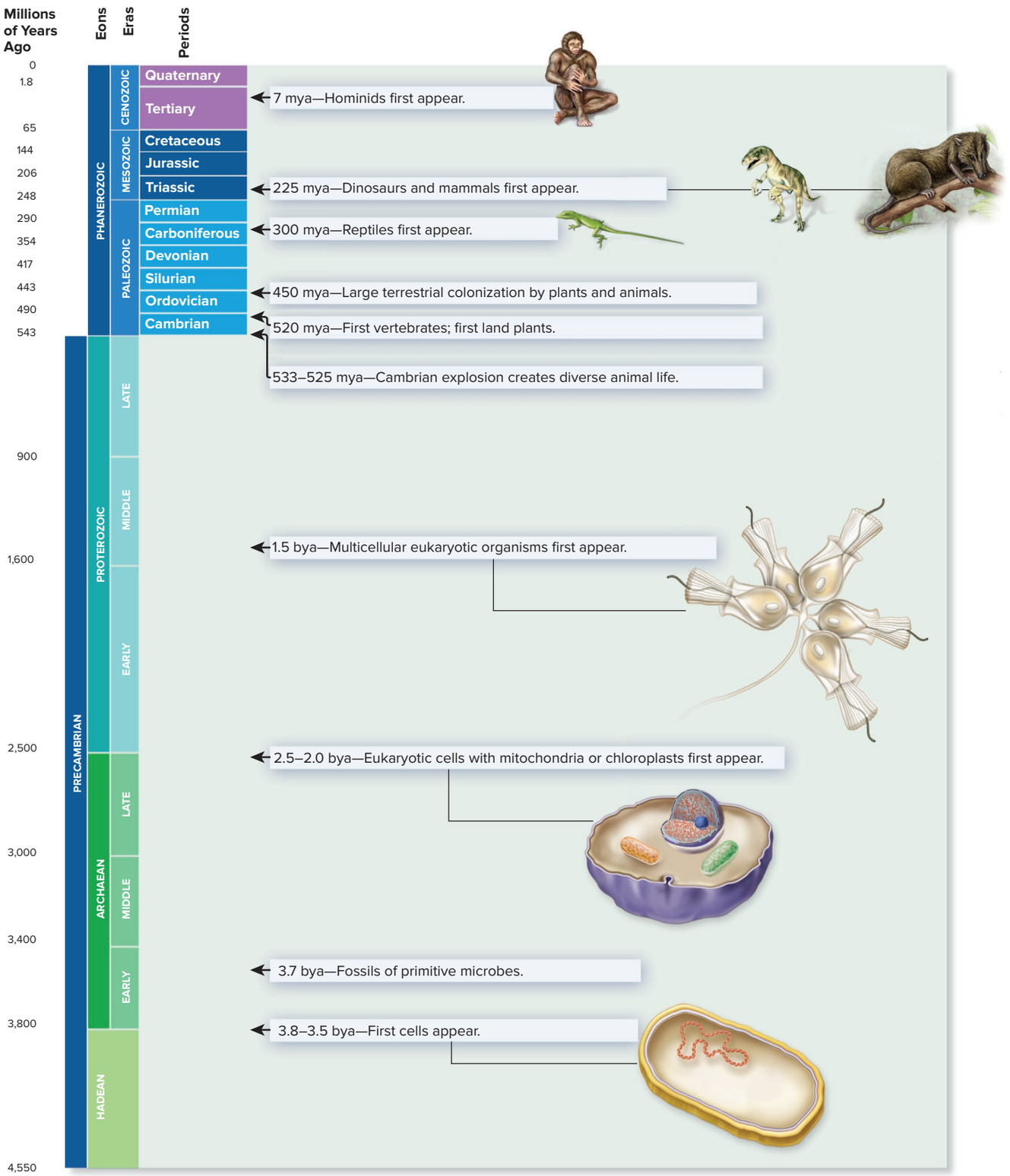


Figure 1.4 **An Overview of the History of Life on Earth.** mya = million years ago; bya = billion years ago.

put together in an attempt to get a coherent picture to emerge, much as for a jigsaw puzzle.

Early Life Was Probably RNA Based

The origin of life rests on a single question: How did early cells, sometimes called *probionts,* arise? At a minimum, modern cells consist of a plasma membrane enclosing water in which numerous chemicals are dissolved and subcellular structures float. It seems likely that the first self-replicating entity was much simpler than even the most primitive modern living cells. Before there was life, most evidence suggests that Earth was a very different place: hot and anoxic, with an atmosphere rich in water vapor, carbon dioxide, and nitrogen. In the oceans, hydrogen, methane, and carboxylic acids were formed by geological and chemical processes. Areas near hydrothermal vents or in shallow pools may have provided the conditions that allowed chemicals to react with one another, randomly "testing" the usefulness of the reaction and the stability of its products. Some reactions released energy and would eventually become the basis of modern cellular metabolism. Other reactions generated molecules that functioned as catalysts, some aggregated with other molecules to form the predecessors of modern cell structures, and others were able to replicate and act as units of hereditary information.

In modern cells, three different molecules fulfill the roles of catalysts, structural molecules, and hereditary molecules. Proteins have two major roles in modern cells: catalytic and structural. Catalytic proteins are **enzymes** and structural proteins serve a myriad of functions such as transport, attachment, and motility. DNA stores hereditary information that is replicated and passed on to the next generation. RNA converts the information stored in DNA into protein. Any hypothesis about the origin of life must account for the evolution of these molecules, but the very nature of their relationships to each other in modern cells complicates attempts to imagine how they evolved. Proteins can do cellular work, but their synthesis involves other proteins and RNA, and uses information stored in DNA. DNA cannot do cellular work and proteins are needed for its replication. RNA synthesis requires both DNA as the template and proteins as the catalysts for the reaction.

Based on these considerations, it is hypothesized that at some time in the evolution of life, there must have been a single molecule that could do both cellular work and replicate. This idea was supported in 1981 when Thomas Cech discovered an RNA molecule in a protist (*Tetrahymena* sp.) that also had catalytic activity. Since then, other catalytic RNA molecules have been discovered, including an RNA found in ribosomes that is responsible for forming peptide bonds—the bonds that hold together amino acids, the building blocks of proteins. Catalytic RNA molecules are now called **ribozymes.**

The discovery of ribozymes suggested that RNA at some time was capable of storing, copying, and expressing genetic information, as well as catalyzing other chemical reactions. In 1986 Nobel laureate Walter Gilbert coined the term **RNA world** to describe this precellular stage in the evolution of life. However, for this precellular RNA-based stage to proceed to the evolution of cellular life forms, a lipid membrane must have formed around the RNA (figure 1.5). This important evolutionary step is easier to imagine than other events in the origin of cellular life forms because lipids, major structural components of the membranes of modern organisms, spontaneously form liposomes—vesicles bounded by a lipid bilayer. ▶| *Lipids (appendix I)*

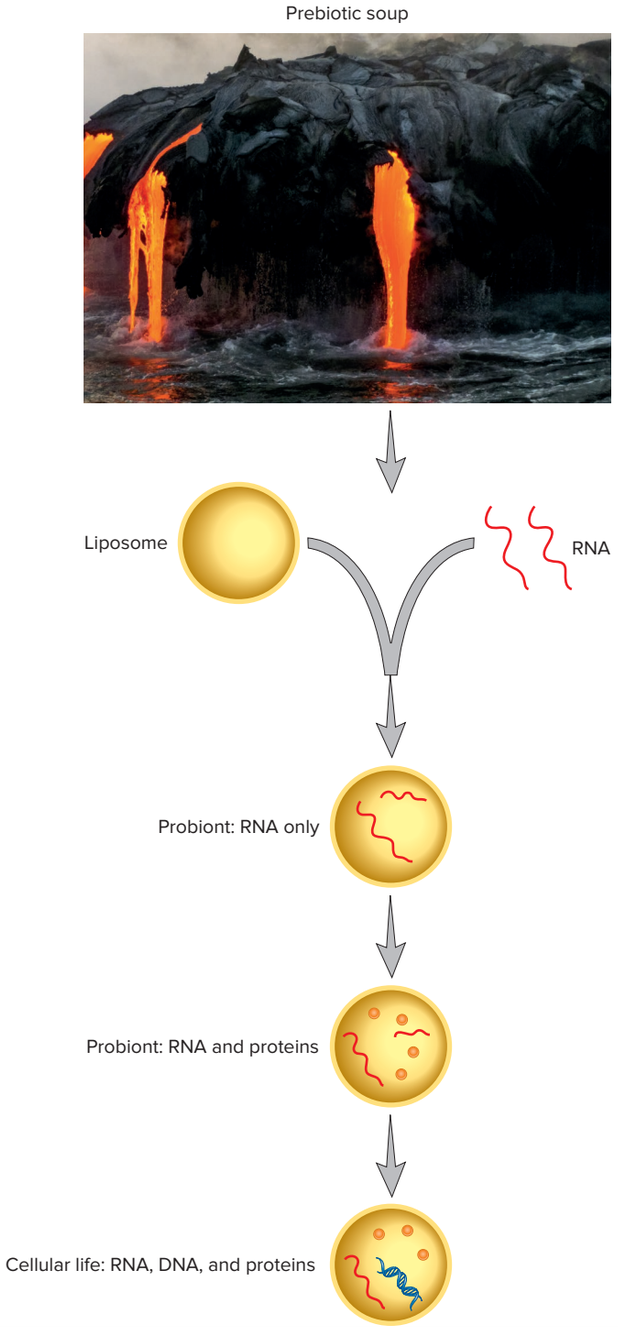


Figure 1.5 The RNA World Hypothesis for the Origin of Life. Benny Marty/Shutterstock

MICRO INQUIRY *Why are the probionts pictured above not considered cellular life?*

Jack Szostak, also a Noble laureate, is a leader in exploring how cells containing only RNA, so-called protocells, may have formed. When his group created liposomes using simpler fatty acids than those found in membranes today, the liposomes were leaky. These leaky liposomes allowed single RNA nucleotides to move into the liposome, but prevented large RNA chains from moving out. Furthermore, researchers could prod the liposomes into growing and dividing. Dr. Szostak's group has also been able to create conditions in which an RNA molecule could serve as a template for synthesis of a complementary RNA strand. These experiments may have recapitulated early steps in the evolution of cells. As seen in figure 1.5, several other processes need to occur to reach the level of complexity found in extant cells.

Apart from its ability to perform catalytic activities, the function of RNA suggests its ancient origin. Consider that much of the cellular pool of RNA in modern cells exists in the ribosome, a structure that consists largely of ribosomal RNA (rRNA) and uses messenger RNA (mRNA) and transfer RNA (tRNA) to construct proteins. Also rRNA itself catalyzes peptide bond formation during protein synthesis. Thus RNA seems to be well poised for its importance in the development of proteins. Because RNA and DNA are structurally similar, RNA could have given rise to double-stranded DNA. It is suggested that once DNA evolved, it became the storage facility for genetic information because it provides a more chemically stable structure. Two other pieces of evidence support the RNA world hypothesis: the fact that the energy currency of cells, ATP, is a ribonucleotide and the discovery that RNA can regulate gene expression. So it would seem that proteins, DNA, and cellular energy can be traced back to RNA. ▶| *ATP: the major energy currency of cells (section 6.2); Riboswitches: effector-mRNA interactions regulate transcription (section 11.3); Translational riboswitches (section 11.4)*

Despite evidence supporting the RNA world hypothesis, it is not without problems. Another area of research also fraught with considerable debate is the evolution of metabolism, in particular the evolution of energy-conserving metabolic processes. The early Earth was a hot environment that lacked oxygen. Thus the cells that arose there must have been able to use the available energy sources under these harsh conditions. Today there are heat-loving archaea capable of using inorganic molecules such as FeS as a source of energy. Some suggest that this interesting metabolic capability is a remnant of the first form of energy metabolism. Another metabolic strategy, oxygen-releasing photosynthesis (oxygenic photosynthesis), appears to have evolved perhaps as early as 2.7 billion years ago. This is supported by the discovery of ancient stromatolites (**figure 1.6**). Stromatolites are layered rocks formed by the incorporation of mineral sediments into layers of cyanobacteria growing in thick mats on surfaces. The oxygen released by these early cyanobacteria is thought to have altered Earth's atmosphere to its current oxygen-rich state, allowing the evolution of additional energy-capturing strategies such as aerobic respiration, the oxygen-consuming metabolic process that is used by many microbes and animals.

Evolution of the Three Domains of Life

Phylogenetic or **phyletic classification systems** seek to compare organisms on the basis of evolutionary relationships. The

Figure 1.6 Stromatolites. Modern stromatolites from Western Australia. Each stromatolite is a rocklike structure, typically 1 m in diameter, containing layers of cyanobacteria. Horst Mahr/imagebroker/age fotostock

term **phylogeny** (Greek *phylon,* tribe or race; *genesis,* generation or origin) refers to the evolutionary development of organisms. Because the microbial fossil record is so scant and hard to interpret, microbial phylogeny relies on comparisons of multiple features found in extant organisms. These include biomolecules such as fatty acids and certain conserved enzymes (e.g., DNA polymerase), cell wall construction, and nucleotide sequences, particularly of small subunit rRNA molecules (SSU rRNA) (**table 1.1**). In practice, this has resulted in the development of a **universal phylogenetic tree** (figure 1.2) based on comparisons of SSU rRNA, the rRNA found in the small subunit of the ribosome. ▶| *Ribosomes (section 3.6)*

Comparing SSU rRNA Molecules

Figure 1.2 is an example of a **phylogenetic tree.** The general concept of phylogenetic tree construction is not difficult to understand. In one approach, the sequences of nucleotides in the genes that encode SSU rRNAs from diverse organisms are aligned, and pair-wise comparisons of the sequences are made. The rRNAs from small ribosomal subunits (16S from bacterial and archaeal cells and 18S from eukaryotes) have become the molecules of choice for inferring microbial phylogenies and making taxonomic assignments. The SSU rRNAs are widely applicable in studies of microbial evolution, relatedness, and identification for several important reasons. First, although not identical, they play the same role in all microorganisms. In addition, because ribosomes are absolutely necessary for survival, the genes encoding SSU rRNAs cannot tolerate large changes in nucleotide sequence. The utility of SSU rRNAs is extended by the presence of certain sequences within SSU rRNA genes that vary among organisms as well as other regions that are quite similar. The variable regions enable comparison between closely related microbes, whereas the stable sequences allow the comparison of distantly related microorganisms.

Comparative analysis of SSU rRNA sequences from thousands of organisms has demonstrated the presence of **oligonucleotide signature sequences** (**figure 1.7**). These are short, conserved nucleotide sequences specific for phylogenetically

Table 1.1 Comparison of Bacteria, Archaea, and Eukarya

Property	Bacteria	Archaea	Eukarya
Organization of Genetic Material			
True membrane-bound nucleus	No	No	Yes
DNA complexed with histones	No	Some	Yes
Chromosomes	Usually one circular chromosome; chromosomes have single origin of replication; some are polyploid	One circular chromosome; some have chromosomes with multiple origins of replication; some are polyploid	Multiple, linear chromosomes with multiple origins of replication
Plasmids	Very common	Very common	Rare
Introns in genes	Rare	Rare	Yes
Nucleolus	No	No	Yes
Mitochondria, Chloroplasts, Endoplasmic Reticulum, Golgi, and Lysosomes Observed	No	No	Yes
Plasma Membrane Lipids	Ester-linked phospholipids; some have sterols	Glycerol diethers and diglycerol tetraethers	Ester-linked phospholipids and sterols
Flagella	Submicroscopic in size; filament composed of single type of flagellin protein	Submicroscopic in size; some filaments composed of more than one type of archaellin protein	Microscopic in size; membrane bound; usually 20 microtubules in 9 + 2 pattern
Peptidoglycan in Cell Walls	Yes	No	No
Ribosome Size and Structure	70S; 3 rRNAs; ~55 ribosomal proteins	70S; most have 3 rRNAs; ~68 ribosomal proteins	80S; 4 rRNAs and ~80 ribosomal proteins
Cytoskeleton	Rudimentary	Rudimentary	Yes

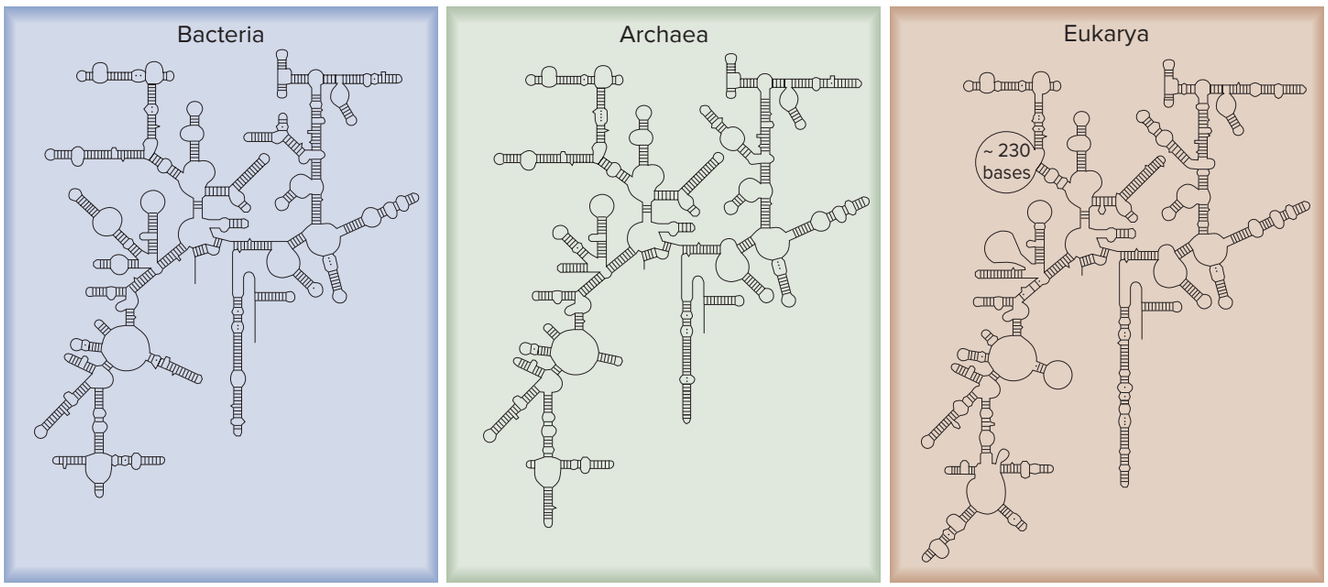


Figure 1.7 Signature rRNA Help Identify Microbes. Representative examples of rRNA secondary structures from the three domains: Bacteria *(Escherichia coli),* Archaea *(Methanococcus vannielii),* and Eukarya *(Saccharomyces cerevisiae).*

defined groups of organisms. Thus the signature sequences found in bacterial rRNAs are rarely or never found in archaeal rRNAs and vice versa. Likewise, the 18S rRNA of eukaryotes bears signature sequences that are specific to the domain Eukarya. In addition, signature sequences for a lower taxon, for example, the genus *Pseudomonas,* may be found in the variable regions of a higher taxon such as Bacteria.

When comparing SSU rRNA sequences from two different organisms, the number of differences between the nucleotide sequences is counted (**figure 1.8**). This value serves as a measure of the evolutionary distance between the organisms; the more differences counted, the greater the evolutionary distance. The evolutionary distances from many comparisons are used by sophisticated computer programs to construct the tree. The tip of each branch in the tree represents one of the organisms used in the comparison. The distance from the tip of one branch to the tip of another is the evolutionary distance between the two organisms.

Two things should be kept in mind when examining phylogenetic trees developed in this way. The first is that they are molecular trees, not organismal trees. In other words, they represent, as accurately as possible, the evolutionary history of a molecule (e.g., rRNA) and the gene that encodes it. Second, the distance between branch tips is a measure of relatedness, not of time. If the distance along the lines is very long, then the two organisms are more evolutionarily diverged (i.e., less related). However, we do not know when they diverged from each other. This concept is analogous to a printed map that accurately shows the distance between two cities but because of many factors (traffic, road conditions, etc.) cannot show the time needed to travel that distance.

Importantly, a tree may be unrooted or rooted. An unrooted tree (**figure 1.9*a***) represents phylogenetic relationships but does not indicate which organisms are more primitive relative to the others. Figure 1.9*a* shows that A is more closely related to C than it is to either B or D, but it does not indicate which of the four species might be the oldest. In contrast, the rooted tree (figure 1.9*b*) includes a node (taxonomic unit) that serves as the common ancestor and shows the development of the four species from this root. It is much more difficult to develop a rooted tree. For example, there are 15 possible rooted trees that connect four species but only three possible unrooted trees.

An unrooted tree can be rooted by adding data from an outgroup—a species known to be very distantly related to all the species in the tree (figure 1.9*c*). The root is determined by the point of the tree where the outgroup joins. This provides a point of reference to identify the oldest node on the tree, which is the node closest to the outgroup. So, for example, in figure 1.9*c,* organism Z is the outgroup and the oldest node on the tree is marked with an arrow.

LUCA

What does the universal phylogenetic tree tell us about the evolution of life? At the center of the tree is a line labeled "Origin" (figure 1.2). This is where data indicate the *l*ast *u*niversal

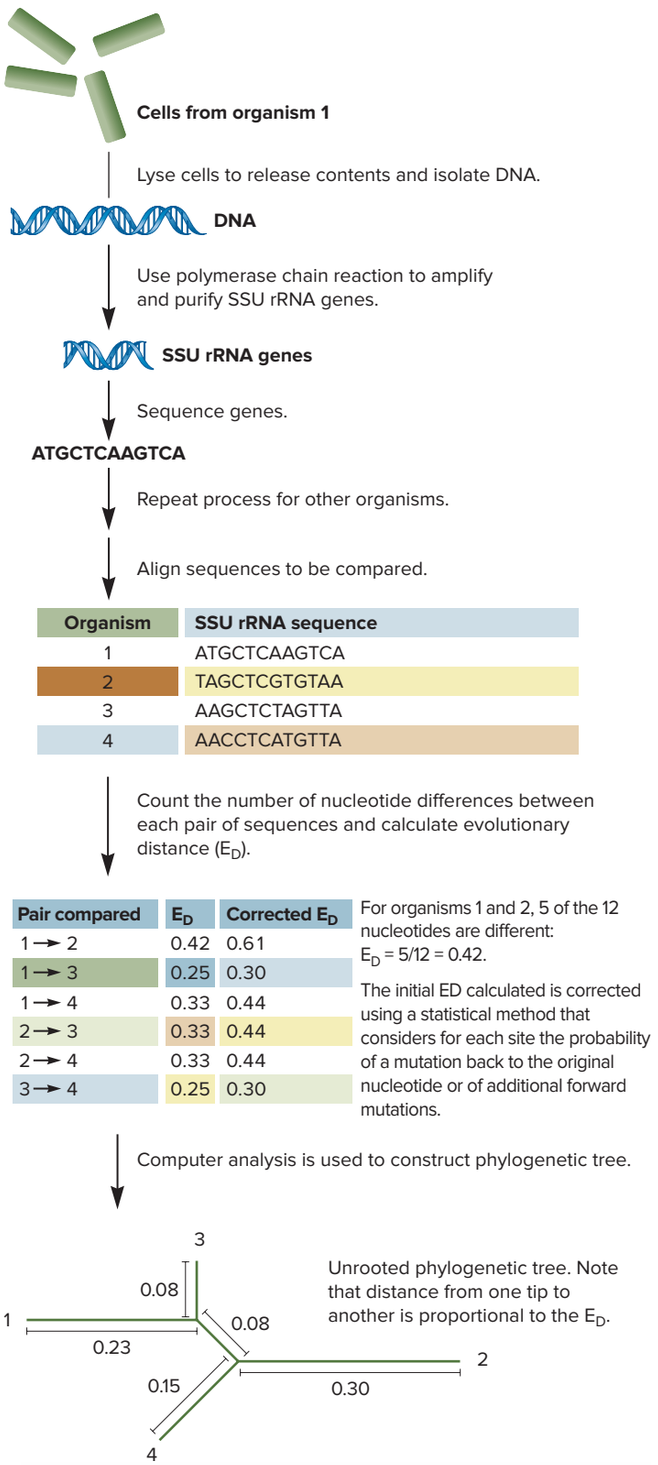


Figure 1.8 The Construction of a Phylogenetic Tree Using a Distance Method. The polymerase chain reaction is described in chapter 31.

MICRO INQUIRY *Why does the branch length indicate amount of evolutionary change but not the time it took for that change to occur?*

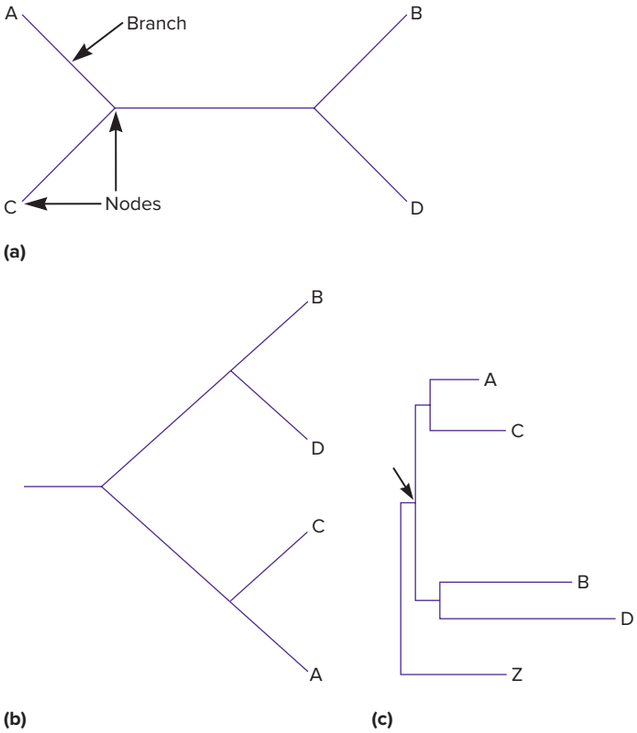


Figure 1.9 Phylogenetic Tree Topologies. (a) Unrooted tree joining four taxonomic units. (b) Rooted tree. (c) The tree shown in (a) can be rooted by adding an outgroup, represented by Z. The arrow indicates a speciation event that resulted in the develop of new species from an ancestral organism.

*c*ommon *a*ncestor (LUCA) to all three domains should be placed. LUCA is on the bacterial branch, which means that Archaea and Eukarya evolved independently, separate from Bacteria. Thus the universal phylogenetic tree presents a picture in which all life, regardless of eventual domain, arose from a single common ancestor. One can envision the universal tree of life as a real tree that grows from a single seed.

The evolutionary relationship of Archaea and Eukarya is still the matter of considerable debate. According to the universal phylogenetic tree we show here, Archaea and Eukarya shared common ancestry but diverged and became separate domains. Other versions suggest that Eukarya evolved out of Archaea. The close evolutionary relationship of these two forms of life is still evident in the manner in which they process genetic information. For instance, certain protein subunits of archaeal and eukaryotic RNA polymerases, the enzymes that catalyze RNA synthesis, resemble each other to the exclusion of those of bacteria. However, archaea have other features that are most similar to their counterparts in bacteria (e.g., mechanisms for conserving energy). This has further complicated and fueled the debate. The evolution of the nucleus and endoplasmic reticulum is also controversial. However, hypotheses regarding the evolution of other membrane-bound organelles are more widely accepted and are considered next.

Mitochondria, Mitochondria-Like Organelles, and Chloroplasts Evolved from Endosymbionts

The **endosymbiotic hypothesis** is generally accepted as the origin of several eukaryotic organelles, including mitochondria, chloroplasts, and hydrogenosomes. **Endosymbiosis** is an interaction between two organisms in which one organism lives inside the other. The original endosymbiotic hypothesis proposed that over time a bacterial endosymbiont of an ancestral cell in the eukaryotic lineage lost its ability to live independently, becoming either a mitochondrion, if the intracellular bacterium used aerobic respiration, or a chloroplast, if the endosymbiont was a cyanobacterium (**figure 1.10**).

Although the mechanism by which the endosymbiotic relationship was established is unknown, there is considerable evidence to support this hypothesis. Mitochondria and chloroplasts contain DNA and ribosomes; both are similar to bacterial DNA and ribosomes. Peptidoglycan, the unique bacterial cell wall molecule, has even been found between the two membranes that enclose the chloroplasts of some algae. Indeed, inspection of figure 1.2 shows that both organelles belong to the bacterial lineage based on SSU rRNA analysis. More specifically, mitochondria are most closely related to bacteria called proteobacteria.

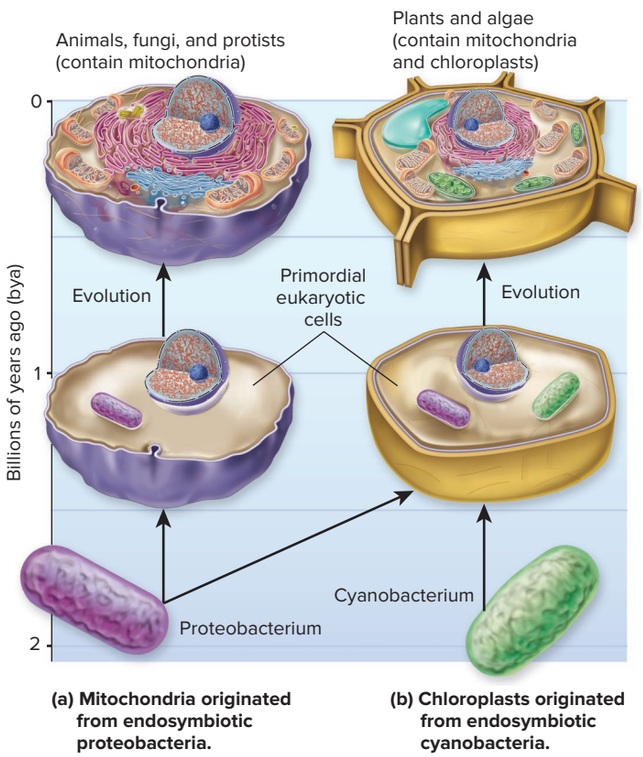


Figure 1.10 The Endosymbiotic Theory. (a) According to this hypothesis, mitochondria derived from a bacterium in the phylum Proteobacteria. (b) A similar phenomenon occurred for chloroplasts, which derived from cyanobacteria.

The chloroplasts of plants and green algae are thought to have descended from an ancestor of the cyanobacterial genus *Prochloron,* which contains species that live within marine invertebrates. ▶| *Phylum Cyanobacteria: oxygenic photosynthetic bacteria (section 15.4)*

The endosymbiotic hypothesis for mitochondria has been refined by the **hydrogen hypothesis.** This asserts that the endosymbiont was an anaerobic bacterium that produced H_2 and CO_2 as end products of its metabolism. Over time, the host became dependent on the H_2 produced by the endosymbiont. Ultimately the endosymbiont evolved into one of several organelles (*see figure 4.11*). If the endosymbiont developed the capacity to perform aerobic respiration, it evolved into a mitochondrion. Other endosymbionts evolved into other organelles such as a hydrogenosome—an organelle found in some extant protists that produces ATP by a process called fermentation *(see figure 4.13).*

Evolution of Cellular Microbes

Although the history of early cellular life forms may never be known, we know that once they arose, they were subjected to the same evolutionary processes as modern organisms. The ancestral bacteria, archaea, and eukaryotes possessed genetic information that could be duplicated, lost, or mutated in other ways. These mutations could have many outcomes. Some led to the death of the microbe, but others allowed new functions and characteristics to evolve. Those mutations that allowed the organism to increase its reproductive ability were selected and passed on to subsequent generations. In addition to selective forces, geographic isolation of populations allowed some groups to evolve separately from others. Thus selection and isolation led to the eventual development of new collections of genes (i.e., genotypes) and many new species.

In addition to mutation, other mechanisms exist for reconfiguring the genotypes of a species and therefore creating genetic diversity. Most eukaryotic species increase their genetic diversity by reproducing sexually. Thus each offspring of the two parents has a mixture of parental genes and a unique genotype. Bacteria and archaea do not reproduce sexually. They increase their genetic diversity by mutation and horizontal (or lateral) gene transfer (HGT). During HGT, genetic information from a donor organism is transferred to a recipient, creating a new genotype. Thus genetic information is passed between individuals of the same generation and even between species found in different domains of life. Genome sequencing has revealed that HGT has played an important role in the evolution of all microbial species. Importantly, HGT still occurs in bacteria and archaea leading to the rapid evolution of microorganisms with antibiotic resistance, new virulence properties, and novel metabolic capabilities. The outcome of HGT is that most microbes have mosaic genomes composed of bits and pieces of the genomes of other organisms. ▶| *Horizontal gene transfer: creating genetic variation the asexual way (section 12.4)*

Microbial Taxonomy

The science of classifying living things is called **taxonomy.** Taxonomy consists of three separate but interrelated parts: classification, nomenclature (naming), and identification. A taxonomic scheme is used to arrange organisms into groups called taxa (s., **taxon**) based on mutual similarity. Microbes are placed in taxonomic levels arranged in a nonoverlapping hierarchy so that each level includes not only the traits that define the rank above it but also a new set of more restrictive traits (**figure 1.11**). Thus within each domain—Bacteria, Archaea, or Eukarya—each organism is assigned (in descending order) to a phylum, class, order, family, genus, and species epithet or name. Some microbes are also given a subspecies designation. Microbial groups at each level have a specific suffix that indicates rank or level.

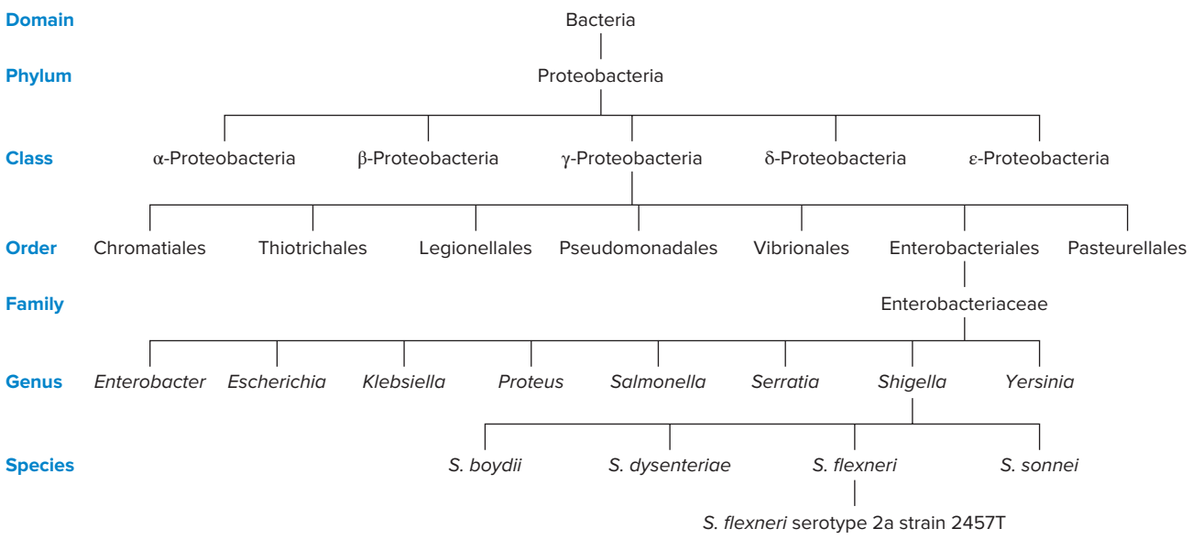


Figure 1.11 Hierarchical Arrangement in Taxonomy. In this example, members of the genus *Shigella* are placed within higher taxonomic ranks. Not all classification possibilities are given for each rank to simplify the diagram. Note that *-ales* denotes order and *-ceae* indicates family.

Microbiologists name microbes using the binomial system of the eighteenth-century biologist and physician Carl Linnaeus. The Latin, italicized name consists of two parts. The first part, which is capitalized, is the generic name (i.e., the name of the genus to which the microbe belongs), and the second is the uncapitalized species epithet. For example, the bacterium that causes plague is called *Yersinia pestis*. Often the name of an organism will be shortened by abbreviating the genus name with a single upper case letter (e.g., *Y. pestis*).

This straightforward organizational approach is complicated by the fact that bacteria and archaea do not reproduce sexually. You may recall from a general biology class that plant and animal **species** are defined as a group of interbreeding or potentially interbreeding natural populations reproductively isolated from other groups. This definition also is appropriate for the many eukaryotic microbes that reproduce sexually. However, bacterial and archaeal species cannot be defined by this criterion, since they do not reproduce sexually. Therefore, comparisons of genome sequences are often used to distinguish one species from another. An appropriate definition is currently hotly debated. A common definition is that bacterial and archaeal species are a collection of strains that share many stable properties and differ significantly from other groups of strains. A **strain** consists of the descendants of a single, pure microbial culture. Strains within a species may be described in a number of different ways. Biovars are variant strains characterized by biochemical or physiological differences, morphovars differ morphologically, serovars have distinctive properties that can be detected by antibodies, and pathovars are pathogenic strains distinguished by the plants in which they cause disease.

Although microbiologists continue to use Linnaeus's classification system, the ongoing explosion in metagenomic analysis has had an impact on this historically accepted hierarchy. Within the last few decades, thousands of 16S rRNA genes and protein-coding genes have been sequenced that do not belong to any previously defined taxa. This data explosion has led to the recent development of the taxonomic classification **superphylum,** below domain and above phylum (e.g., in figure 1.11, superphylum would be placed between Bacteria and Proteobacteria). Ideally a superphylum includes organisms of several phyla that share a number of distinctive characteristics, such as unusual morphological or metabolic features. However, some feel that the term is being loosely applied based on insufficient data—for instance, to SSU rRNA sequences alone.

Comprehension Check

1. Describe two reasons RNA is thought to be the first self-replicating biomolecule.
2. Why and how is SSU rRNA used to determine the relatedness between microorganisms?
3. Does a rooted or unrooted phylogenetic tree provide more information? Explain your answer.
4. Explain the endosymbiotic hypothesis of the origin of mitochondria, hydrogenosomes, and chloroplasts. List two pieces of evidence that support this hypothesis.
5. What is the difference between mutation and horizontal gene transfer?
6. What is the correct way to write this microbe's name: *bacillus subtilis,* Bacillus subtilis, *Bacillus Subtilis,* or *Bacillus subtilis?* Identify the genus name and the species epithet.
7. What is a superphylum and why is it controversial?

1.3 Microbiology Advanced as New Tools for Studying Microbes Were Developed

After reading this section, you should be able to:

- **a.** Evaluate the importance of the contributions to microbiology made by Hooke, Leeuwenhoek, Pasteur, Lister, Koch, Beijerinck, von Behring, Kitasato, Metchnikoff, and Winogradsky
- **b.** Outline a set of experiments that might be used to decide if a particular microbe is the causative agent of a disease
- **c.** Predict the difficulties that might arise when using Koch's postulates to determine if a microbe causes a disease unique to humans

Even before microorganisms were seen, some investigators suspected their existence and role in disease. Among others, the Roman philosopher Lucretius (about 98–55 BCE) and the physician Girolamo Fracastoro (1478–1553) suggested that disease was caused by invisible living creatures. However, until microbes could actually be seen and studied in some other way, their existence remained a matter of conjecture. Therefore **microbiology** is defined not only by the organisms it studies but also by the tools used to study them. The development of microscopes was the critical first step in the evolution of the discipline. However, microscopy alone is unable to answer the many questions scientists ask about microbes. A distinct feature of microbiology is that microorganisms are usually removed from their normal habitats and grown in isolation, apart from all other microbes. This is called a **pure** or **axenic culture.** Although the development of techniques for isolating microbes in pure culture was another critical step in microbiology's history, it is now recognized as having limitations. Microbes in pure culture are in some ways like animals in a zoo; just as a zoologist cannot fully understand animals by studying them in zoos, microbiologists cannot fully understand microbes by studying them in pure culture. Today molecular genetic techniques and genomic analyses are providing new insights into the lives of microbes.

Here we describe how the tools used by microbiologists have influenced the development of the field. As microbiology evolved as a science, it contributed greatly to the well-being of humans. The historical context of some of the important discoveries in microbiology is shown in **figure 1.12**.

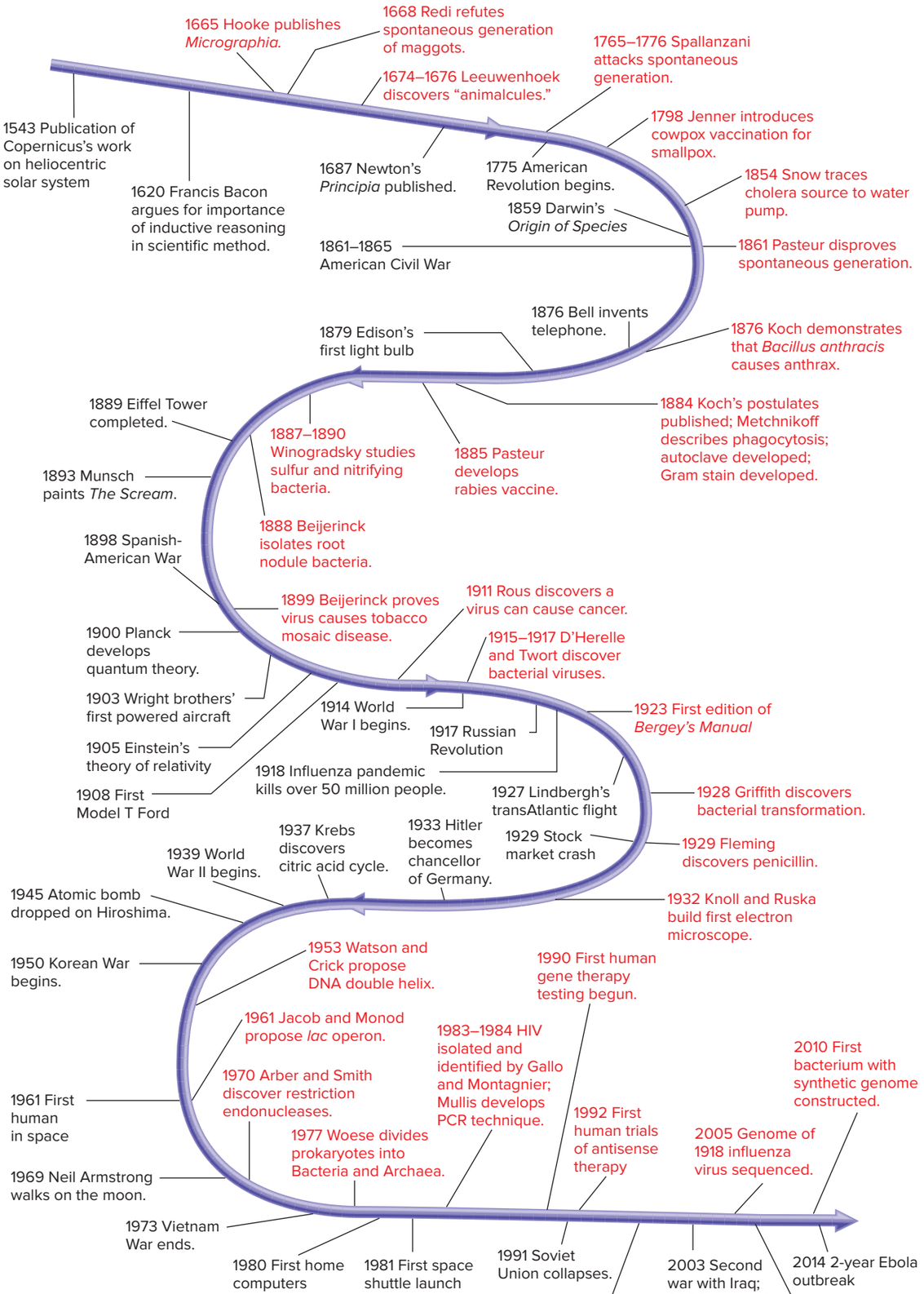
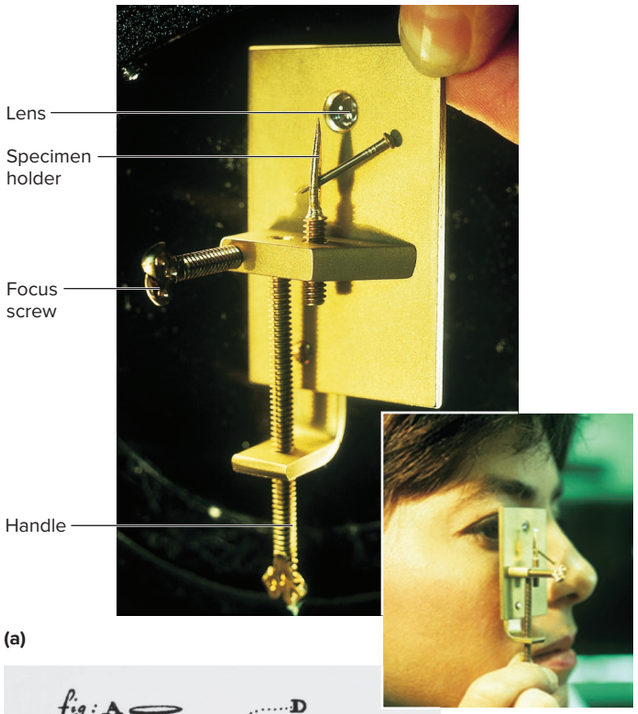


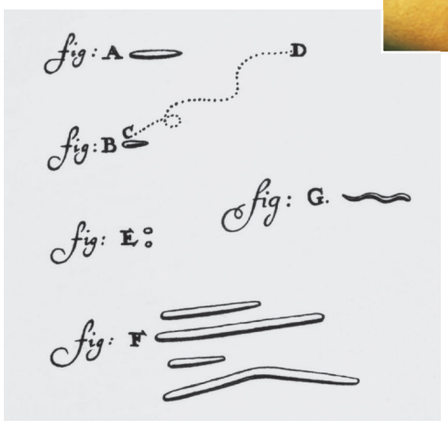
Figure 1.12 **Some Important Events in the Development of Microbiology.**
Milestones in microbiology are marked in red and are discussed within this textbook; other historical events are in black.

Microscopy Led to the Discovery of Microorganisms

The earliest microscopic observations of organisms appear to have been made between 1625 and 1630 on bees and weevils by the Italian Francesco Stelluti (1577–1652), using a microscope probably supplied by Galileo (1564–1642). Robert Hooke (1635–1703) is credited with publishing the first drawings of microorganisms in the scientific literature. In 1665 he published a highly detailed drawing of the fungus *Mucor* in his book *Micrographia. Micrographia* is important not only for its exquisite drawings but also for the information it provided on building microscopes. One design discussed in *Micrographia* was probably a prototype for the microscopes built and used by the amateur microscopist Antony van Leeuwenhoek (1632–1723) of Delft, the Netherlands. Leeuwenhoek earned his living selling men's clothing and accessories but spent much of his spare time constructing simple microscopes composed of double convex glass lenses held between two silver plates (**figure 1.13*a***). His microscopes could magnify about 50 to 300 times, and he may have illuminated his liquid specimens by placing them between two pieces of glass and shining light on them at a 45-degree angle to the specimen plane. This would have provided a form of dark-field illumination whereby organisms appeared as bright objects against a dark background. Beginning in 1673, Leeuwenhoek sent detailed letters describing his discoveries to the Royal Society of London. It is clear from his descriptions that he saw both bacteria and protists (figure 1.13*b*). ▶| *Dark-field microscope: bright object, dark background (section 2.2)*



(a)



(b)

Figure 1.13 van Leeuwenhoek's Microscope and Drawings. (a) A brass replica of the Leeuwenhoek microscope. Inset photo shows how it is held. (b) Leeuwenhoek's drawings of bacteria from the human mouth. (a) Kathy Park Talaro/Pasadena City College; (b) Dr. Jeremy Byrgess/SPL/Getty Images

Culture-Based Methods for Studying Microorganisms Were a Major Development

As important as Leeuwenhoek's observations were, the development of microbiology essentially languished for the next 200 years until techniques for isolating and culturing microbes in the laboratory were formulated. Many of these techniques were developed as scientists grappled with the conflict over the theory of spontaneous generation. This conflict and the subsequent studies on the role played by microorganisms in causing disease ultimately led to what is now called the golden age of microbiology.

Spontaneous Generation

From earliest times, people had believed in **spontaneous generation**—that living organisms could develop from nonliving matter. This view finally was challenged by the Italian physician Francesco Redi (1626–1697), who carried out a series of experiments on decaying meat, which was thought to produce maggots spontaneously. Using covered and uncovered containers of meat, Redi clearly demonstrated that maggots on decaying meat resulted from the presence of fly eggs, and meat did not spontaneously generate maggots. Other experiments helped discredit the theory for larger organisms. However, Leeuwenhoek's communications on microorganisms renewed the controversy. Some proposed that microbes arose by spontaneous generation but larger organisms did not. They pointed out that boiled extracts of hay or meat gave rise to microorganisms after sitting for a while. In 1748 the English priest John Needham (1713–1781) suggested that the organic matter in these extracts contained a "vital force" that could confer the properties of life on nonliving matter.

A few years after Needham's experiments, the Italian priest and naturalist Lazzaro Spallanzani (1729–1799) sealed glass flasks that contained water and seeds and then placed the flasks in boiling water for about 45 minutes. He found that no growth took place as long as the flasks remained sealed. He proposed that air carried germs to the culture medium but also commented that external air might be required for growth of animals already

Figure 1.14 Louis Pasteur.
Pixtal/age fotostock

in the medium. The supporters of spontaneous generation responded that heating the air in sealed flasks destroyed its ability to support life, and therefore did not discredit the theory of spontaneous generation.

In the mid-1800s, several investigators attempted to counter such arguments. These experiments involved allowing air to enter a flask containing a nutrient solution after boiling. The air was either also very hot or it was filtered through sterile cotton wool. In all cases, no microbial growth occurred in the medium. Despite these experiments, the French naturalist Felix Pouchet (1800–1872) claimed in 1859 to have carried out experiments conclusively proving that microbial growth could occur without contact with air.

Pouchet's claim provoked Louis Pasteur (1822–1895) to settle the matter of spontaneous generation. Pasteur (**figure 1.14**) first filtered air through cotton and found that objects resembling plant spores had been trapped. If a piece of the cotton was placed in sterile medium after air had been filtered through it, microbial growth occurred. Next he placed nutrient solutions in flasks, heated their necks in a flame, and pulled them into a variety of curves. The swan-neck flasks he produced in this way remained open to the atmosphere (**figure 1.15**). Pasteur then boiled the solutions and allowed them to cool. No growth took place even though the contents of the flasks were exposed to the air. Pasteur inferred that growth did not occur because dust and germs had been trapped on the walls of the curved necks. If the necks were broken, growth commenced immediately. Pasteur had not only resolved the controversy by 1861 but also had shown how to keep solutions sterile.

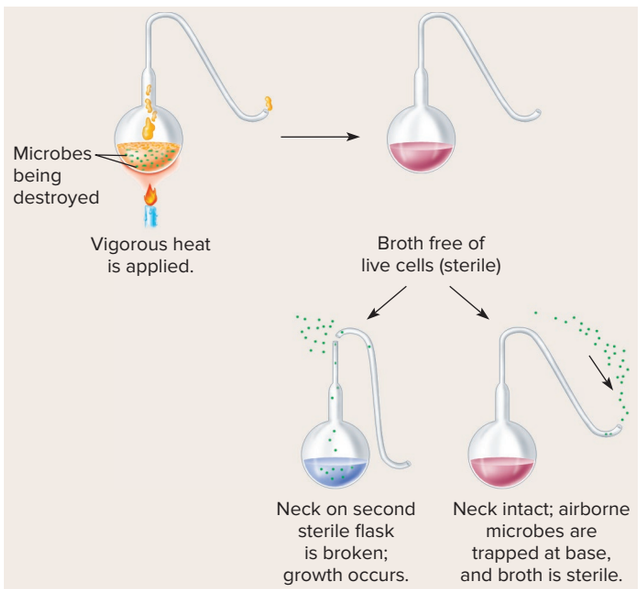


Figure 1.15 Pasteur's Experiments with Swan-Neck Flasks.

The English physicist John Tyndall (1820–1893) and the German botanist Ferdinand Cohn (1828–1898) dealt the final blow to spontaneous generation. In 1877 Tyndall demonstrated that dust did indeed carry germs and that if dust was absent, broth remained sterile even if directly exposed to air. During the course of his studies, Tyndall provided evidence for the existence of exceptionally heat-resistant forms of bacteria. Working independently, Cohn discovered that the heat-resistant bacteria recognized by Tyndall were species capable of producing bacterial endospores. Cohn later played an instrumental role in establishing a classification system for bacteria based on their morphology and physiology. ▶| *Bacterial endospores are a survival strategy (section 3.9)*

These early microbiologists not only disproved spontaneous generation but also contributed to the rebirth of microbiology. They developed liquid media and the methods for sterilizing it so that microbes could be cultured. These techniques were next applied to understanding the role of microorganisms in disease.

Microorganisms and Disease

For hundreds of years, most people believed that disease was caused by supernatural forces, poisonous vapors, and imbalances among the four humors thought to be present in the body. The role of the four humors (blood, phlegm, yellow bile [choler], and black bile [melancholy]) in disease had been widely accepted since the time of the Greek physician Galen (129–199). Support for the idea that microorganisms cause disease—that is, the germ theory of disease—began to accumulate in the early nineteenth century from diverse fields. Agostino Bassi (1773–1856) demonstrated in 1835 that a silkworm disease was due to a fungal infection. In 1845 M. J. Berkeley (1803–1889) proved that the great potato blight of Ireland was caused by a protozoan (then thought to be a fungus), and in 1853 Heinrich de Bary (1831–1888) showed that fungi caused cereal crop diseases.

Pasteur also contributed to this area of research in what may seem an unlikely way. Pasteur was trained as a chemist and spent many years studying the fermentations that yield ethanol and are used in the production of wine and other alcoholic beverages. When he began his work, leading chemists were convinced that fermentation was due to a chemical instability in sugars that resulted in their breakdown into alcohol. Pasteur did not agree; he believed that fermentations were carried out by living organisms.

In 1856 M. Bigo, an industrialist in Lille, France, where Pasteur worked, requested Pasteur's assistance. His business produced ethanol from the fermentation of beet sugars, and the alcohol yields had recently declined and the product had

become sour. Pasteur discovered that the fermentation was failing because the yeast normally responsible for alcohol formation had been replaced by bacteria that produced acid rather than ethanol. In solving this practical problem, Pasteur demonstrated that fermentations were due to the activities of yeasts and bacteria.

Pasteur was also called upon by the wine industry in France for help. For several years, poor-quality wines had been produced. Pasteur referred to the wines as diseased and demonstrated that particular wine diseases were linked to particular microbes contaminating the wine. He eventually suggested a method for heating the wines to destroy the undesirable microbes. The process is now called pasteurization.

Indirect evidence for the germ theory of disease came from the work of the English surgeon Joseph Lister (1827–1912) on the prevention of wound infections. Lister, impressed with Pasteur's studies on fermentation, developed a system of antiseptic surgery designed to prevent microorganisms from entering wounds. Instruments were heat sterilized, and phenol was used on surgical dressings and at times sprayed over the surgical area. The approach was remarkably successful in reducing the rate of surgical infection. It also provided strong indirect evidence for the role of microorganisms in disease.

Koch's Postulates

The first direct demonstration that bacteria cause disease came from the study of anthrax by the German physician Robert Koch (pronounced "Koke"; 1843–1910). Koch (**figure 1.16**) used the criteria proposed by his former teacher Jacob Henle (1809–1885) and others to establish the relationship between *Bacillus anthracis* and anthrax. In these studies, published in 1876, Koch used mice as his model or test organism. Following the steps outlined in **figure 1.17**, he next used guinea pigs to show that *Mycobacterium tuberculosis* causes tuberculosis (TB), which at that time was a leading cause of death in Europe. In 1905 Koch was awarded the Nobel Prize in Physiology or Medicine and his criteria for proving the causal relationship between a microorganism and a specific disease are known as **Koch's postulates.** Koch's postulates have since been used to discover the causative microorganisms for many infectious diseases.

Figure 1.16 Robert Koch. Koch examining a specimen in his laboratory.
Bettmann/Getty Images

While Koch's postulates are still widely used, their application is at times not feasible. For instance, viruses and organisms that must live within host cells such as *Mycobacterium leprae,* the causative agent of leprosy, cannot be isolated in pure culture. Some human diseases lack an appropriate animal model so the postulates cannot be fully met. To avoid some of these difficulties, microbiologists sometimes use molecular and genetic evidence. For instance, molecular methods might be used to detect the nucleic acid of a microorganism in body tissues, rather than isolating it, or the genes thought to be associated with the virulence of a disease-causing microbe (pathogen) might be mutated. In this case, the mutant organism should have decreased ability to cause disease. Introduction of the normal gene back into the mutant should restore the pathogen's virulence.

Our focus thus far has been on the discovery of bacteria, fungi, and protists. But viral pathogens were also being studied during this time. The discovery of viruses and their role in disease was made possible when Charles Chamberland (1851–1908), one of Pasteur's associates, constructed a porcelain filter to remove bacteria. Dimitri Ivanowski (1864–1920) and Martinus Beijerinck (pronounced "by-a-rink"; 1851–1931) used the filter to study tobacco mosaic disease. They found that plant extracts and sap from diseased plants were infectious, even after being filtered with Chamberland's filter. Because the infectious agent passed through a filter that trapped bacterial cells, they reasoned that the agent must be something smaller than a bacterium. Beijerinck proposed that the agent was a "filterable virus" (Latin *virus,* slimy liquid, poison). Eventually viruses were shown to be tiny, acellular infectious agents.

Immunology

The ability to culture microbes also played an important role in early immunological studies. During studies on the bacterium that causes chicken cholera, Pasteur and Pierre Roux (1853–1933) discovered that bacteria incubated in cultures for long periods of time lost their ability to cause disease. These bacteria were said to be attenuated or weakened. When chickens were injected with attenuated bacteria, they remained healthy and were surprisingly able to resist the disease when exposed to virulent bacteria. Pasteur called the attenuated bacteria a vaccine (Latin *vacca,* cow) in honor of Edward Jenner (1749–1823) because, many years earlier, Jenner had used material from cowpox lesions to protect people against smallpox (*see Historical Highlights 26.5*). Shortly after this, Pasteur and Chamberland developed an attenuated anthrax vaccine. ▶| *Vaccines immunize susceptible populations (section 26.6)*

Postulate	Experimentation
1. The microorganism must be present in every case of the disease but absent from healthy organisms.	Koch developed a staining technique to examine human tissue. *Mycobacterium tuberculosis* could be identified in diseased tissue.
2. The suspected microorganisms must be isolated and grown in a pure culture.	Koch grew *M. tuberculosis* in pure culture on coagulated blood serum.
3. The same disease must result when the isolated microorganism is inoculated into a healthy host.	Koch injected cells from the pure culture of *M. tuberculosis* into guinea pigs. The guinea pigs subsequently died of tuberculosis.
4. The same microorganisms must be isolated again from the diseased host.	Koch isolated *M. tuberculosis* in pure culture on coagulated blood serum from the dead guinea pigs.

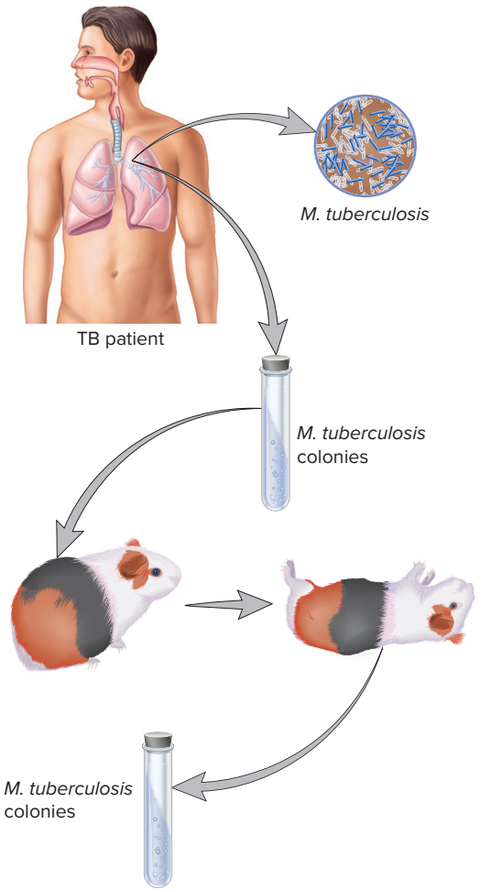


Figure 1.17 **Koch's Postulates Applied to Tuberculosis.**

MICRO INQUIRY *Why is the fourth postulate necessary?*

Pasteur also prepared a vaccine using an attenuated strain of rabies virus. During the course of these studies, a nine-year-old boy named Joseph Meister was bitten by a rabid dog and was brought to Pasteur. Since Joseph's death was certain in the absence of treatment, Pasteur agreed to try vaccination. Joseph was injected 13 times over the next 10 days with increasingly virulent preparations of the attenuated virus. His survival marked a huge advance in the use of vaccines. To thank Pasteur, people from around the world contributed to the construction of the Pasteur Institute in Paris, France. One of the initial tasks of the institute was vaccine production.

These early advances in immunology were made without any concrete knowledge about how the immune system works. Immunologists now know that white blood cells and the chemicals they produce play a central role in immunity. Among the chemicals are soluble proteins called antibodies, found in blood, lymph, and other body fluids. The role of soluble substances in preventing disease was recognized by Emil von Behring (1854–1917) and Shibasaburo Kitasato (1852–1931). After the discovery that diphtheria was caused by a toxin produced by bacteria, they injected inactivated diphtheria toxin into rabbits. The inactivated toxin induced rabbits to produce an antitoxin, which protected against the disease. Antitoxins are now known to be antibodies that specifically bind and neutralize toxins. The first immune system cells were discovered when Élie Metchnikoff (1845–1916) found that some white blood cells could engulf disease-causing bacteria. He called these cells phagocytes and the process phagocytosis (Greek *phagein,* eating).

Microbial Ecology

Early microbial ecologists studied microbial involvement in the carbon, nitrogen, and sulfur cycles. The Russian microbiologist Sergei Winogradsky (1856–1953) made many contributions to soil microbiology, including the discovery that soil bacteria could oxidize iron, sulfur, and ammonia to obtain energy and that many of these bacteria could incorporate CO_2 into organic matter much as photosynthetic organisms do. Winogradsky also isolated anaerobic nitrogen-fixing soil bacteria and studied the decomposition of cellulose. Martinus Beijerinck made fundamental

contributions to microbial ecology as well as virology. He isolated several kinds of nitrogen-fixing bacteria and sulfate-reducing bacteria. Beijerinck and Winogradsky also developed enrichment culture techniques and selective media, which have been of great importance in microbiology. ▶| *Biogeochemical cycling sustains life on Earth (section 20.1); Culture media (section 5.7); Enrichment cultures (section 5.7)*

Comprehension Check

1. What did Pasteur prove when he showed that a cotton plug that had filtered air would trigger microbial growth when transferred to a sterile medium? What argument made previously was he addressing?
2. Discuss the contributions of Lister, Pasteur, and Koch to the germ theory of disease and the treatment or prevention of diseases.
3. What role did the ability to grow bacteria in pure culture play in the develoment of Koch's postulates?
4. What did Jenner, Pasteur, von Behring, Kitasato, and Metchnikoff contribute to the development of immunology?
5. How did Winogradsky and Beijerinck contribute to the study of microbial ecology? What new culturing techniques did they develop in their studies?

1.4 Microbiology Encompasses Many Subdisciplines

After reading this section, you should be able to:

a. Construct a concept map, table, or drawing that illustrates the diverse nature of microbiology and how it has improved human conditions

b. Discuss the opinion held by many microbiologists that microbiology is experiencing its second golden age

Microbiology today is as diverse as the organisms it studies. It has both basic and applied aspects. The basic aspects are concerned with the biology of microorganisms themselves. The applied aspects are concerned with practical problems such as disease, water and wastewater treatment, food spoilage and food production, and industrial uses of microbes. Despite this apparent dichotomy, the basic and applied aspects of microbiology are intertwined. Basic research is often conducted in applied fields, and applications often arise out of basic research.

An important development in microbiology is the increasing use of molecular and genomic methods to study microbes and their interactions with other organisms. These methods have led to a time of rapid advancement that rivals the golden age of microbiology. Indeed, many feel that microbiology is in its second golden age. ▶| *Microbial DNA technologies (chapter 31); Microbial genomics (chapter 32)*

Major Fields in Microbiology

Microbiology is commonly divided into subdisciplines based on the type of microbe studied. Thus microbiology encompasses bacteriology and virology as well as other microbe-specific fields. Microbiology can also be divided based on the activities of microbes—for instance, environmental microbiology and agricultural microbiology. Finally, microbiologists may study only one aspect of the biology of microbes, leading to subdisciplines such as microbial genetics and microbial physiology.

One of the most active and important fields in microbiology is medical microbiology, which deals with diseases of humans. Medical microbiologists investigate agents causing infectious diseases and measures for their control and elimination. They are involved in tracking down new, unidentified pathogens such as those causing hantavirus pulmonary syndrome, West Nile encephalitis, and Zika virus. These microbiologists also study how microorganisms cause disease. Clinical laboratory scientists, the microbiologists who work in hospital and other clinical laboratories, use culture and molecular techniques to provide information needed by physicians to diagnose and treat infectious disease.

Major epidemics have regularly affected human history. For example, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) has killed over 35 million people since the beginning of the HIV epidemic in 1981. Public health microbiology is concerned with the control and spread of such communicable diseases. Public health microbiologists and epidemiologists monitor the amount of disease in populations. Based on their observations, they can detect outbreaks and epidemics as they begin, and implement appropriate control measures. They also conduct surveillance for new diseases as well as bioterrorism events. Public health microbiologists working for local governments monitor community food establishments and water supplies to ensure they are safe and free from pathogens. ▶| *Epidemiology and public health microbiology (chapter 26)*

To understand, treat, and control infectious disease, it is important to understand how the immune system protects the body from pathogens; this question is the concern of immunology. Immunology is one of the fastest growing areas in science. Many advances have been in response to the discovery of HIV, which specifically targets cells of the immune system. Immunology also deals with the nature and treatment of allergies and autoimmune diseases such as rheumatoid arthritis. ▶| *Innate host resistance (chapter 22); Adaptive immunity (chapter 23)*

Microbial ecology is another important field in microbiology. Microbial ecologists employ a variety of culture and molecular approaches to describe the vast diversity of microbes in terms of their morphology, physiology, and relationships with organisms and the components of their habitats. The importance of microbes in local and global cycling of carbon, nitrogen, and sulfur has been long studied; however, these studies have acquired new urgency as our climate changes. Of particular interest is the role of microbes in both the production and removal of greenhouse gases such as carbon dioxide and methane. Microbial ecologists also are employing microorganisms in bioremediation to reduce pollution. An exciting frontier in microbial ecology is the study of the microbes normally associated with the human body—the human microbiome. ▶| *Global climate change (section 20.2); Human microbiome and host*

interactions (chapter 24); Biodegradation and bioremediation harness microbes to clean the environment (section 30.7)

Agricultural microbiology is a field related to both medical microbiology and microbial ecology. It is concerned with the impact of microorganisms on food production, such as nitrogen-fixing bacteria, which affect soil fertility. Other microbes live in the digestive tracts of ruminants such as cattle and break down the plant materials these animals ingest. There are also plant and animal pathogens that have significant economic impact if not controlled. Furthermore, some pathogens of domestic animals also cause human disease. Agricultural microbiologists work on methods to increase soil fertility and crop yields, study rumen microorganisms to increase meat and milk production, and try to combat plant and animal diseases. Currently many agricultural microbiologists are studying the use of bacterial and viral insect pathogens as substitutes for chemical pesticides. ▶| *Microorganisms in natural ecosystems (chapter 21)*

In addition to agricultural microbiology, food microbiology has contributed to the ready supply of high-quality foods. Food microbiologists study the microbes used to make food and beverages (e.g., yogurt, cheese, beer) as well as the microbes that cause food spoilage or are pathogens that are spread through food. For example, periodic outbreaks of certain *Escherichia coli* strains have led to renal failure and death. To protect the public, the specific strain must be traced back to the contaminated food source. Food microbiologists also work to prevent microbial spoilage of food and conduct research on the use of microorganisms as nutrient sources for livestock and humans. ▶| *Microbiology of food (chapter 29)*

Industrial microbiology involves the use of microbes to make products helpful to humans. An important advance occurred in 1929 when Alexander Fleming rediscovered that the fungus *Penicillium* sp. produced what he called penicillin, the first antibiotic that could successfully control bacterial infections. Although it took World War II for scientists to learn how to mass-produce penicillin, scientists soon found other microorganisms capable of producing additional antibiotics. Today industrial microbiologists also use microorganisms to make products such as vaccines, steroids, alcohols and other solvents, vitamins, amino acids, enzymes, and biofuels. These alternative fuels are renewable and may help decrease pollution associated with burning fossil fuels. ▶| *Biofuel production is a dynamic field (section 30.2); Microbial fuel cells: batteries powered by microbes (section 30.3)*

The advances in medical microbiology, agricultural microbiology, food and dairy microbiology, and industrial microbiology are outgrowths of the labor of many microbiologists doing basic research in areas such as microbial physiology, microbial genetics, molecular biology, and bioinformatics. Microbial physiologists study many aspects of the biology of microorganisms, including their diverse metabolic capabilities. They also study the synthesis of antibiotics and toxins, the ways in which microorganisms survive harsh environmental conditions, and the effects of chemical and physical agents on microbial growth and survival. Microbial geneticists, molecular biologists, and bioinformaticists study the nature of genetic information and how it regulates the development and function of cells and organisms. The bacteria *E. coli* and *Bacillus subtilis,* the yeast *Saccharomyces cerevisiae* (baker's yeast), and bacterial viruses such as T4 and lambda continue to be important model organisms used to understand biological phenomena.

The future of microbiology is bright. Genomics has revolutionized biology, as scientists are now beginning to understand organisms holistically, rather than in a reductionist, piecemeal manner. How the genomes of microbes evolve, the nature of host-pathogen interactions, the minimum set of genes required for an organism to survive, and many more topics are aggressively being examined by molecular and genomic analyses. This is an exciting time to be a microbiologist. Enjoy the journey.

Comprehension Check

1. Briefly describe the major subdisciplines in microbiology. Which do you consider to be applied fields? Which are basic?
2. Log all the microbial products you use in a week. Be sure to consider all foods and medications (including vitamins). Consider how your life might be different without these items.
3. List all the activities or businesses you can think of in your community that directly depend on microbiology.

Key Concepts

1.1 Members of the Microbial World

- Microbiology studies microscopic cellular organisms that are often unicellular or, if multicellular, do not have highly differentiated tissues. Microbiology also focuses on biological entities that are acellular (**figure 1.1**).
- Microbiologists divide cellular organisms into three domains: Bacteria, Archaea, and Eukarya (**figure 1.2**).
- Microbes in the domains Bacteria and Archaea lack organelles. Eukaryotic microbes (protists and fungi) are placed in Eukarya. Viruses, viroids, satellites, and prions are acellular entities not placed in any domain but are classified by a separate system.

1.2 Microbes Have Evolved and Diversified for Billions of Years

- Earth is approximately 4.5 billion years old. Within the first 1 billion years of its existence, life arose (**figure 1.4**).
- The RNA world hypothesis posits that the earliest self-replicating entity had RNA enclosed in a lipid bilayer. RNA stores genetic information and conducts cellular processes (**figure 1.5**).

- Comparisons of small subunit (SSU) rRNA genes have been useful in creating universal phylogenetic trees. These trees provide information about the origin and evolution of life (**figure 1.7**).
- Phylogenetic relationships often are shown as branched diagrams called phylogenetic trees. Trees are based on pairwise comparison of amino acid or nucleotide sequences, followed by computer analysis (**figure 1.8**).
- Trees may be either rooted or unrooted. Unrooted trees can be rooted by including an outgroup when the tree is constructed (**figure 1.9**).
- The last universal common ancestor (LUCA) is placed on the bacterial branch of the universal phylogenetic tree (figure 1.2). Thus Bacteria are thought to have diverged first, and Archaea and Eukarya arose later.
- Mitochondria, chloroplasts, and hydrogenosomes are thought to have evolved from bacterial endosymbionts of ancestral cells in the eukaryotic lineage (**figure 1.10**).
- Taxonomic ranks are arranged in a nonoverlapping hierarchy (**figure 1.11**). Species are named using the binomial system of Linnaeus.
- It is difficult to define a bacterial or archaeal species because these microbes do not reproduce sexually.

1.3 Microbiology Advanced as New Tools for Studying Microbes Were Developed

- Microbiology is defined not only by the organisms it studies but also by the tools it uses. Microscopy and culture-based techniques play important roles in the evolution of the discipline.
- Antony van Leeuwenhoek used simple microscopes and was the first person to extensively describe microorganisms (**figure 1.13**).
- Culture-based techniques for studying microbes began to develop as scientists debated the theory of spontaneous generation. Experiments by Francesco Redi and others disproved the theory of spontaneous generation of larger organisms. The spontaneous generation of microorganisms was disproved by Louis Pasteur and others (**figure 1.15**).
- Koch's postulates are used to prove a direct relationship between a suspected pathogen and a disease. Robert Koch and his coworkers developed techniques to grow bacteria on solid media and isolate pure cultures of pathogens (**figure 1.17**).
- Viruses were discovered following the invention of a bacterial filter by Charles Chamberland. Dimitri Ivanowski and Martinus Beijerinck were important contributors to the field of virology.
- Microbiologists founded the field of immunology; they created vaccines and discovered antibodies and phagocytic cells.
- Microbial ecology grew out of the work of Sergei Winogradsky and Beijerinck. They studied the role of microorganisms in carbon, nitrogen, and sulfur cycles and developed enrichment culture techniques and selective media.

1.4 Microbiology Encompasses Many Subdisciplines

- There are many fields in microbiology. These include medical, public health, industrial, and food and dairy microbiology. Microbial ecology, physiology, and genetics are important subdisciplines of microbiology.

Active Learning

1. Why aren't viruses, viroids, satellites, and prions included in the three-domain system?
2. Some individuals can be infected by a pathogen yet not develop disease. In fact, some become chronic carriers of the pathogen. How does this observation affect Koch's postulates? How might the postulates be modified to account for the existence of chronic carriers?
3. Support this statement: "Vaccinations against various childhood diseases have contributed to the entry of women, particularly mothers, into the full-time workplace."
4. For many years, the oldest stromatolites with compelling evidence of ancient life on Earth were found in Western Australia. The microbial communities that built these sedimentary structures were dated at roughly 3.5 billion years old. However, in 2016 stromatolites in Greenland were reported to be 3.7 billion years old. Interestingly, the complexity and morphology of the Greenland stromatolites is similar to that of those in Australia. Why is it of interest to document the oldest evidence of life on Earth? What might this suggest about the evolution of life during the intervening 200 million years? What is the significance, if any, of the geographic distribution of these stromatolites?

   Read the original paper: Nutman, A.P., et al. 2016. Rapid emergence of life shown by discovery of 3,700-million-year-old microbial structures. *Nature*. 537:535–539. doi:10.1038/nature19355.

2

Microscopy

Kenneth Lambert/AP Images

Anthrax Bioterrorism Attack

While on a trip to North Carolina in late September 2001, a sixty-three-year-old photojournalist from Florida began feeling ill. His muscles ached, he felt nauseous, and he had a fever. After returning home, his symptoms worsened considerably. On October 2, he awoke with vomiting and mental confusion. When his wife took him to a local emergency room, doctors performed a spinal tap and collected cerebral spinal fluid (CSF). The fluid was sent to the hospital laboratory where it was stained using the Gram-stain technique. Much to the surprise of the clinical lab scientists, the CSF contained long, Gram-positive rods that formed chains, a morphology unlike that of typical meningitis-causing bacteria. Based on the Gram-stain results, an initial diagnosis of inhalation anthrax was made. This was very surprising because inhalation anthrax is extremely rare in the United States. Doctors immediately began treatment with antibiotics, but the photojournalist's condition worsened, and he died October 5.

Thus began the first anthrax bioterrorism attack in the United States. Over the next several weeks, 16 other people developed inhalation anthrax; four of these individuals died. Much was learned about the disease from this attack. Much was also learned about the nation's readiness to deal with bioterrorism. This event is also a reminder of the continuing importance of microscopy in microbiology and in diagnosing disease.

In this chapter, we introduce some of the most commonly used types of microscopy. We begin with light microscopes and then describe other common types of microscopes, as well as how specimens are prepared for examination by microscopes.

Readiness Check:

Based on what you have learned previously, you should be able to:

- ✓ Explain why microscopy is a major tool used by microbiologists (sections 1.1 and 1.3)
- ✓ Define magnification
- ✓ Express the size of organisms using the metric system (**table 2.1**)

Table 2.1 Common Units of Measurement

Unit	Abbreviation	Value
1 centimeter	cm	10^{-2} meter or 0.394 inches
1 millimeter	mm	10^{-3} meter
1 micrometer	µm	10^{-6} meter
1 nanometer	nm	10^{-9} meter
1 Angstrom	Å	10^{-10} meter

2.1 Lenses Create Images by Bending Light

After reading this section, you should be able to:

- **a.** Relate the refractive indices of glass and air to the path light takes when it passes through a prism or convex lens
- **b.** Correlate lens strength and focal length

Light microscopes were the first microscopes invented and they continue to be the most common. To understand light microscopy, we must consider the way lenses bend and focus light to form images. When a ray of light passes from one medium to another, refraction occurs; that is, the ray is bent at the interface. The **refractive index** is a measure of how much a substance slows the velocity of light; the direction and magnitude of bending are determined by the refractive indices of the two media forming the interface. For example, when light passes from air into glass, which has a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface (**figure 2.1**). As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal.

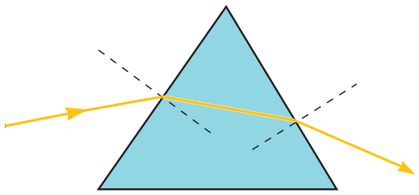


Figure 2.1 The Bending of Light by a Prism. Lines perpendicular to the surface of the prism are called normal; they are indicated by dashed lines. As light enters the glass, it is bent toward the first normal. When light leaves the glass and returns to air, it is bent away from the second normal.

Thus a prism bends light because glass has a different refractive index from air and the light strikes its surface at an angle.

Lenses act like a collection of prisms operating as a unit. When the light source is distant so parallel rays of light strike the lens, a convex lens focuses the rays at a specific point, the focal point (*F* in figure 2.2). The distance between the center of the lens and the focal point is called the focal length (*f* in figure 2.2).

Our eyes cannot focus on objects nearer than about 25 cm (i.e., about 10 inches). This limitation may be overcome by using a convex lens as a simple magnifier (or microscope) and holding it close to an object. A magnifying glass provides a clear image at much closer range, and the object appears larger. Lens strength is related to focal length; a lens with a short focal length magnifies an object more than a lens having a longer focal length.

Comprehension Check

1. How do refraction, refractive index, focal point, and focal length differ?
2. Draw the path of a light ray through a thin and thick lens.
3. If the lenses in question 2 were in corrective eyeglasses, which would be used for reading and which would improve distance vision?

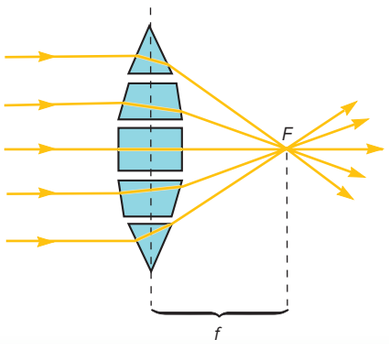


Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point *F*. The focal point lies a distance *f*, the focal length, from the lens center.

MICRO INQUIRY *How would the focal length change if the lens shown here were thicker?*

2.2 There Are Several Types of Light Microscopes

After reading this section, you should be able to:

- **a.** Evaluate the parts of a light microscope in terms of their contributions to image production and use of the microscope
- **b.** Predict the relative degree of resolution based on light wavelength and numerical aperture of the lens used to examine a specimen
- **c.** Create a table that compares and contrasts the various types of light microscopes in terms of their uses, how images are created, and the quality of images produced

Microbiologists currently employ a variety of light microscopes in their work, each designed for specific applications. Modern microscopes are compound microscopes; that is, they have two sets of lenses. The **objective lens** is the lens closest to the specimen. It forms a magnified image that is further enlarged by one or more additional lenses.

Bright-Field Microscope: Dark Object, Bright Background

The **bright-field microscope** is routinely used to examine both stained and unstained specimens. It forms a dark image against a lighter background, thus it has a "bright field." It consists of a metal stand composed of a base and an arm to which the remaining parts are attached (figure 2.3). A light source, either a mirror or an electric illuminator, is located in the base. Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and move either the stage or the nosepiece vertically to focus the image.

The stage is positioned about halfway up the arm. Microscope slides are clipped to the stage, which can be moved during viewing by rotating control knobs. The substage **condenser lens** (or simply, condenser) is within or beneath the stage and focuses a cone of light on the slide. Its position may be fixed in simpler microscopes but can be adjusted vertically in more complex models.

The curved upper part of the arm holds the body assembly, to which a nosepiece and one or more **ocular lenses** (also called **eyepieces**) are attached. Binocular microscopes have eyepieces for both eyes. The body assembly contains a series of mirrors and prisms so the barrel holding the eyepiece may be tilted for ease in viewing. The nosepiece holds three to five objective lenses of differing magnifying power and can be rotated to change magnification. Ideally a microscope should be **parfocal;** that is, the image should remain in focus when objective lenses are changed.

The image seen when viewing a specimen with a compound microscope is created by the objective and ocular lenses working together. Light from the illuminated specimen is focused by the objective lens, creating an enlarged image within the microscope.

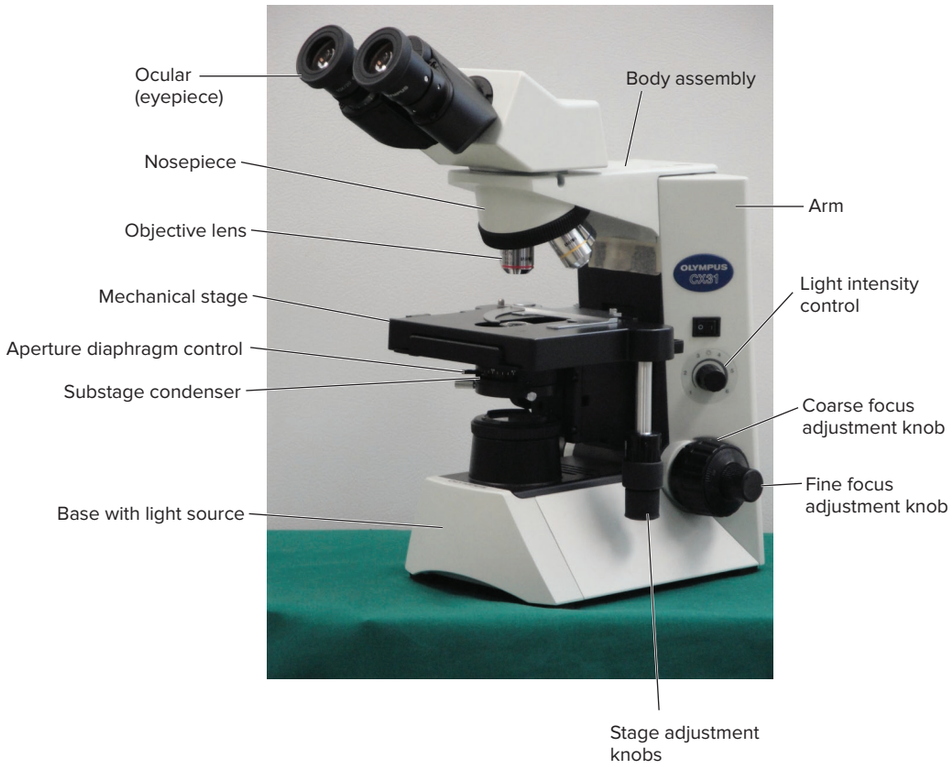


Figure 2.3 **A Bright-Field Microscope.** James Redfearn/McGraw-Hill Education

The ocular lens further magnifies this primary image. The total magnification is calculated by multiplying the objective and eyepiece magnifications. For example, if a 45× objective lens is used with a 10× eyepiece, the overall magnification of the specimen is 450×.

Better Microscope Resolution Means a Clearer Image

The most important part of the microscope is the objective lens, which must produce a clear image, not just a magnified one. **Resolution** is the ability of a lens to separate or distinguish between small objects that are close together. At best, the resolution of a bright-field microscope is 0.2 µm, which is about the size of a very small bacterium. Why is this the case? Resolution is in part dependent on the **numerical aperture** (n sin θ) of a lens. Numerical aperture is defined by two components: n is the refractive index of the medium in which the lens works (e.g., air = 1) and θ is 1/2 the angle of the cone of light entering an objective (figure 2.4). A cone with a narrow angle does not adequately separate the rays of light emanating from closely packed objects, and the images are not resolved. A cone of light with a very wide angle is able to separate the rays, and the closely packed objects appear widely separated and resolved. Recall that the refractive indices of all materials through which light waves pass determine the direction of the light rays emanating from the specimen. Some objective lenses work in air; since sin θ cannot be greater than 1 (the maximum θ is 90° and sin 90° is 1.00), no lens working in air can have a numerical aperture greater than 1.00. The only practical way to raise the numerical aperture above 1.00, and therefore achieve

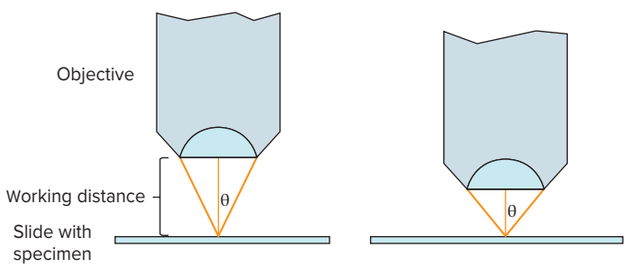


Figure 2.4 **Numerical Aperture in Microscopy.** The numerical aperture of a lens is related to a value called the angular aperture (symbolized by θ), which is 1/2 the angle of the cone of light that enters a lens from a specimen. The equation for numerical aperture is n sin θ. In the right-hand illustration, the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

Table 2.2 Properties of Objective Lenses

	OBJECTIVE			
Property	**Scanning**	**Low Power**	**High Power**	**Oil Immersion**
Magnification	4×	10×	40–45×	90–100×
Numerical aperture	0.10	0.25	0.55–0.65	1.25–1.4
Approximate focal length (*f*)	40 mm	16 mm	4 mm	1.8–2.0 mm
Working distance	17–20 mm	4–8 mm	0.5–0.7 mm	0.1 mm
Approximate resolving power with light of 450 nm (blue light)	2.3 µm	0.9 µm	0.35 µm	0.18 µm

higher resolution, is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass (**table 2.2**). If air is replaced with immersion oil, many light rays enter the objective that would have otherwise escaped due to reflection and refraction at the surfaces of the objective lens (**figure 2.5**). This results in an increase in numerical aperture and resolution.

Resolution is described mathematically by an equation developed in the 1870s by Ernst Abbé (1840–1905), a German physicist responsible for much of the optical theory underlying microscope design. The Abbé equation states that the minimal distance (*d*) between two objects that reveals them as separate entities depends on the wavelength of light (λ) used to illuminate the specimen and on the numerical aperture of the lens ($n \sin \theta$), which is the ability of the lens to gather light.

$$d = \frac{0.5\,\lambda}{n \sin \theta}$$

The smaller *d* is, the better the resolution, and finer detail can be discerned in a specimen; *d* becomes smaller as the wavelength of light used decreases and as the numerical aperture increases. Thus the greatest resolution is obtained using a lens with the largest possible numerical aperture and light of the shortest wavelength, at the blue end of the visible spectrum (in the range of 450 to 500 nm).

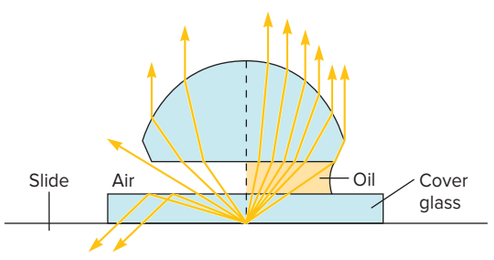


Figure 2.5 The Oil Immersion Objective. An oil immersion objective operating in air and with immersion oil.

Examination of figure 2.4 shows that one objective is much closer to the specimen than the other. Specifically, the objective on the right has a higher numerical aperture and a shorter working distance. The working distance of an objective is the distance between the surface of the lens and the surface of the cover glass (if one is used) or the specimen when it is in sharp focus. As illustrated here, objectives with large numerical apertures and great resolving power have short working distances (table 2.2).

Numerical aperture is also an important feature of a microscope's condenser. The condenser is a large, light-gathering lens used to project a wide cone of light through the slide and into the objective lens. Although most condensers have a numerical aperture between 1.2 and 1.4, the numerical aperture will not exceed about 0.9 unless the top of the condenser is oiled to the bottom of the slide. During routine microscope operation, the condenser usually is not oiled, and this limits the overall resolution, even with an oil immersion objective.

The most accurate calculation of a microscope's resolving power considers both the numerical aperture of the objective lens and that of the condenser, as is evident from the following equation, where NA is the numerical aperture.

$$d_{\text{microscope}} = \frac{\lambda}{(\text{NA}_{\text{objective}} + \text{NA}_{\text{condenser}})}$$

However, in most cases the limit of resolution of a light microscope is calculated using the Abbé equation, which considers the objective lens only. We now see why the maximum theoretical resolving power of a microscope when viewing a specimen using an oil immersion objective (numerical aperture of 1.25) and blue-green light is approximately 0.2 µm.

$$d = \frac{(0.5)(530\text{ nm})}{1.25} = 212\text{ nm or } 0.2\,\mu\text{m}$$

Given the limit of resolution of a light microscope, the largest useful magnification—the level of magnification needed to increase the size of the smallest resolvable object to be visible with the light microscope—can be determined. Our eye can just

detect a speck 0.2 mm in diameter. When the acuity of the eye and the resolution of the microscope are considered together, it is calculated that the useful limit of magnification is about 1,000 times the numerical aperture of the objective lens. Most standard microscopes have 10× eyepieces and have an upper limit of about 1,000× with oil immersion. A 15× eyepiece may be used with good objective lenses to achieve a useful magnification of 1,500×. Any further magnification does not enable a person to see more detail. Indeed, a light microscope can be built to yield a final magnification of 10,000×, but it would simply be magnifying a blur. Only the electron microscope provides sufficient resolution to make higher magnifications useful.

Visualizing Living, Unstained Microbes

Bright-field microscopes are probably the most common microscope found in teaching, research, and clinical laboratories. However, many microbes are unpigmented so are not clearly visible because there is little difference in contrast between the cells, subcellular structures, and water. As we discuss in section 2.3, one solution to this problem is to stain cells before observation. Unfortunately staining procedures usually kill cells. But what if an investigator wishes to view living cells? Three types of light microscopes create detailed, clear images of living specimens: dark-field microscopes, phase-contrast microscopes, and differential interference contrast microscopes.

Dark-Field Microscope: Bright Object, Dark Background

The **dark-field microscope** produces detailed images of living, unstained cells and organisms by simply changing the way in which they are illuminated. A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective. Only light that has been reflected or refracted by the specimen forms an image (**figure 2.6**). The field surrounding a specimen appears black, while the object itself is brightly illuminated (**figure 2.7**). The dark-field microscope can reveal considerable internal structure in larger eukaryotic microorganisms (figure 2.7*b*). It also can be used to identify certain bacteria such as the thin and distinctively shaped *Treponema pallidum,* the causative agent of syphilis (figure 2.7*a*).

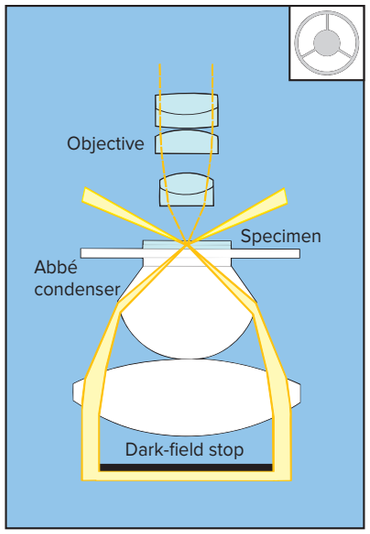
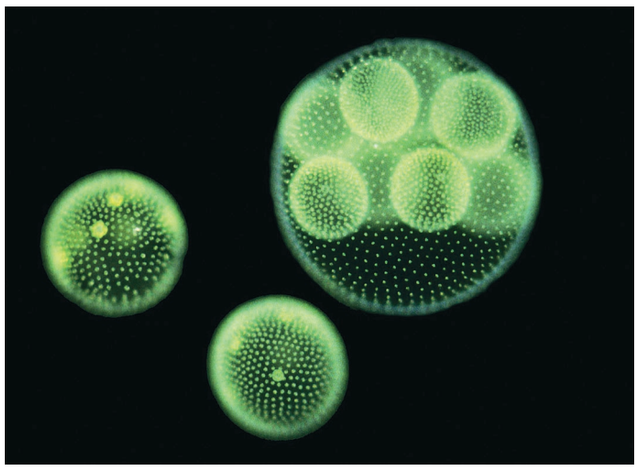


Figure 2.6 Dark-Field Microscopy. In dark-field microscopy, a dark-field stop (inset) is placed underneath the condenser lens system. The condenser then produces a hollow cone of light so that the only light entering the objective is reflected or refracted by the specimen.

(a) *T. pallidum*



(b) *Volvox*

Figure 2.7 Examples of Dark-Field Microscopy. (a) *Treponema pallidum,* the spirochete that causes syphilis (×400). (b) The protist *Volvox.* Note daughter colonies within the mature *Volvox* colony. (a) Source: Schwartz/CDC; (b) Stephen Durr/McGraw-Hill Education

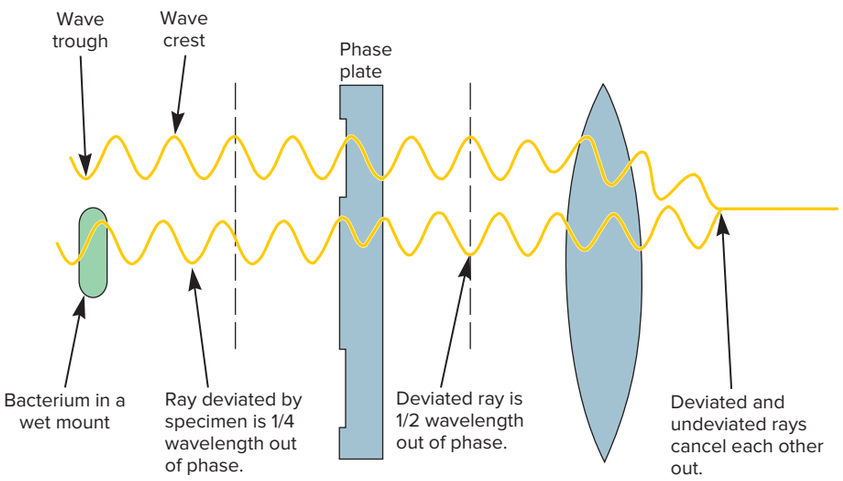


Figure 2.8 The Production of Contrast in Phase-Contrast Microscopy. The behavior of deviated and undeviated (i.e., undiffracted) light rays in the positive-phase-contrast microscope. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background.

Phase-Contrast Microscope

To understand phase-contrast microscopy, consider a bacterium in a drop of water on a microscope slide (i.e., a wet mount) as illustrated in **figure 2.8**. The refractive indices of bacterial cell structures are greater than that of water. Therefore, light waves passing through a cell structure will be diffracted and slowed more than light waves passing through the water inside and outside the cell. Thus both deviated light waves that interact with bacterial cell structures and undeviated light waves that pass around and through the cell are produced. Because the deviated light waves are slowed relative to the undeviated light waves, they are said to be out of phase. That is, the crests and troughs of the deviated and undeviated waves do not align. Typically the deviated light waves are slowed by about ¼ wavelength compared to the undeviated light (figure 2.8).

Phase-contrast microscopes take advantage of this phenomenon to create differences in light intensity that provide contrast to allow the viewer to see a clearer, more detailed image of the specimen (**figure 2.9**). They do so by separating the two types of light so that the undeviated light (primarily from the surroundings) can be manipulated and then recombined with the deviated light (from the bacterium) to form an image. Two components allow this to occur: a condenser annulus and a phase plate (**figure 2.10**). The condenser annulus is an opaque disk with a thin transparent ring. A ring of light is directed by the condenser annulus to the condenser, which focuses the light on the specimen as shown in figure 2.10. Deviated and undeviated light then pass through the objective toward the phase plate. The phase plate has a thin ring through which the undeviated light (i.e., from the surroundings) is focused (figure 2.8). In a common type of phase-contrast microscopy (positive phase contrast), the ring is coated with a substance that advances the phase of the undeviated light by ¼ wavelength. The deviated light is focused on the rest of the phase plate, which lets the deviated light pass through unchanged. After leaving the phase plate, the deviated and undeviated light are now out of phase by ½ wavelength (figure 2.8). When the two rays of light recombine to form an image, they cancel each other out, a phenomenon called destructive interference. Destructive interference is also seen if the crest of a wave of water meets the trough of another wave—the two cancel each other out and the surface of the water remains calm at the point where they meet. In our example, the resulting image consists of a darker bacterium against a lighter background.

Phase-contrast microscopy is especially useful for studying microbial motility, determining the shape of living cells, and detecting bacterial structures such as endospores and inclusions. Endospores are clearly visible because they have refractive indices markedly different from that of water. Phase-contrast microscopes also are widely used to study eukaryotic cells. ▶| *Inclusions (section 3.6); Bacterial endospores are a survival strategy (section 3.9)*

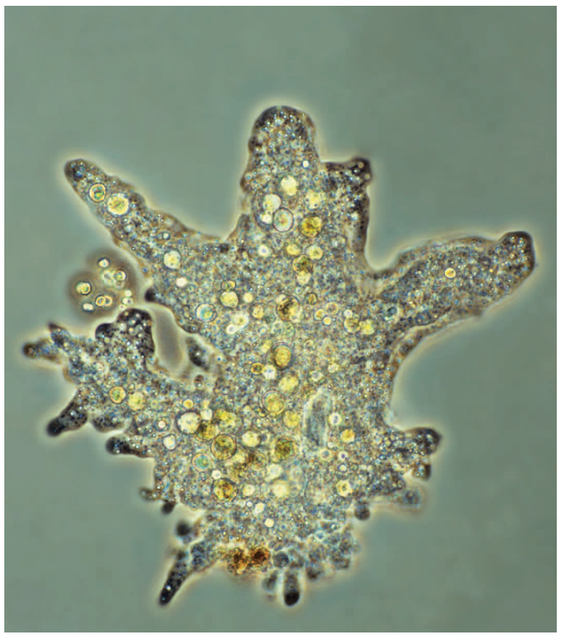


Figure 2.9 Example of Phase-Contrast Microscopy. An amoeba, a eukaryotic microbe that moves by means of pseudopodia, which extend out from the main part of the cell body. Stephen Durr/McGraw-Hill Education

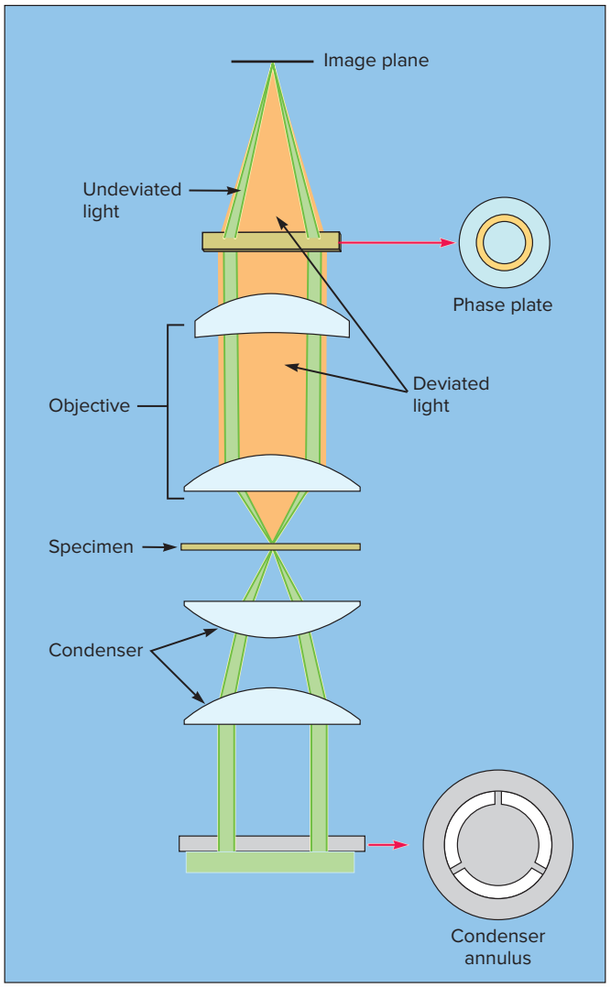


Figure 2.10 Phase-Contrast Microscopy. The optics of a positive-phase-contrast microscope.

MICRO INQUIRY *What is the purpose of the condenser annulus in a phase-contrast microscope?*

Differential Interference Contrast Microscope

The **differential interference contrast** (**DIC**) **microscope** is similar to the phase-contrast microscope in that it creates an image by detecting differences in refractive indices and thickness. Two beams of plane-polarized light at right angles to each other are generated by prisms. In one design, the object beam passes through the specimen, while the reference beam passes through a clear area of the slide. After passing through the specimen, the two beams combine and interfere with each other to form an image. A live, unstained specimen appears brightly colored and seems to pop out from the background, giving the viewer the sense that a three-dimensional image is being viewed (**figure 2.11**). Structures such as cell walls, endospores, granules, vacuoles, and nuclei are clearly visible.

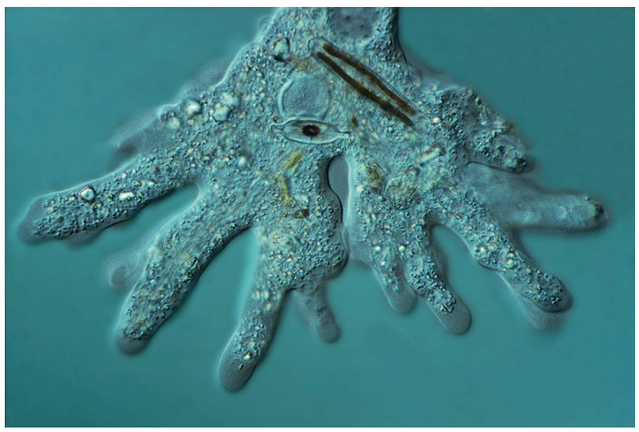


Figure 2.11 Differential Interference Contrast Microscopy. This image of the protozoan *Amoeba proteus* appears three-dimensional and contains considerable detail. Stephen Durr/McGraw-Hill Education

Fluorescence Microscopes Use Emitted Light to Create Images

The light microscopes thus far considered produce an image from light that passes through a specimen. An object also can be seen because it emits light. This is the basis of fluorescence microscopy. When some molecules absorb radiant energy, they become excited and release much of their trapped energy as light. Any light emitted by an excited molecule has a longer wavelength (i.e., has lower energy) than the radiation originally absorbed. **Fluorescent light** is emitted very quickly by the excited molecule as it gives up its trapped energy and returns to a more stable state.

The **fluorescence microscope** excites a specimen with a specific wavelength of light that triggers the emission of fluorescent light by the object, which forms the image. The most commonly used fluorescence microscopy is epifluorescence microscopy, also called incident light or reflected light fluorescence microscopy. Epifluorescence (Greek *epi,* upon) microscopes illuminate specimens from above. The objective lens also acts as a condenser (**figure 2.12**). A mercury vapor arc lamp or other source produces an intense beam of light that passes through an exciter filter. The exciter filter transmits only the desired wavelength of light. The excitation light is directed down the microscope by the dichromatic mirror. This mirror reflects light of shorter wavelengths (i.e., the excitation light) but allows light of longer wavelengths to pass through. The excitation light continues down, passing through the objective lens to the specimen, which is usually stained with molecules called **fluorochromes** (**table 2.3**). The fluorochrome absorbs

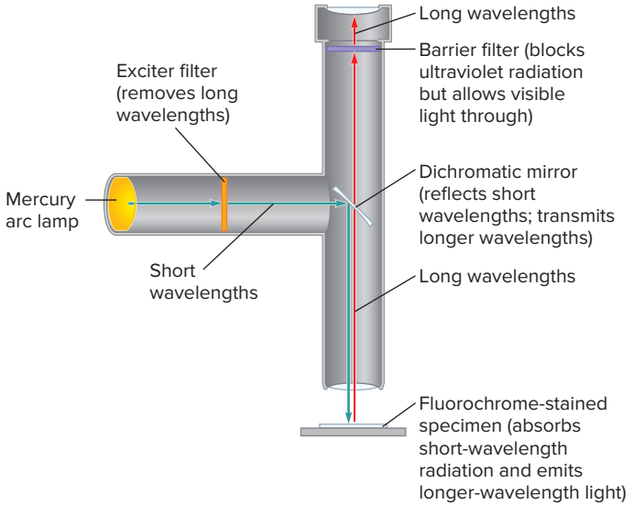


Figure 2.12 Epifluorescence Microscopy. The principles of operation of an epifluorescence microscope.

light energy from the excitation light and emits fluorescent light that travels up through the objective lens into the microscope. Because the emitted fluorescent light has a longer wavelength, it passes through the dichromatic mirror to a barrier filter, which blocks out any residual excitation light. Finally, the emitted light passes through the barrier filter to the eyepieces.

The fluorescence microscope has become an essential tool. Bacterial pathogens can be identified after staining with fluorochromes or specifically tagging them with fluorescently labeled antibodies using immunofluorescence procedures. In ecological studies, fluorescence microscopy is used to observe microorganisms stained with fluorochrome-labeled probes or fluorochromes that bind specific cell constituents (table 2.3). In addition, microbial ecologists use epifluorescence microscopy to visualize photosynthetic microbes, as their pigments naturally fluoresce when excited by light of specific wavelengths. It is even possible to distinguish live bacteria from dead bacteria by the color they fluoresce after treatment with a specific mixture of stains (**figure 2.13*a***). ▶| *Methods in microbial biology (chapter 33); Identification of microorganisms from specimens (section 34.2)*

Table 2.3 Commonly Used Fluorochromes

Fluorochrome	Uses
Acridine orange	Stains DNA
Diamidino-2-phenyl indole (DAPI)	Stains DNA
Fluorescein isothiocyanate (FITC)	Often attached to DNA probes or to antibodies that bind specific cellular components
Tetramethyl rhodamine isothiocyanate (TRITC or rhodamine)	Often attached to antibodies that bind specific cellular components

Another important use of fluorescence microscopy is the localization of specific proteins within cells. One approach is to use genetic engineering techniques that fuse the gene for the protein of interest to a gene isolated from jellyfish belonging to the genus *Aequorea.* This jellyfish gene encodes a protein that naturally fluoresces green when exposed to light of a particular wavelength and is called green fluorescent protein (GFP). Thus when the protein is made by the cell, it is fluorescent. GFP has been used extensively in studies on bacterial cell division and related phenomena (**figure 2.14**). In fact, the 2008 Nobel Prize in Chemistry was awarded to Osamu Shimomura of Japan and Americans Martin Chalfie and Roger Tsien for their development of this important tool. ▶| *Fluorescence labeling (section 31.4)*

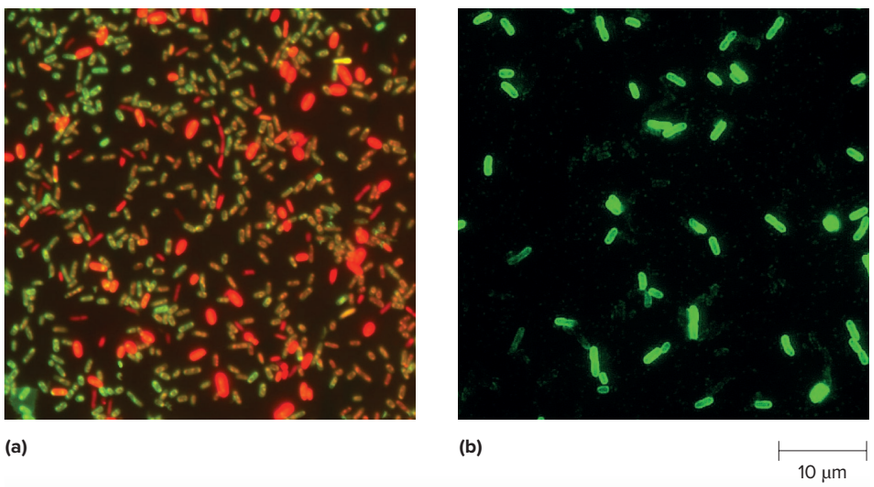


Figure 2.13 Fluorescent Dyes and Tags. (a) A sample of bacteria stained with dyes that cause live cells to fluoresce green and dead ones red. (b) Fluorescent antibodies tag specific molecules. In this case, the antibody binds to a molecule that is unique to *Yersinia pestis,* the bacterium that causes plague. (a) Dr. Rita B. Moyes; (b) Source: Larry Stauffer/Oregon State Public Health Laboratory/CDC

MICRO INQUIRY *How might fluorescently labeled antibody be used to diagnose an infectious disease?*

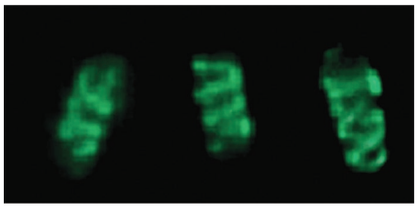
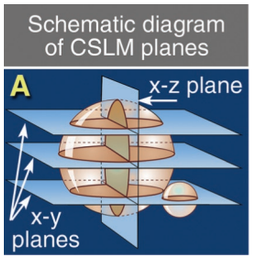


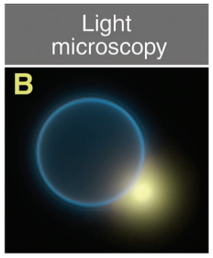
Figure 2.14 Green Fluorescent Protein. Visualization of MbI, a cytoskeletal protein of *Bacillus subtilis*. The MbI protein has been fused with green fluorescent protein and therefore fluoresces green. Jeff Errington/Centre for Bacterial Cell Biology/Newcastle University

Confocal Microscopy

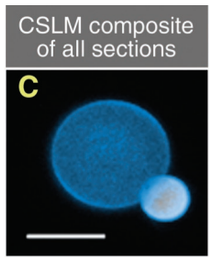
Like the large and small beads illustrated in **figure 2.15*a***, biological specimens are three-dimensional. When three-dimensional objects are viewed with traditional light microscopes, light from all areas of the object, not just the plane of focus, enters the microscope and is used to create an image. The resulting image is murky and fuzzy (figure 2.15*b*). This problem has been solved by the confocal scanning laser microscope (CSLM), or simply, **confocal microscope.** The confocal microscope uses a laser beam to illuminate a specimen that has been fluorescently stained. A major component of the confocal microscope is an opening (that is, an aperture) placed above the objective lens. The aperture eliminates stray light from parts of the specimen that lie above and below the plane of focus (**figure 2.16**). Thus the only light used to create the image is from the plane of focus, and a much sharper image is formed.



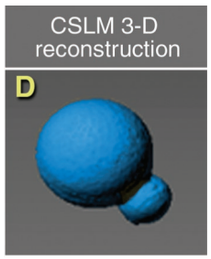
(a) All objects are defined by three axes: x, y, and z. The confocal scanning laser microscope (CSLM) creates images of planes formed by the x and y axes (x-y planes) and planes formed by the x and z axes (x-z planes).



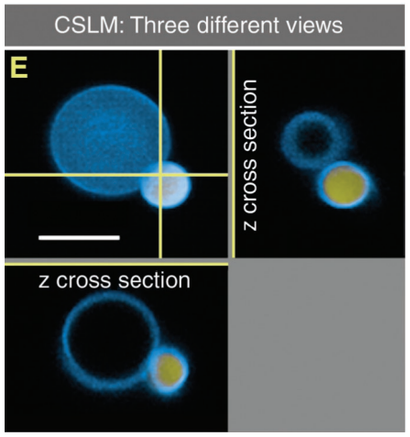
(b) The light microscope image of the two beads shown in **(a)**. Note that neither bead is clear, because the image is generated from light emanating from multiple planes of focus.



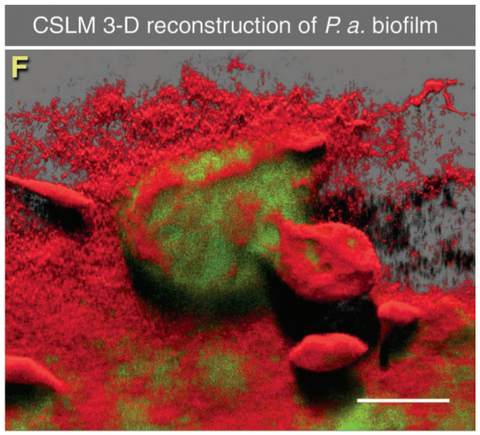
(c) A CSLM uses light from a single plane of focus to generate an image. A composite image of the two beads is generated using digitized information collected from multiple planes within the beads.



(d) Digitized information is collected from multiple planes within the beads to generate a three-dimensional reconstruction.



(e) Views are also generated using different planes. The top left panel is the image of a single x-y plane (i.e., looking down from the top of the beads). The two lines represent the two x-z planes imaged in the other two panels. The vertical line indicates the x-z plane shown in the top right panel (i.e., a view from the right side of the beads) and the horizontal line indicates the x-z plane shown in the bottom panel (i.e., a view from the front face of the beads).



(f) A three-dimensional reconstruction of a *Pseudomonas aeruginosa* biofilm. The biofilm was exposed to an antibacterial agent and then stained with dyes that distinguish living (green) from dead (red) cells. The cells on the surface of the biofilm have been killed, but those in the lower layers are still alive. This image clearly demonstrates the difficulty of killing all the cells in a biofilm.

Figure 2.15 Light and Confocal Microscopy. (a–e) Two beads examined by light and confocal microscopy. (f) Three-dimensional reconstruction of a biofilm. (b–f) P. Dirckx/Center for Biofilm Engineering/Montana State University

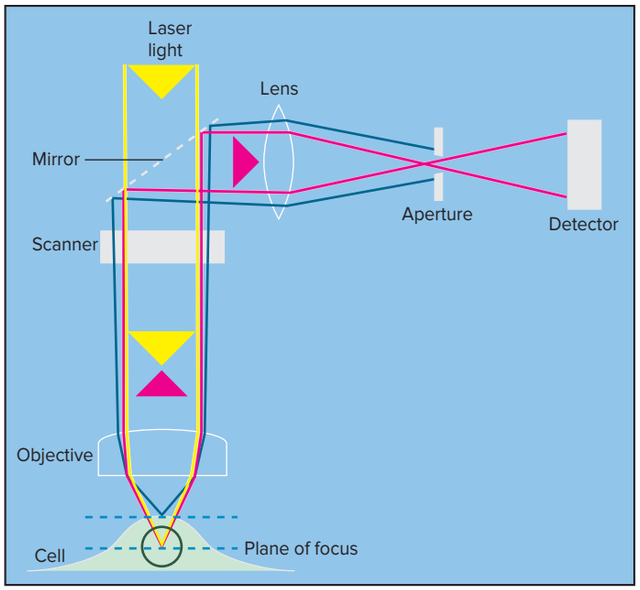


Figure 2.16 Ray Diagram of a Confocal Microscope. The yellow lines represent laser light used for illumination. Red lines symbolize the light arising from the plane of focus, and the blue lines stand for light from parts of the specimen above and below the focal plane.

MICRO INQUIRY *How does the light source differ between a confocal light microscope and other light microscopes?*

To generate a confocal image, a computer interfaced with the confocal microscope receives digitized information from each plane in the specimen. This information can be used to create a clear and detailed composite image (figure 2.15*c*) or a three-dimensional reconstruction of the specimen (figure 2.15*d*). Images of x-z plane cross sections of the specimen can also be generated, giving the observer views of the specimen from three perspectives (figure 2.15*e*). Confocal microscopy has numerous applications. One is the study of biofilms, which can form on many different types of surfaces, including indwelling medical devices such as hip joint replacements. As shown in figure 2.15*f*, it is difficult to kill all cells in a biofilm. This makes biofilms a particular concern because their formation on medical devices can result in infections that are difficult to treat. ▶| *Biofilms are common in nature (section 5.6)*

Comprehension Check

1. Compare and contrast the function of the condenser, objective, and eyepiece lenses.
2. What happens to resolution if each of the following increase: wavelength of light, refractive index, and numerical aperture? How are resolution and magnification related?
3. What might a microscopist see through an oil immersion objective if no immersion oil is present?
4. Why don't most light microscopes use 30× ocular lenses for greater magnification?
5. Compare and contrast how dark-field, phase-contrast, differential interference contrast, epifluorescence, and confocal microscopes work and the kind of image or images provided by each. Give a specific use for each type.
6. Which type of microscope(s) would be used to examine the following specimens? Photosynthetic bacteria, swimming bacteria, GFP-labeled microorganisms, unstained microbes, and pond scum; explain each of your answers.

2.3 Staining Helps to Visualize and Identify Microbes

After reading this section, you should be able to:

a. Recommend a fixation process to use when the microbe is a bacterium or archaeon and when the microbe is a protist

b. Plan a series of appropriate staining procedures to describe an unknown bacterium as fully as possible

c. Compare what happens to Gram-positive and Gram-negative bacterial cells during each step of the Gram-staining procedure

As noted in section 2.2, specimens examined by bright-field microscopy are often fixed and stained before being examined. Such preparation serves to increase the visibility of the microorganisms, accentuate specific morphological features, and preserve them for future study. Importantly, some staining procedures help microbiologists identify the organism being examined.

Fixation

Stained cells seen in a microscope should resemble living cells as closely as possible. **Fixation** is the process by which the internal and external structures of specimens are preserved and fixed in position. It inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. A microorganism usually is killed and attached firmly to the microscope slide during fixation.

There are two fundamentally different types of fixation: heat fixation and chemical fixation. **Heat fixation** is routinely used to observe bacteria and archaea. Typically, a film of cells (a smear) is gently heated. Heat fixation preserves overall morphology and inactivates enzymes. However, it also destroys proteins in subcellular structures, which may distort their appearance. **Chemical fixation** is used to protect fine cellular substructure as well as morphology. It is used when examining microorganisms by many electron microscopy techniques. Chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble, and immobile. Common fixative

mixtures contain ethanol, acetic acid, mercuric chloride, formaldehyde, and glutaraldehyde.

Dyes and Simple Staining

The many types of dyes used to stain microorganisms have two features in common. First, they have **chromophore groups**—chemical moieties with conjugated double bonds that give the dye its color. Second, they bind cells by ionic, covalent, or hydrophobic bonds.

Dyes that bind cells by ionic interactions are probably the most common. Ionizable dyes may be divided into two general classes based on the nature of their charged group: basic dyes and acidic dyes (**table 2.4**). The staining effectiveness of ionizable dyes may be altered by pH because the nature and number of the charged moieties on cell components change with pH. Thus acidic dyes stain best under acidic conditions when proteins and many other molecules carry a positive charge; basic dyes are most effective at higher pH values.

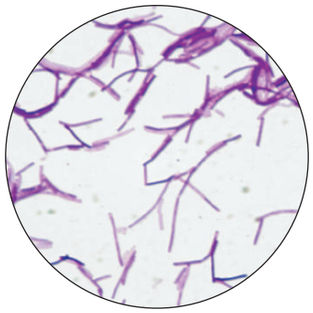
Dyes that bind through covalent bonds or that have certain solubility characteristics are also useful. For instance, DNA can be stained by the Feulgen procedure in which the staining compound (Schiff's reagent) is covalently attached to the deoxyribose sugars of DNA. Sudan III (Sudan Black) selectively stains lipids because it is lipid soluble but does not dissolve in aqueous portions of the cell.

Microorganisms can be stained by **simple staining,** in which a single dye is used (**figure 2.17**). Simple staining's value lies in its ease of use. The fixed smear is covered with stain for a short time, excess stain is washed off with water, and the slide is blotted dry. Basic dyes such as crystal violet, methylene blue, and carbolfuchsin are frequently used in simple staining to determine the size, shape, and arrangement of bacterial and archaeal cells.

While most dyes directly stain the cell or object of interest, some dyes (e.g., India ink and nigrosin) are used in **negative staining.** In negative staining, the background is stained, not the cell; instead, the unstained cells appear as bright objects against a dark background.

Differential Staining

The **Gram stain,** developed in 1884 by the Danish physician Christian Gram, is the most widely employed staining method in bacteriology. The Gram stain is an example of **differential staining**—procedures that distinguish organisms based on their staining properties. Use of the Gram stain classifies most bacteria into one of two groups—Gram negative or Gram positive, based on the composition of their cell wall.



Crystal violet stain
of slender, rod shaped bacteria.

Figure 2.17 **Simple Staining Illustrates Cell Size and Morphology.**
Dr. Rita B. Moyes

The Gram-stain procedure is illustrated and described in **figure 2.18**. The result of Gram staining is pink to red Gram-negative bacteria and purple Gram-positive bacteria (**figure 2.19*a***). ▶| *Overview of bacterial cell wall structure (section 3.4)* *Gram staining*

Acid-fast staining is another important differential staining procedure. It can be used to identify *Mycobacterium tuberculosis* and *M. leprae* (figure 2.19*b*), the pathogens responsible for tuberculosis and leprosy, respectively. These bacteria, as well as other mycobacteria, have cell walls containing lipids constructed from mycolic acids, a group of branched-chain hydroxy fatty acids, which prevent dyes from readily binding to the cells (*see figure 13.8*). A commonly used staining procedure, the cold Ziehl-Neelsen method, uses high concentrations of phenol and carbol fuchsin, as well as a wetting agent, to drive the stain carbol fuchsin into mycobacterial cells. Once this dye has penetrated, the cells are not easily decolorized by acidified alcohol (acid-alcohol) and thus are said to be acid-fast. Non-acid-fast bacteria are easily decolorized by acid-alcohol and thus are stained another color by a second dye called a counterstain.

One of the simplest staining procedures is capsule staining (figure 2.19*c*), a technique that reveals the presence of capsules, a

Table 2.4 Ionizable Dyes

Type of Dye	Examples	Chromophore Characteristics
Basic dyes	Methylene blue, basic fuchsin, crystal violet, safranin, malachite green	Have positively charged groups; bind to negatively charged molecules such as nucleic acids, many proteins, and the surfaces of bacterial and archaeal cells
Acidic dyes	Eosin, rose bengal, and acid fuchsin—possess groups such as carboxyls (—COOH) and phenolic hydroxyls (—OH).	Have negatively charged groups; bind to positively charged cell structures

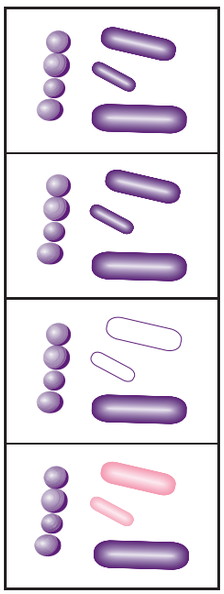
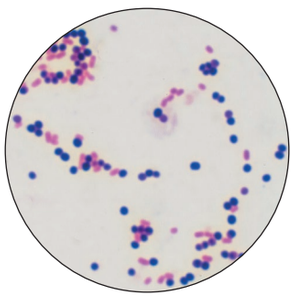
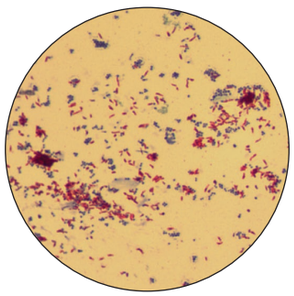
	Steps in Staining	State of Bacteria	Explanation
	Step 1: Crystal violet (primary stain) for 1 minute. Water rinse.	Cells stain purple.	The dye is taken up equally well by all cells present.
	Step 2: Iodine for 1 minute. Water rinse.	Cells remain purple.	Iodine is a mordant, a compound that enhances binding of dye to cell walls.
	Step 3: Alcohol (decolorizer) for 10–30 seconds. Water rinse.	Gram-positive cells remain purple. Gram-negative cells become colorless.	Gram-negative cell walls are thin and cannot retain the dye.
	Step 4: Safranin (counterstain) for 30–60 seconds. Water rinse. Blot dry.	Gram-positive cells remain purple. Gram-negative cells appear red.	Only unstained Gram-negative cells can take up the dye; crystal violet in the Gram-negative cells repels the counterstain.

Figure 2.18 Gram Stain. Steps in the Gram staining procedure.

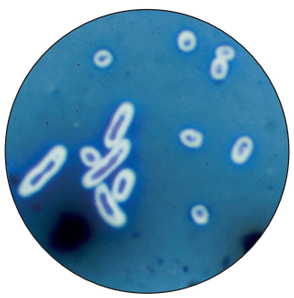
MICRO INQUIRY *Why is the decolorization step considered the most critical in the Gram staining procedure?*



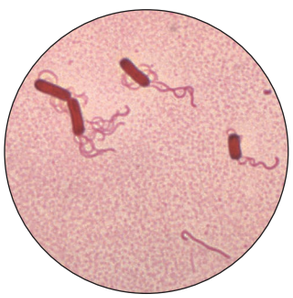
(a) Gram stain
Purple cells are Gram positive.
Red cells are Gram negative.



(b) Acid-fast stain
Red cells are acid-fast.
Blue cells are non-acid-fast.



(c) Capsule stain of *Klebsiella pneumoniae*



(d) Flagellar stain of *Bacillus brevis*

Figure 2.19 Differential Stains. (a, c) Lisa Burgess/McGraw-Hill Education; (b) James Redfearn/McGraw-Hill Education; (d) Source: Dr. William A. Clark/CDC

network usually made of polysaccharides that surrounds many bacteria and some fungi. Cells are mixed with India ink or nigrosin dye and spread out in a thin film on a slide. After air-drying, cells appear as bodies surrounded by a halo of capsule in the midst of a blue-black background because ink and dye particles cannot penetrate either the cell or its capsule. Thus capsule staining is an example of negative staining. There is little distortion of cell shape, and the cell can be counterstained for even greater visibility. ▶| *Capsules and slime layers (section 3.5)*

Flagella staining provides taxonomically valuable information about the presence and distribution pattern of flagella on bacterial and archaeal cells (figure 2.19*d; see also figure 3.37*). Their flagella are fine, threadlike organelles of locomotion that are so slender (about 10 to 30 nm in diameter) they can only be seen directly using an electron microscope (although bundles of flagella can be visualized by darkfield microscopy). To observe bacterial flagella with a light microscope, their thickness is increased by coating them with mordants such as tannic acid and potassium alum and then staining with a dye such as basic fuchsin. ▶| *Bacterial flagella (section 3.7)*

Comprehension Check

1. Describe the two general types of fixation. Which would you use when Gram staining a bacterium? Which would you use before observing the organelles of a protist?
2. Why are basic dyes more effective under alkaline conditions?
3. What procedural error(s) might be responsible if you Gram stained a mixture of bacteria known to be Gram positive and Gram negative and see only red cells?
4. Are capsular and flagellar staining differential staining procedures? Explain your answer.

2.4 Electron Microscopes Use Beams of Electrons to Create Highly Magnified Images

After reading this section, you should be able to:

a. Create a concept map, illustration, or table that compares transmission electron microscopes (TEMs) to light microscopes

b. Decide when it would be best to examine a microbe by TEM, scanning electron microscopy (SEM), and electron cryotomography

As we have seen, light microscopes have a resolution limit of about 0.2 µm. This greatly compromises their usefulness for detailed studies of many microorganisms (**figure 2.20**). Viruses, for

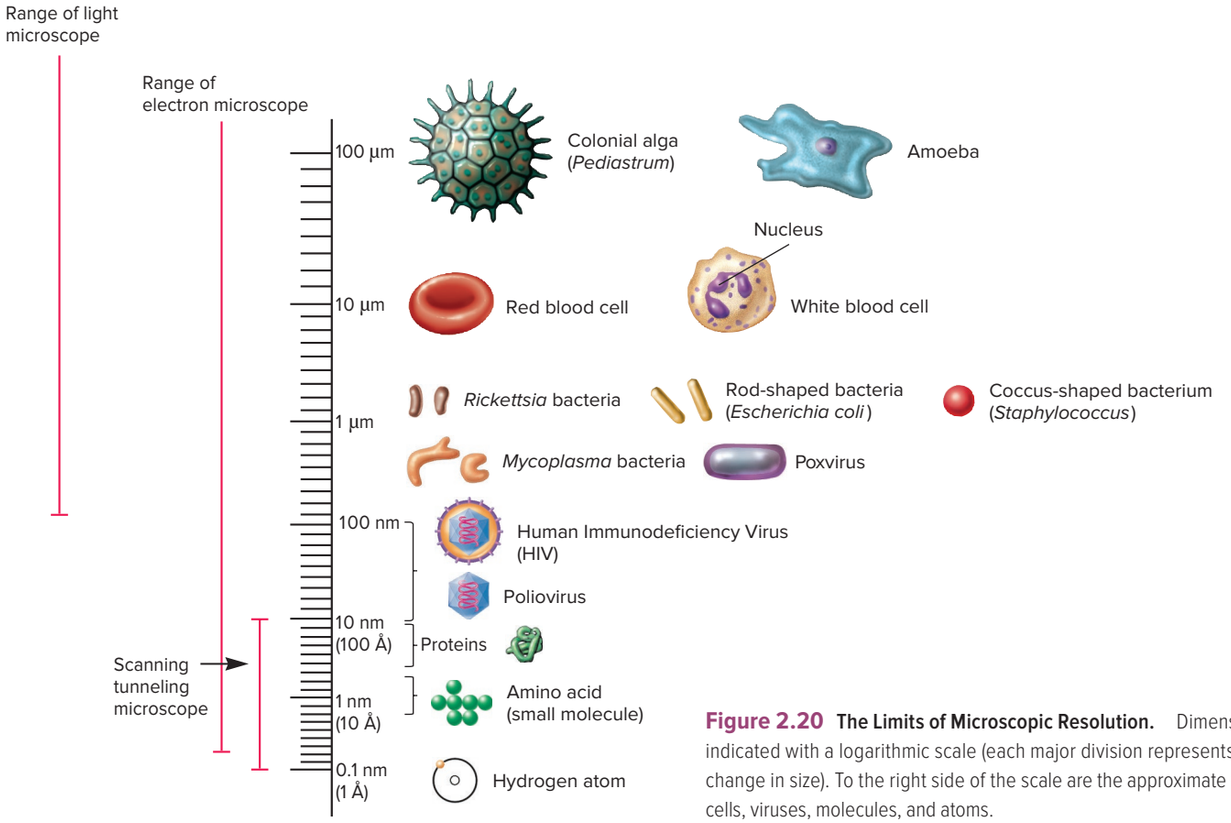


Figure 2.20 The Limits of Microscopic Resolution. Dimensions are indicated with a logarithmic scale (each major division represents a 10-fold change in size). To the right side of the scale are the approximate sizes of cells, viruses, molecules, and atoms.

example, are too small to be seen with light microscopes (with the exception of some recently discovered giant viruses). Although the general shape and cellular arrangement of bacteria and archaea can be observed, their internal structure cannot be effectively studied by light microscopy.

Recall that the resolution of a light microscope increases as the wavelength of the light it uses for illumination decreases. In electron microscopes, electrons replace light as the illuminating beam. The electron beam can be focused, much as light is in a light microscope, but its wavelength is about 100,000 times shorter than that of visible light. Therefore electron microscopes have a practical resolution roughly 1,000 times better than the light microscope; with many electron microscopes, points closer than 0.5 nm can be distinguished, and the useful magnification is well over 100,000× (figure 2.20). The value of the electron microscope is evident on comparison of the photographs in **figure 2.21**. Microbial morphology can now be studied in great detail. We briefly review the most common types of electron microscopy.

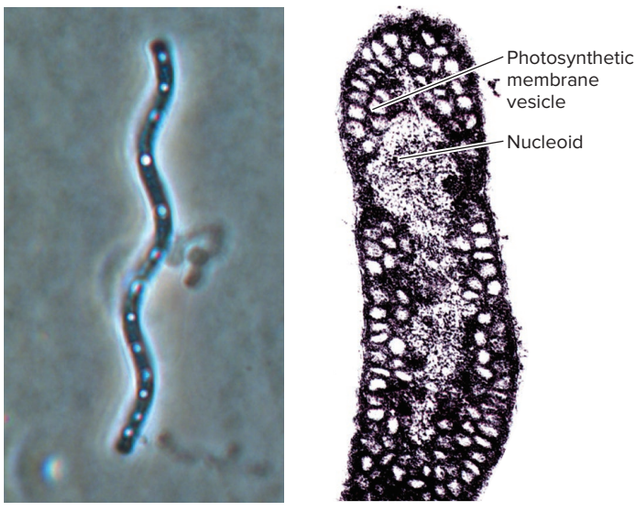


Figure 2.21 Light and Electron Microscopy. A comparison of light and electron microscopic resolution. (a) The proteobacterium *Spirillum volutans* in phase-contrast light microscope (×1,000). (b) A thin section of another spiral-shaped proteobacterium *Rhodospirillum rubrum* in transmission electron microscope (×100,000). (a) James Redfearn/McGraw-Hill Education; (b) Biology Media/Science Source

Transmission Electron Microscope

A **transmission electron microscope** (**TEM**) uses a heated tungsten filament in the electron gun to generate a beam of electrons that is focused on the specimen by the condenser

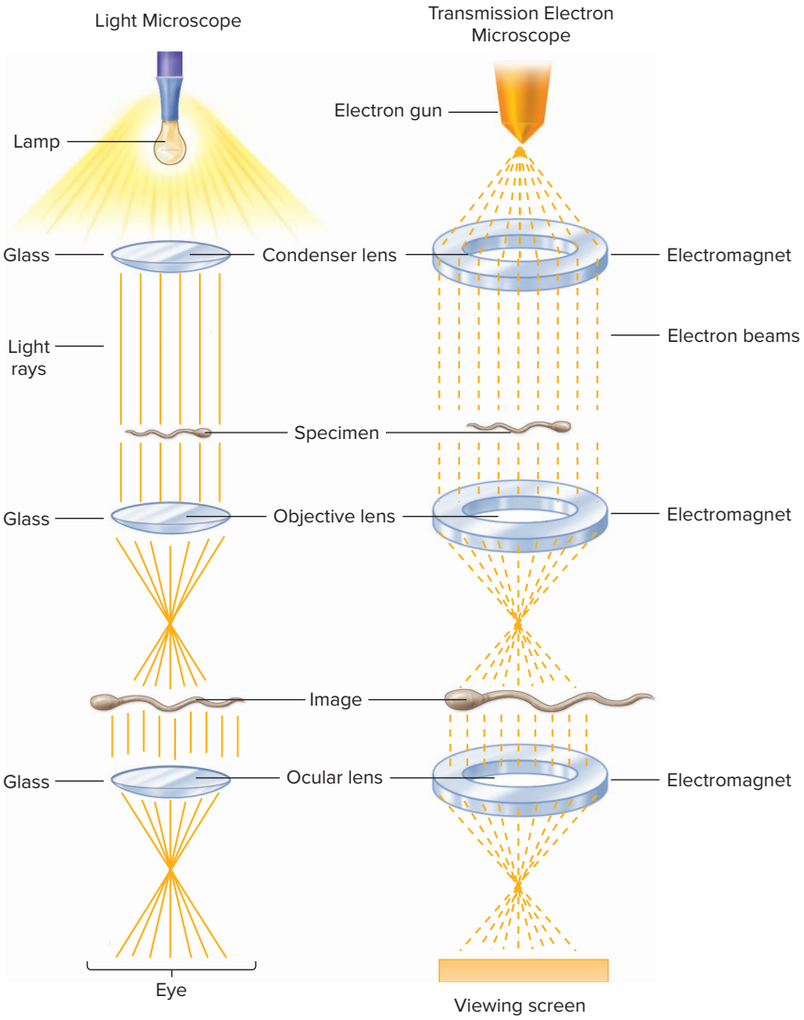


Figure 2.22 **A Comparison of Light and Transmission Electron Microscopes.**

Figure 2.23 **A Transmission Electron Microscope.** The electron gun is at the top of the central column, and the magnetic lenses are within the column. The image on the fluorescent screen is also viewed on a computer monitor.
Holly Curry/McGraw-Hill Education

(**figure 2.22**). Since electrons cannot pass through a glass lens, doughnut-shaped electromagnets called magnetic lenses focus the beam. To obtain a clear image, the column containing the lenses and specimen must be under vacuum because electrons are deflected by collisions with air molecules. The specimen scatters some electrons, but those that pass through are used to form an enlarged image of the specimen on a fluorescent screen that interfaces with a computer monitor (**figure 2.23**). Denser regions in the specimen scatter more electrons and therefore appear darker because fewer electrons strike that area of the screen; these regions are said to be "electron dense." In contrast, electron-transparent regions are brighter.

Table 2.5 compares some of the important features of light and transmission electron microscopes. The TEM has distinctive features that place harsh restrictions on the nature of samples that can be viewed and the means by which those samples must be prepared. Specimens must be viewed in a vacuum and only extremely thin slices (20 to 100 nm) of a specimen can be viewed because electron beams are easily absorbed and scattered by solid matter. To cut such a thin slice, specimens must be embedded in a supportive plastic matrix. To prepare specimens, they are first fixed with chemicals such as glutaraldehyde and osmium tetroxide to stabilize cell structure. The specimen is then dehydrated with organic solvents (e.g., acetone or ethanol). Next the specimen is soaked in unpolymerized, liquid epoxy plastic until it is completely permeated, and then the plastic is hardened to form a solid block. Thin sections are skillfully cut from the block with a glass or diamond knife using a device called an ultramicrotome.

Samples are usually stained so they can be seen clearly with a TEM. The probability of electron scattering is determined by the density (atomic number) of atoms in the specimen. Biological molecules are composed primarily of atoms with low atomic numbers (H, C, N, and O), and electron scattering is fairly constant throughout an unstained cell or virus. Therefore specimens are further prepared by soaking thin sections with solutions of heavy metal salts such as lead citrate and uranyl acetate. The lead and uranium ions bind to structures in the specimen and make them more electron opaque, thus increasing contrast in the material. Heavy osmium atoms from the osmium tetroxide fixative also stain specimens and increase their contrast. The stained thin sections are then mounted on tiny copper grids and viewed.

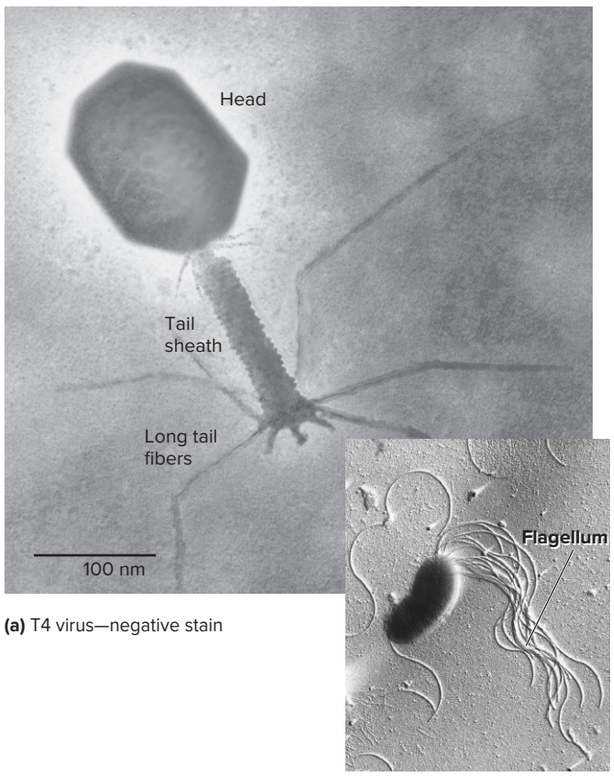
Table 2.5 Characteristics of Light and Transmission Electron Microscopes

Feature	Light Microscope	Transmission Electron Microscope
Highest practical magnification	About 1,000–1,500	Over 100,000
Best resolution[1]	0.2 μm	0.2 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Focusing mechanism	Adjust lens position mechanically	Adjust current to the magnetic lens
Method of changing magnification	Switch the objective lens or eyepiece	Adjust current to the magnetic lens
Specimen mount	Glass slide	Metal grid (usually copper)

[1]The resolution limit of a human eye is about 0.2 mm.

Two other important techniques for preparing specimens for TEM include negative staining and shadowing. In negative staining, the specimen is spread out in a thin film with either phosphotungstic acid or uranyl acetate. Just as in negative staining for light microscopy, the specimen appears bright against a dark background, in this case because the heavy metals do not penetrate biological material. Negative staining enables visualization of viruses and cellular microbes, but unlike thin sections, internal structures cannot be discerned (**figure 2.24*a***). In shadowing, a specimen is coated with a thin film of platinum or other heavy metal by evaporation at an angle of about 45 degrees from horizontal so that the metal strikes the microorganism on only one side. In one commonly used imaging method, the area coated with metal appears dark in photographs, whereas the uncoated side and the shadow region created by the object are light (figure 2.24*b*). This technique is particularly useful in studying virus particle morphology, flagella, and DNA.

The process of chemical fixation and dehydration can introduce artifacts that can alter cellular morphology. This can be minimized or avoided by using a freeze-etching procedure. When cells are rapidly frozen in liquid nitrogen, they become very brittle and can be broken along lines of greatest weakness, usually down the middle of internal membranes (**figure 2.25**). The exposed surfaces are then shadowed and coated with layers of platinum and carbon to form a replica of the surface. After the specimen has been removed chemically, this replica is studied in the TEM, providing a detailed view of intracellular structure.



(a) T4 virus—negative stain

(b) *P. fluorescens*—after shadowing

Figure 2.24 Stained Microorganisms Visualized by TEM. (a) T4 is a virus that infects *Escherichia coli*. (b) *Pseudomonas fluorescens* with its polar flagella. (a) Ami Images/Science Source; (b) Dr. Tony Brain/SPL/Science Source

MICRO INQUIRY *Why are all electron micrographs black and white (although they are sometimes artificially colorized after printing)?*

Scanning Electron Microscope

Transmission electron microscopes form an image from radiation that has passed through a specimen. The **scanning electron**