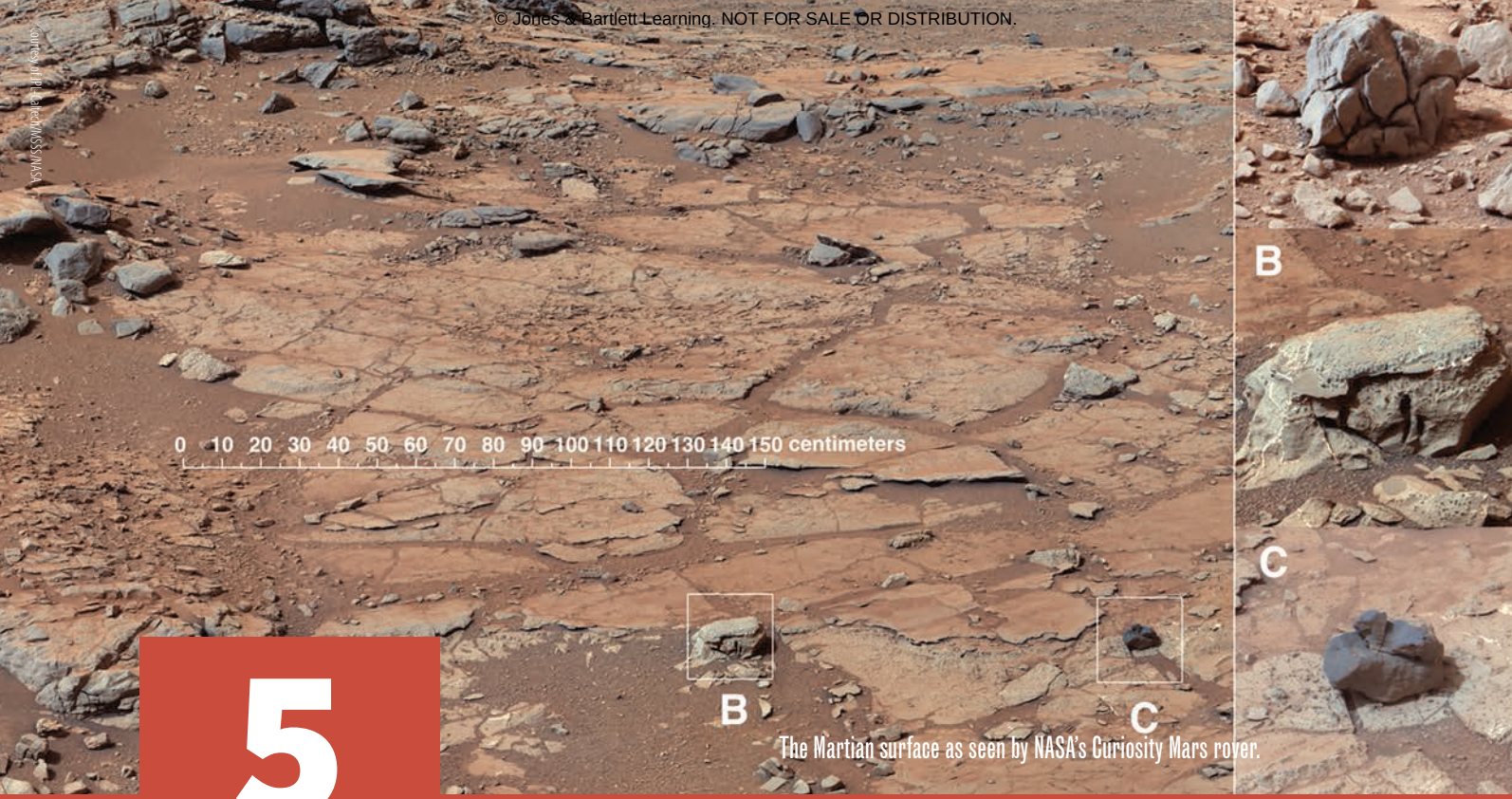


Courtesy of JPL-Caltech/MSSS/NASA

The Martian surface as seen by NASA's Curiosity Mars rover.

5

CHAPTER PREVIEW

Microbial Growth and Nutrition

Bacteria © NIAID

Books have been written about it; movies have been made; even a radio play in 1938 about it frightened thousands of Americans. What is it? Martian life. In 1877 the Italian astronomer, Giovanni Schiaparelli, saw lines on Mars, which he and others assumed were canals built by intelligent beings. It wasn't until well into the twentieth century that this notion was disproved. Still, when we gaze at the red planet, we wonder: Did life ever exist there?

We are not the only ones wondering. Astronomers, geologists, and many other scientists have asked the same question. Today microbiologists have joined their scientific colleagues, wondering if microbial life once existed on the Red Planet or, for that matter, elsewhere in our solar system.

So, could microbes, as we know them here on Earth, survive on Mars, where temperatures are far below 0°C, the atmosphere contains little oxygen gas, and ultraviolet (UV) radiation bombards the planet surface? Researchers, using a device to simulate the Martian soil environment, placed in the soil microbes known to survive extremely cold environments here on Earth. Their results suggested that members of the Archaea, specifically the methanogens, could grow in the cold, low-oxygen atmosphere, especially if protected from UV light by being buried just under the soil surface.

TABLE 5.1 Some Microbial Record Holders (and Their Taxonomic Domain)

Hottest environment (Juan de Fuca ridge)—121°C: Strain 121 (Archaea)
Coldest environment (Antarctica)— –15°C: *Planococcus cryohalophilus* (Bacteria)
Highest radiation survival—5 MRad, or 5000× what kills humans: *Deinococcus radiodurans* (Bacteria)
Deepest—3.2 km underground: Many bacterial and archaeal species
Most acid environment (Iron Mountain, CA)—pH 0.0: *Ferroplasma acidarmanus* (Archaea)
Most alkaline environment (Lake Calumet, IL)—pH 12.8: Proteobacteria (Bacteria)
Longest in space (NASA satellite)—6 years: *Bacillus subtilis* (Bacteria)
High-pressure environment (Mariana Trench)—1200 times atmospheric pressure: *Moritella, Shewanella*, and others (Bacteria)
Saltiest environment (Eastern Mediterranean basin)—47% salt: Several bacterial and archaeal species
Loneliest (South Africa)—3.3 kilometers below Earth's surface: *Desulforudis audaxviator*

Source: http://www.astrobio.net/news/

So, microbiologists have joined the search for extraterrestrial life. This seems a valid pursuit because the **extremophiles** found here on Earth survive, and even require, living in extreme environments (TABLE 5.1)—some not so different from Mars (FIGURE 5.1). If life (as we know it) did or does exist on Mars, it almost certainly was or is bacterial/archaeal.

Extremophile: A microorganism that lives in climatic or environmental extremes, such as extremely high/low temperatures, high acidity, or high salt environments.

FIGURE 5.1 The Martian Surface? This barren-looking landscape is not Mars but the Atacama Desert in Chile. It looks similar to photos taken by the Mars rovers *Spirit* and *Opportunity*. **» Does this area look like a habitable place for life, even microbial life?**

In 2004, NASA sent two spacecraft to Mars to look for indirect signs of past life. Scientists here on Earth monitored instruments on the Mars rovers, *Spirit* and *Opportunity,* designed to search for signs suggesting water once existed on the planet. Some findings suggest there are areas where salty seas once washed over the plains of Mars, creating a life-friendly environment.

Opportunity found evidence for ancient shores on what once was believed to be a sea. Scientists reported in 2008 that a more recent spacecraft, the *Phoenix Mars Lander,* detected water ice near the Martian soil surface.

Since 2012, another exploration of the Red Planet has been underway, as the Mars rover *Curiosity* is searching to see if there are any chemical signs suggesting life may have existed on the Red planet.

Whether microorganisms are here on Earth in moderate or extreme environments or on Mars, there are certain physical and chemical requirements they must possess to survive, grow, and reproduce. In this chapter, we explore the process of microbial cell reproduction, examine the physical and chemical conditions required for growth, and discover the ways that microbial growth can be measured.

CHAPTER CHALLENGE

The United States has sent several spacecraft to Mars since the first Viking landers in 1976. Recently, an international team of scientists carried out studies suggesting terrestrial microbes could hitch a ride to Mars on such a craft, and they could even survive the journey. The team believes most spacecraft that have touched down on Mars were not thoroughly sterilized, so they could have carried living microbes from Earth. NASA scientists have assumed Mars' thin atmosphere, which allows intense UV radiation to reach the planet's surface—triple Earth's intensity—would kill any life inadvertently carried on the spacecraft. However, in laboratory tests, here on Earth the scientists came across some spore-forming bacterial species that could survive UV bombardment at a level equivalent to that on Mars—if the spores were buried just a few millimeters in the soil. Could such an earthly extremophile survive on Mars, or could there actually be Martian microbes on the planet? Are there conditions here on Earth that might give us an idea? Let's examine what it takes for a bacterial species to survive both "normal" and "extreme" earthly environments.

Microbial Reproduction Is Part of the Cell Cycle

Growth in the microbial world usually refers to an increase in the numbers of individuals; that is, an increase in the population size with each cell carrying the identical genetic instructions of the parent cell. **Asexual reproduction** is the process to maintain this genetic constancy. In eukaryotic microbes, an elaborate interaction of microtubules and proteins with pairs of chromosomes in the cell nucleus allows for the precise events of mitosis and cytokinesis. Bacterial and archaeal cells divide without this elaborate division apparatus but still possess similar protein filaments to use in the reproduction process.

Binary Fission Is Part of the Cell Cycle

The series of events involving growth, DNA replication, and cell division is called the **cell cycle**. For a bacterial cell like *E. coli*, the cycle can be divided into three periods (FIGURE 5.2A).

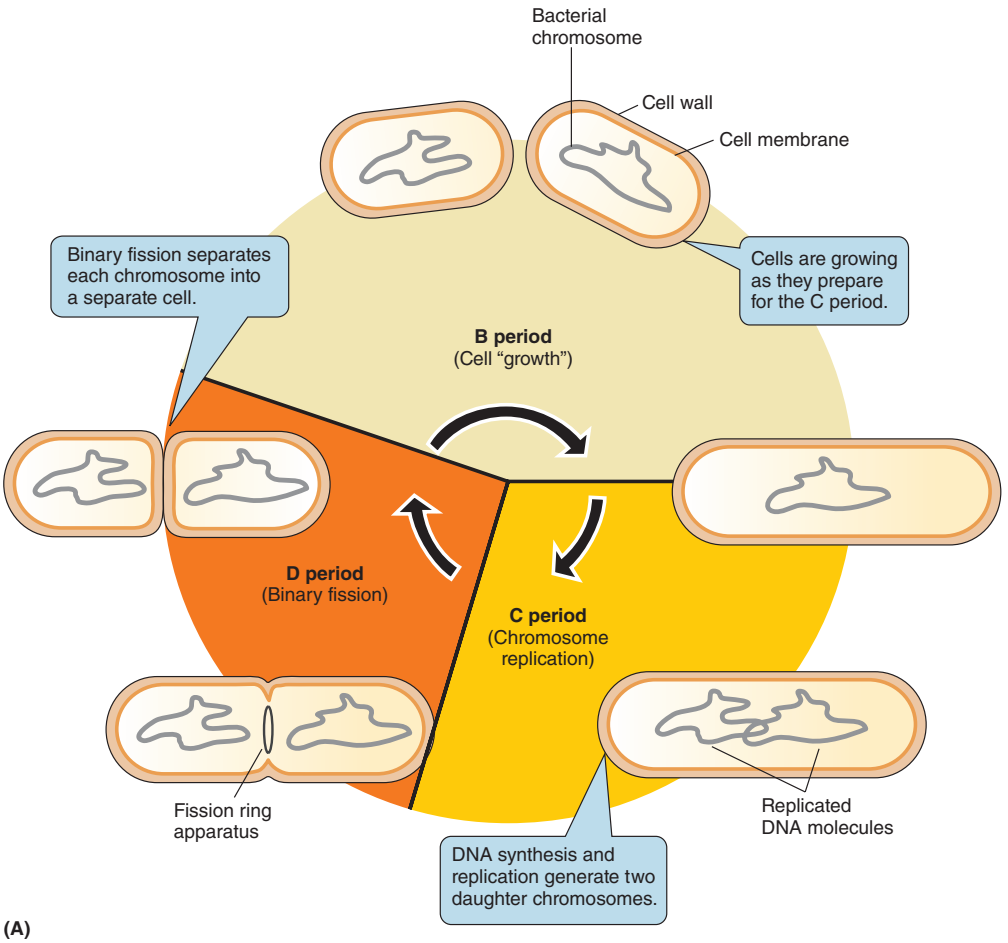
B Period. Before a bacterial cell actually divides into two cells, it goes through a phase of metabolic "growth," called the **B period**, where the cell increases in cell mass and cell size. During this period, the cell is preparing for chromosome replication, so the chromosome remains a single, circular DNA molecule.

C Period. Once the cell has increased in size, DNA replication starts. During this **C period**, DNA synthesis occurs and the bacterial chromosome is copied, which will ensure each daughter cell has one complete set of genetic information when cell division is completed. Chromosome segregation involves the cell's cytoskeleton but, unlike eukaryotic cells, lacks the mitotic spindle to segregate replicated chromosomes. DNA replication will be examined in a later chapter.

D Period. As DNA replication ends, the cell undergoes **binary fission**. During this **D period**, a partition or septum forms at midcell (FIGURE 5.2B). The splitting of the cell in half (**cytokinesis**) is coordinated by cytoskeletal proteins organized into a "fission ring apparatus," which ensures two nearly identical daughter cells are formed. Depending on the growth conditions, the septum material may dissolve at a slow rate allowing pairs, chains, or clusters of cells to form, representing the characteristic arrangements for many bacterial species. With other species, like *E. coli,* the cells completely separate from one another after the septum is complete. Each of the attached or free cells then enters another B period.

Bacterial and Archaeal Cells Can Grow Exponentially

The interval of time between successive binary fissions of a cell or population of cells is known as the **generation time** (or doubling time). Under optimal conditions, some species, especially



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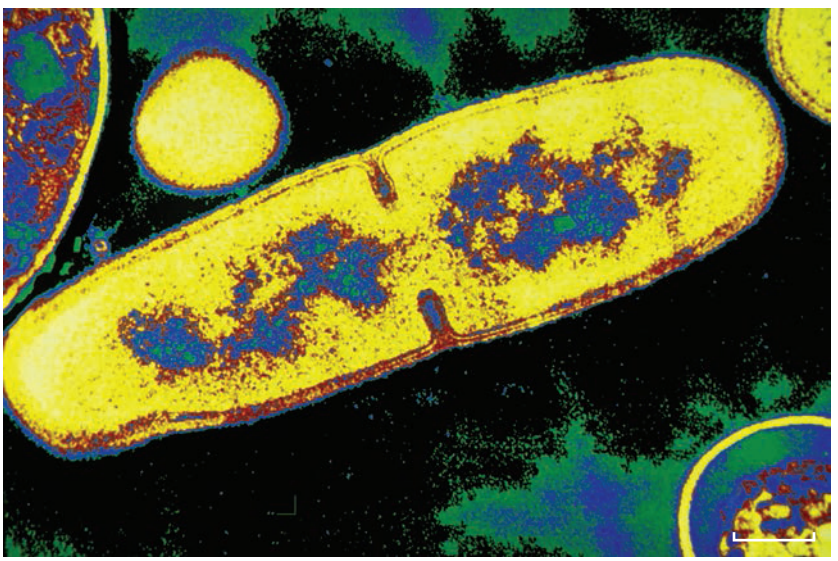


(B)

FIGURE 5.2 **The Bacterial Cell Cycle. (A)** The bacterial cell cycle can be separated into three stages or periods involved with cell growth (B period), chromosome replication (C period), and binary fission (D period). **(B)** A false-color transmission electron micrograph of a cell of *Bacillus licheniformis* undergoing binary fission. The inward growth of the cell envelope is evident at midcell between the segregated chromosomes. (Bar = 0.25 μm.) **How would binary fission differ for a prokaryotic organism having cells arranged in chains and another forming single cells?**

many pathogens, have a very fast generation time; for others, including many environmental species, it is much slower (TABLE 5.2). In fact, for some, it is extremely slow, as **MicroFocus 5.1** reports.

One enterprising mathematician calculated that if *E. coli* binary fissions were to continue at their optimal generation time (15 minutes) for 36 hours, the bacterial cells would cover the surface of the Earth! Thankfully, this will not occur because of the limitation of nutrients and the loss of ideal physical factors required for growth. The majority of the bacterial cells would starve to death or die in their own waste.

The generation time is useful in determining how much time passes before disease symptoms

Bacteria: © NIAID

MICROFOCUS 5.1: Evolution/Environmental Microbiology

A Microbe's Life—Zombie Style

What do *World War Z*, *Zombieland*, *Dawn of the Dead* and some extreme microbial species have in common? All are examples of a zombie state, which for the above-mentioned big screen flicks identifies "creatures" that have died and been reanimated as unconscious, mindless monsters, often apparently arising from deep below the Earth. Well, in the microbial world there also are some microbes that exist in a zombie-like state deep below the Earth.

As you already know, microbes can be found just about anywhere on and in planet Earth where an energy source and nutrients are found. This includes deep below the Earth's surface. In 2013, scientists with the Integrated Ocean Drilling Program, an international marine research program, announced they had discovered bacterial organisms in rocks 2.5 kilometers below the ocean floor. More impressive, these organisms are up to 100 million years old, and the researchers believe the bacterial cells divide only once every 10,000 years! The growth is so slow, scientists originally wondered if these cells were alive—or just undead. However, give them a luxury microbial meal and the absorption of nutrients by the cells can be detected. Thus, the apparent snail's pace for binary fission and growth results from the infinitesimally small amount of energy and nutrients available in their rocky homes. At these rates, it takes thousands of years for the single-celled intraterrestrials (living within the Earth) to produce enough energy to support binary fission. Dr. Beth Orcutt from the Bigelow Laboratory for Ocean Sciences in Maine stated, "Is it really true to call [these microbes] alive when [they're] doubling every thousands of years? It's almost like a zombie state."

Unlike the "human zombies" who attempt to disturb human society (at least in the movies), the ocean scientists believe the rock-dwelling microbial zombies could be beneficially affecting the chemistry in the rocks, the deep Earth, and the planet itself.

Scientifically, these bacterial communities offer an opportunity to learn how cells can survive on so few nutrients and minimal energy sources. Their very existence helps scientists to understand the line between life and death, and to estimate what the bare minimum is for an organism to stay alive.

Dr. Beth Orcutt (front row, second from left) and researchers are trying to understand how life below the sea floor can survive and thrive.

Photo by Jen Magnusson.

TABLE 5.2 Examples of Generation Times—Common Bacterial Species Growing Under Optimal Conditions[1]

Species	Growth Medium	Generation Time (minutes)
Escherichia coli	Synthetic	17
Bacillus megaterium	Synthetic	25
Staphylococcus aureus	Complex	27–30
Streptococcus lactis	Milk	26
Streptococcus lactis	Complex	48
Lactobacillus acidophilus	Milk	66–87
Mycobacterium tuberculosis	Synthetic	792–932 (= 12–15.5 hours)
Treponema pallidum	Rabbit testes	1,980 (= 33 hours)

[1]Table modified from microblog.me.uk/138
Adapted from MicrobLog, Doubling Time

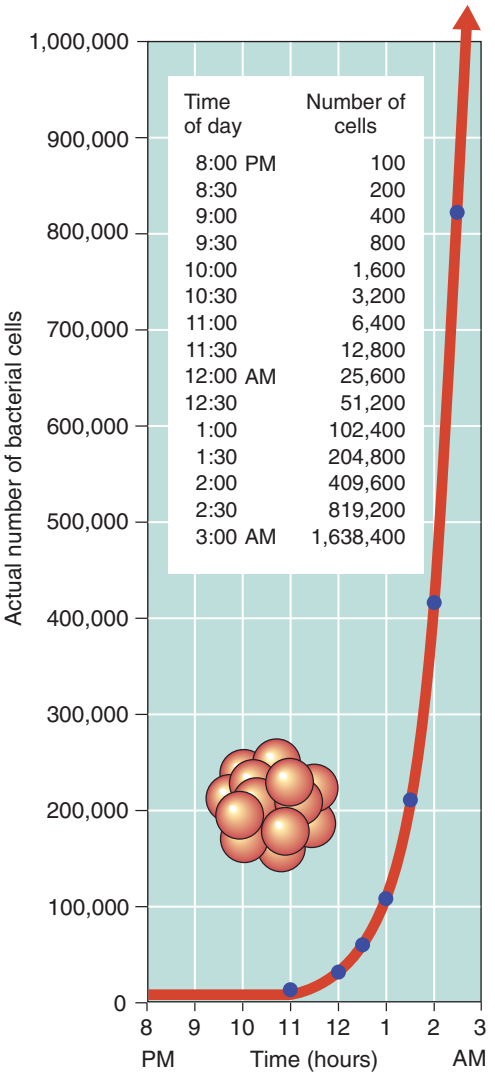


FIGURE 5.3 A Skyrocketing Bacterial Population. The number of *Staphylococcus aureus* cells progresses from 100 cells to almost 2 million cells in a mere 7 hours. The J-shaped growth curve gets steeper and steeper as the hours pass. Only a depletion of food, buildup of waste, or some other limitation will halt the progress of the curve.
What is the generation time for *S. aureus* in this figure?

appear in an infected individual; faster division times often mean a shorter **incubation period** for a disease. For example, suppose you eat an improperly refrigerated chocolate éclair that was contaminated with *Staphylococcus aureus* (FIGURE 5.3). If you ingested 100 cells at 8:00 PM this evening, 200 would be present by 8:30, 400 by 9:00, and 800 by 9:30 PM. You would have more than 25,000 by midnight. By 3:00 AM, the exponential growth will have produced more than 1.6 million cells. Depending on the response of the immune system, it is quite likely that sometime during the night you would know you have food poisoning.

Incubation period: The time from entry of a pathogen into the body until the first symptoms appear.

Bacterial and archaeal organisms are subject to the same controls on growth as all other organisms on Earth. Let's examine the most important growth factors conferring optimal generation times.

CONCEPT AND REASONING CHECKS 1

a. Propose an explanation as to how a bacterial cell "knows" when to divide.
b. If it takes an *E. coli* cell 7 hours of growth to reach some 2 million cells, how long would it take a *T. pallidum* cell to reach the same number under optimal conditions?

CHAPTER CHALLENGE A

We already know some bacterial and archaeal organisms can survive in hostile environments. In fact, these extremophiles not only survive in these environments, they actually can thrive there. This means they need to go through binary fission as part of their cell cycle to maintain their population numbers.

Question A:
Propose how these extremophiles can carry out DNA replication and reproduce in an environment that would otherwise kill a temperate microbe? What are the chances an Earthly extremophile would have been on a spacecraft to Mars? And if it did, would it survive?

Answers can be found in **Appendix F**.

Microbial Growth Progresses Through Distinct Phases

In the previous section, we discovered how fast some bacterial cells can grow under ideal circumstances. Let's look at the dynamics of bacterial growth in a little more detail.

A Bacterial Growth Curve Illustrates the Dynamics of Growth

A typical **bacterial growth curve** for a population illustrates the events occurring over time (FIGURE 5.4). If a sample of bacterial cells is transferred to a tube of fresh broth, four distinct phases of growth occur: the lag phase; the logarithmic phase; the stationary phase; and the decline phase.

The Lag Phase. The first portion of the growth curve, during which time the bacterial cells are adapting to their new environment and compensating for changes in nutritional conditions, is called the **lag phase**. In the broth, some cells may actually die from the shock of transfer or the inability to adapt to the new environment. The actual length of the lag phase (**B period** of the cell cycle) depends on the metabolic activity of the microbial population. They must grow in size, take up nutrients, and replicate their DNA (**C period**)—all in preparation for binary fission (**D period**).

The Log Phase. The population now enters an active, exponential stage of growth called the **logarithmic (log) phase**. In the log phase, all cells are undergoing binary fission and the generation time is dependent on the species and environmental conditions present. The cells exhibit balanced growth because all aspects of metabolism and physiology remain constant.

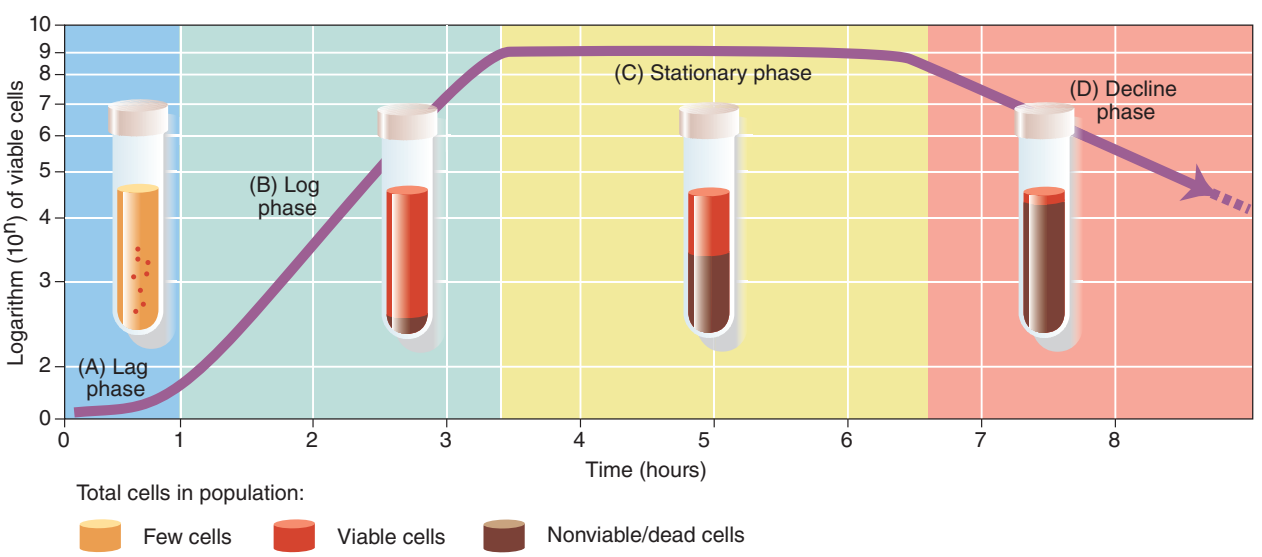


FIGURE 5.4 The Growth Curve for a Bacterial Population. A typical bacterial population goes sequentially through four growth phases. » **Why would antibiotics work best to kill or inhibit cells in the log phase?**

As each generation time passes, the number of cells doubles and the graph rises accordingly on a logarithmic scale.

In a broth tube, the medium becomes cloudy (turbid) due to increasing cell numbers. If plated on solid growth medium, bacterial growth will be so vigorous that visible colonies appear within 24 to 48 hours and each colony may consist of millions of cells (FIGURE 5.5). Vulnerability to antibiotics is also highest at this active stage of growth because many antibiotics affect metabolic processes like protein synthesis in dividing cells.

The Stationary Phase. Because a broth tube represents a "closed system," nutrients are not available indefinitely. Therefore, after some hours in a broth tube, available nutrients become scarce and waste products accumulate. This limitation of nutrients and buildup of waste materials leads to a decline in the growth rate. The vigor of the population changes and the cells enter a plateau, called the **stationary phase**. Now the number of viable cells equals the number of nonviable or dead cells.

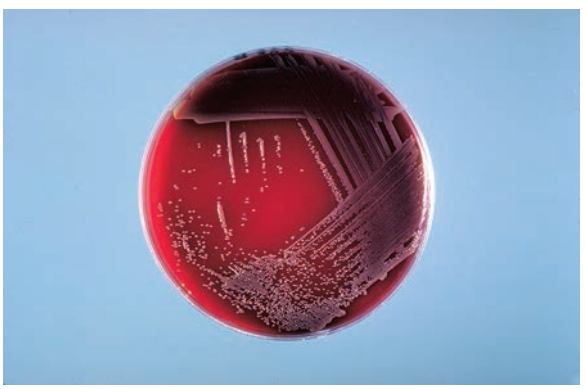
The Decline Phase. If nutrients in the external environment remain limited or the quantities become exceedingly low, the population enters a **decline phase** (or death phase). Here the number of viable cells declines logarithmically. For many species, the history of the population ends with the death of the last cell.

On the other hand, for some bacterial species, a few members of the population can escape death by entering a state of dormancy.

Some Bacterial Cells Can Exist In a Metabolically Inactive State

Environmental conditions vary tremendously and often they might not be favorable to active population growth. Such unfavorable conditions include the presence of toxic chemicals, such as antibiotics, and nutrient limitation (potential starvation). Therefore, throughout the cell cycle microbes must constantly monitor or sense their surroundings to ensure the conditions will support continued growth. For some bacterial species, if the environment is not favorable, the cells respond by entering a dormant (nondividing) state. When environmental conditions improve, the cells "revive" and once again start dividing. Two such dormancy strategies are described here.

Persister Cell Formation. During the log phase of growth, some bacterial populations produce a subset of cells that normally stop dividing and remain in the B period of the cell cycle. However, these so-called **persister**



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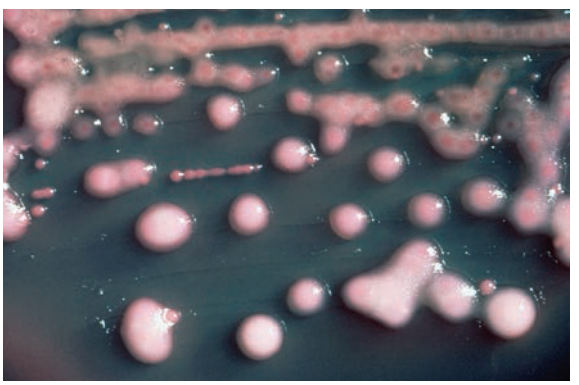


(B)

FIGURE 5.5 **Two Views of Bacterial Colonies. (A)** Bacterial colonies cultured on blood agar in a culture dish. Blood agar is a mixture of nutrient agar and blood cells. It is often used for growing bacterial colonies. **(B)** Close-up of typhoid bacterial (*Salmonella enterica* serotype Typhi) colonies being cultured on a growth medium. **How did each colony in (A) or (B) start?**

(A) Courtesy of Dr. J. J. Farmer/CDC. (B) Courtesy of CDC.

cells maintain a very low rate of metabolism. Should the environment change for the worse, the persister cells in the population survive even though the rest of the population may die. For example, such "altruistic-like" behavior benefits pathogens like *M. tuberculosis.* If antibiotics are used to treat the infection, any persister cells will survive because they are not affected by the antibiotics, which target only actively dividing, log phase cells (FIGURE 5.6). Should the antibiotics be withdrawn at some later date, the persister cells become active again, forming a new actively growing population with some persister cells present.

Endospore Formation. A few medically-significant gram-positive genera, especially *Bacillus* and *Clostridium,* undergo a different dormancy scenario when the cells experience nutrient depletion. Species, such as *Bacillus anthracis* (causative agent of anthrax) and *Clostridium botulinum* (the causative agent of botulism) enter the stationary phase and begin spore formation or **sporulation** that produces dormant structures called **endospores** (FIGURE 5.7).

Unlike persister cells, most endospores are the result of a focused morphological differentiation process specifically tied to nutrient limitation (starvation) in the environment and is initiated by **quorum sensing** within the "starving" population.

Spore formation requires a complex program of gene expression that involves hundreds of genes. The process involves the replication of the bacterial chromosome and binary fission that produces an asymmetric cell division (FIGURE 5.8). The smaller cell, called the prespore, will become the mature endospore, while the larger mother cell will commit itself to maturation of the endospore before rupturing. Depending on the exact asymmetry of cell division, the single endospore may develop at the end of the mother cell, near the end, or at the center of the cell (the position is useful for species identification purposes). The entire process takes about 6 to 8 hours.

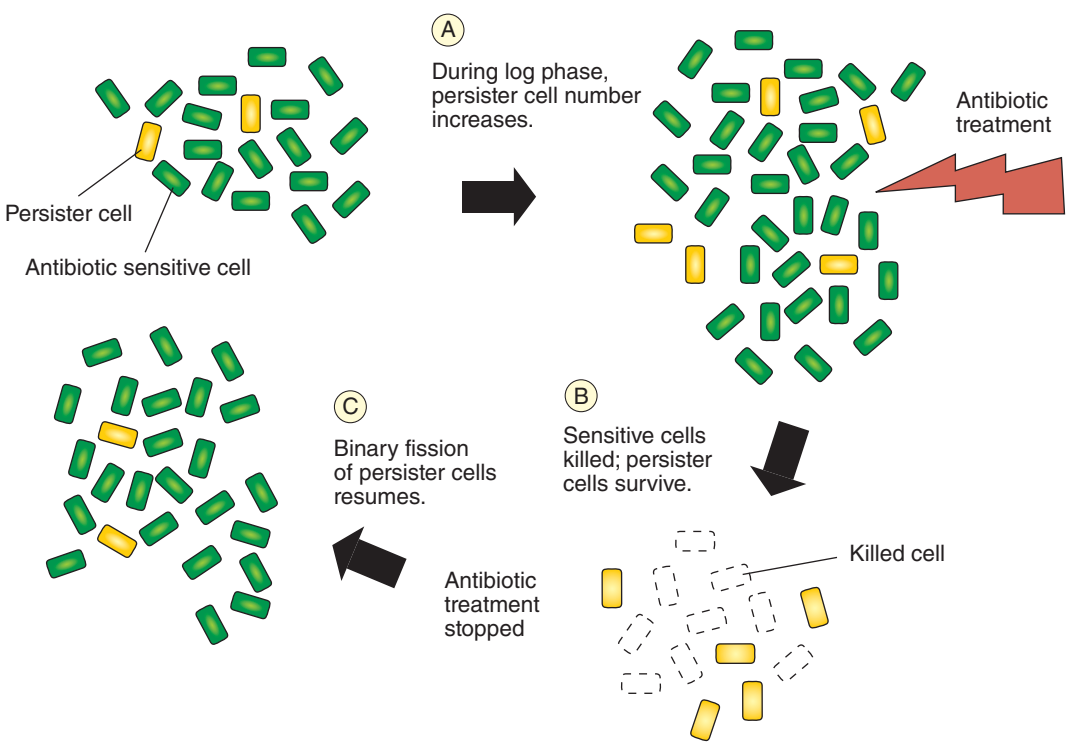
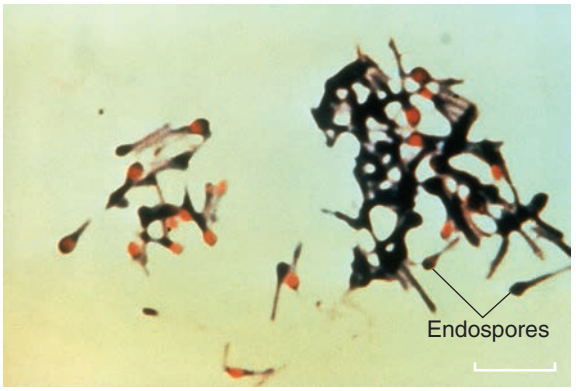


FIGURE 5.6 **Persister Cells.** The presence of persister cells within a bacterial cell population allows the species to survive environmental changes, in this case the presence of an antibiotic. **» What other way could a susceptible bacterial cell population survive in the presence of an antibiotic?**



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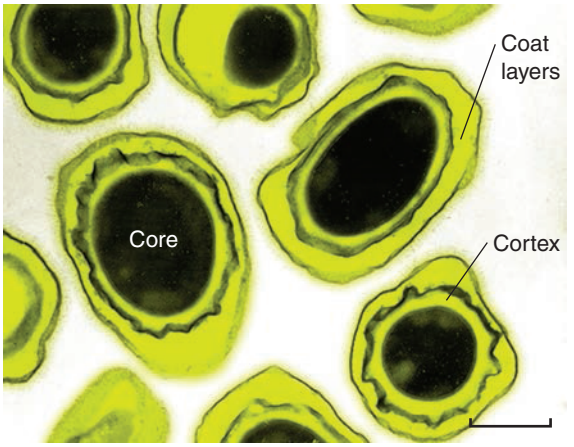

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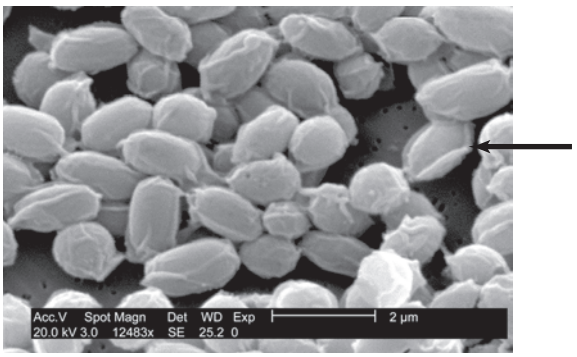


(C)

FIGURE 5.7 Three Different Views of Bacterial Endospores. (A) A light microscope image of *Clostridium* cells showing terminal spore formation. Note the characteristic drumstick appearance of the spores. (Bar = 5.0 µm.) **(B)** A false-color transmission electron micrograph of *Bacillus anthracis* spores. The visible spore structures include the core, cortex, and coat layers. (Bar = 0.5 µm.) **(C)** A scanning electron microscope view of a germinating spore (arrow). Note that the spore coat divides equatorially along the long axis, and as it separates, the vegetative cell emerges. (Bar = 2.0 µm.) **If an endospore is resistant to so many environmental conditions, how does a spore "know" conditions are favorable for germination?**

(A) Courtesy of CDC. (B) © Scott Camazine/Alamy. (C) Courtesy of Janice Carr/CDC.

The highly dehydrated free spore contains cytoplasm, highly compacted DNA, and a large amount of **dipicolinic acid**, a unique chemical compound that helps stabilize the proteins and DNA. Thick layers of peptidoglycan form the cortex and some 70 proteins form the layers of the spore coat to protect the cytoplasmic contents. It is important to emphasize that sporulation is not a reproductive process. Rather, the endospore represents a "resting stage" produced in response to environmental "hard times."

Endospores are probably the most resistant living structures known. Desiccation has little effect on the spore. By containing little water, endospores also are heat resistant and undergo very few chemical reactions. These properties make them difficult to eliminate from contaminated medical materials and food products. For example, endospores can remain viable in boiling water (100°C) for 2 hours. They can survive in 70% ethyl alcohol for 20 years. Humans can barely withstand 500 rems of radiation, but endospores can survive one million rems. In this dormant condition, endospores can "survive" for long periods of time, perhaps millennia, as **MicroFocus 5.2** reveals. No wonder some scientists believe they could survive a trip to Mars!

Rem (Roentgen equivalent in man): A measure of radiation dose related to biological effect.

When the environment is favorable for cell growth, the spore coat and cortex break down over a 90-minute period and each endospore rapidly germinates and grows out as a vegetative cell (see Figure 5.7C).

Killing endospores can be a tough task. Heating them for many hours under high pressure will do the trick. If they contaminate machinery, such as the mail-sorting equipment contaminated in the 2001 anthrax attacks, there are potent but highly dangerous chemical methods to kill the spores. Such methods are discussed in another chapter.

Medical Significance. A few serious diseases in humans are caused by spore formers. The most newsworthy has been *B. anthracis*,

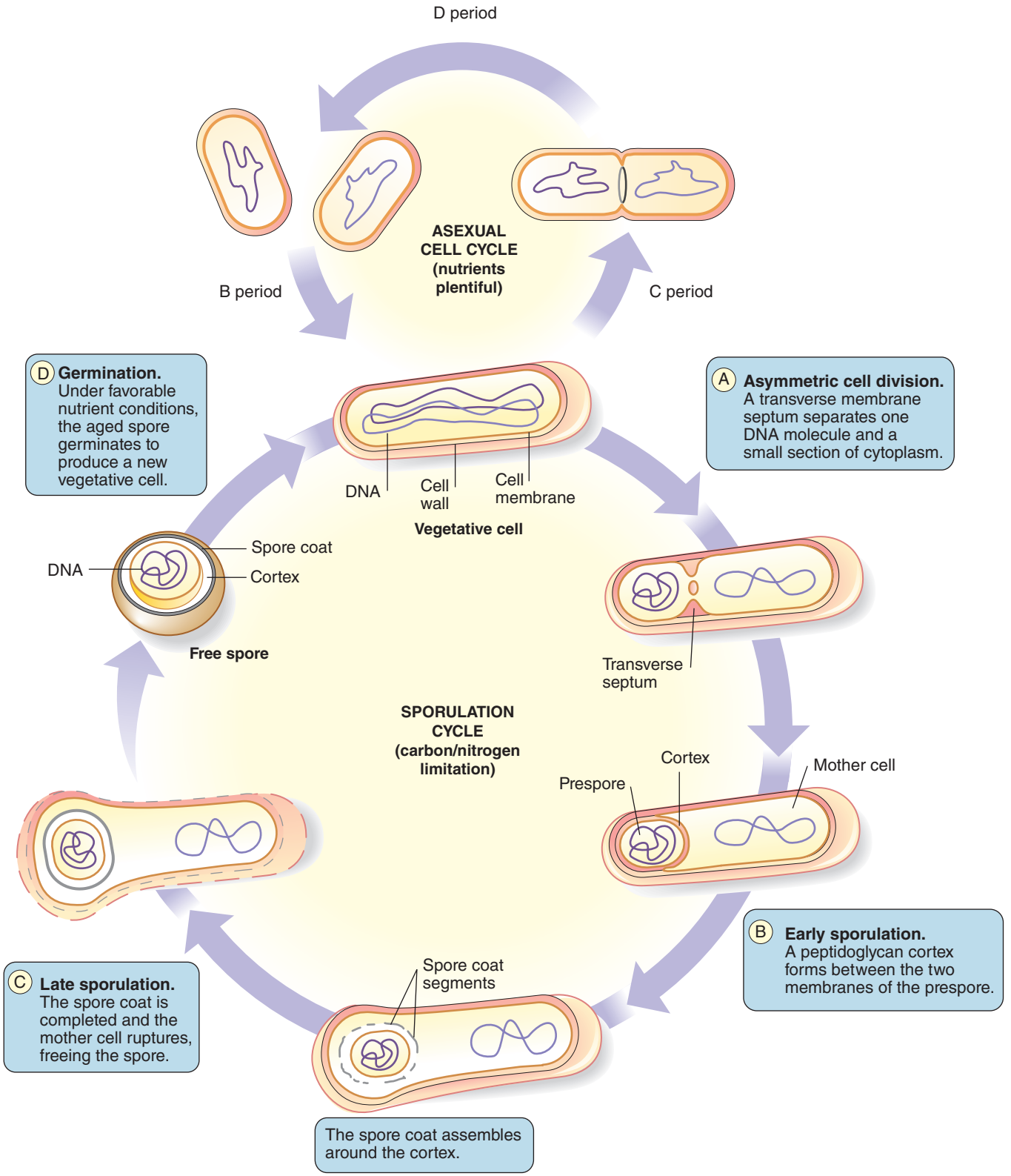


FIGURE 5.8 **The Formation of a Bacterial Spore by *Bacillus subtilis*.** (**A–E**) When nutrient conditions become limiting (e.g., carbon, nitrogen), endospore formers, such as *B. subtilis*, enter the sporulation cycle shown here. **Hypothesize how a vegetative cell "knows" nutrient conditions are limiting.**

MICROFOCUS 5.2: Being Skeptical

Germination of 25 Million-Year-Old Endospores?

Endospores have been discovered in various archaeological sites and environments around the world. Living spores have been recovered and germinated from the intestines of Egyptian mummies several thousand years old. In 1983, archaeologists found viable *Thermoactinomyces* spores in sediment lining Minnesota's Elk Lake. The sediment was over 7,500 years old.

All these reports, though, pale in comparison to the controversial discovery reported in 1995 by researcher Raul Cano of California Polytechnic State University, San Luis Obispo. Cano found bacterial spores in the gut of a fossilized bee trapped in amber—a hardened resin—produced from a tree in the Dominican Republic. The amber with the fossilized bee was dated as being about 25 million years old. When the amber was cracked open and the material from the gut of the bee extracted and placed in nutrient medium, the equally ancient endospores germinated. With microscopy, the cells from a colony were very similar to *Bacillus sphaericus*, which is found today in bees in the Dominican Republic. Is it possible for an endospore to survive for 25 million years—even if it is encased in amber?

Critics were quick to claim the bacterial species may represent a modern-day species that contaminated the amber sample being examined. However, Professor Cano had carried out appropriate and rigorous decontamination procedures and sterilized the amber sample before cracking it open. He also carried out all the procedures in a class II laminar flow hood, which prevents outside contamination from entering the working area. In addition, the hood had never been used for any other bacterial extraction processes. Several other precautions were added to eliminate any chance that the spores were modern-day contaminants from an outside source.

The major question remaining is whether DNA can remain intact and functional after so long a period of dormancy. Does the DNA really have a capability of replication and producing new vegetative growth? Granted, the DNA presumably was protected in a resistant spore, but could DNA remain intact for 25 million years? Research on bacterial DNA suggests the maximum survival time is about 400,000 to 1.5 million years. If true, then the 25 million-year-old spores could not be viable. But that is based on current predictions, and they may be subject to change as more research is carried out with ancient DNA.

The verdict? It seemed unlikely such ancient endospores could germinate after 25 million years. Then, in 2000, another research group using Cano's techniques revived 250 million-year-old bacterial cells from spores trapped in salt crystals. Maybe it is possible.

the agent of the 2001 anthrax bioterror attack through the mail. This potentially deadly disease, originally studied by Koch and Pasteur, develops when inhaled spores germinate in the lower respiratory tract and the resulting vegetative cells secrete two deadly toxins. Other human diseases result from the germination of *Clostridium* endospores—gas gangrene (*C. perfringens*) and tetanus or lockjaw (*C. tetani*). Clostridial endospores often are found in soil and, should they find their way into human tissue through a puncture wound, the spores will germinate in the wound's dead tissue and produce deadly toxins. Botulism, caused by *C. botulinum*, can be a deadly form of food poisoning. Many foods improperly preserved can be contaminated with endospores that on germination, the vegetative cells produce deadly toxins.

In hospital and healthcare facilities, *C. difficile* (often referred to as *C. diff*) can cause serious infections of the colon (colitis) that are often resistant to antibiotic treatment. More than three million *C. difficile* infections occur in U.S. hospitals each year, partly coming from the tissue germination of endospores originally present on improperly cleaned medical equipment and on personal items. (These diseases will be covered in more detail in the appropriate body system chapter).

Optimal Microbial Growth Is Dependent on Several Physical Factors

Having examined the bacterial cell cycle and dormancy, let's examine the essential physical and chemical factors influencing cell growth.

Temperature. Temperature is one of the most important factors governing growth. Every microbial species has an optimal growth temperature. Each species also has an approximate 30°C operating range, from minimum to maximum, over which the cells will grow although they will have a slower generation time (FIGURE 5.9). In general, most microbes can be assigned to one of three temperature groups—psychrophiles, mesophiles, or thermophiles—based on their optimal growth temperature.

Microbes that have their optimal growth rates near 15°C but can still grow at 0°C to 20°C are called **psychrophiles** (*psychro* = "cold"). Because about 70% of the Earth is covered by oceans having deep-water temperatures below 5°C, psychrophiles make up a large portion of the global microbial community. In fact, many psychrophiles can grow as fast at 4°C as *E. coli* does at 37°C. On the other hand, at these low temperatures, psychrophiles would not be human pathogens because they cannot grow at the warmer 37°C body temperature.

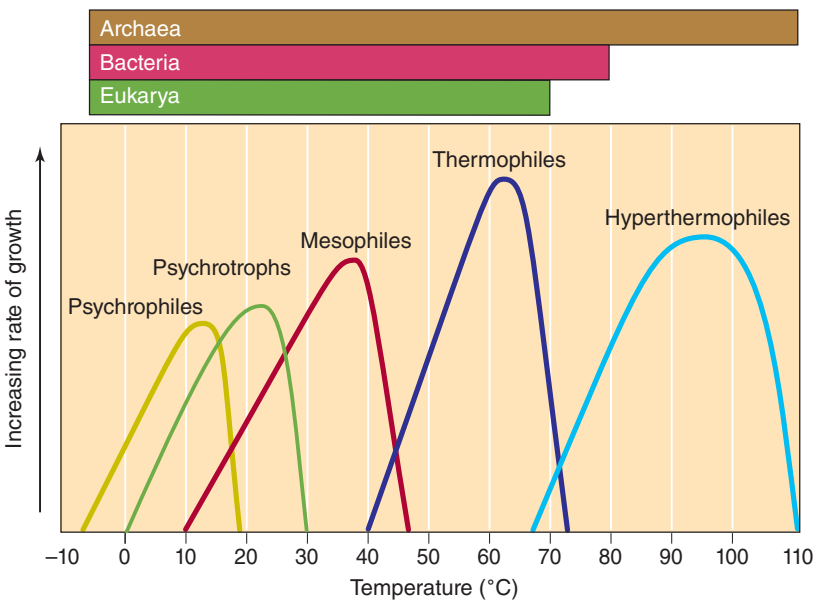


FIGURE 5.9 Growth Rates for Different Microorganisms in Response to Temperature. Temperature optima and ranges define the growth rates for Bacteria, Archaea, and Eukarya. Notice that the growth rates decline quite rapidly to either side of the optimal growth temperature. **» Propose what adaptations are needed for microbes to survive at the psychrophilic or thermophilic extremes.**

Another group of "cold-loving" microbes are the **psychrotrophs** or **psychrotolerant** microorganisms. These species have a slightly higher optimal growth temperature as well as a broader operating range. Psychrotrophs can be found in water and soil in temperate regions of the world but are perhaps most commonly encountered on spoiled refrigerated foods (4°C). Some bacterial and archaeal species are psychrotolerant as are several microbial eukaryotic species, including fungi (molds). When such contaminated foods are consumed without heating, the toxins the microbes produce may cause food poisoning. One example is *Campylobacter,* the most frequently identified cause of infective diarrhea (Clinical Case 5).

At the opposite extreme are the **thermophiles** (*thermo* = "heat") that multiply best at temperatures around 60°C but still multiply over a range from 40°C to 70°C. Thermophiles are present in compost heaps and hot springs, and can be contaminants in dairy products because they survive pasteurization temperatures. However, thermophiles pose little threat to human health because they do not grow well at the cooler temperature of the body.

There also are many archaeal species that grow optimally at temperatures exceeding 80°C and having optima near 95°C. These **hyperthermophiles** have been isolated from seawater near hot-water vents along rifts on the floor of the Pacific Ocean. Because the high pressure keeps the water from boiling, some archaeal species can grow at an astonishing 121°C (see Table 5.1).

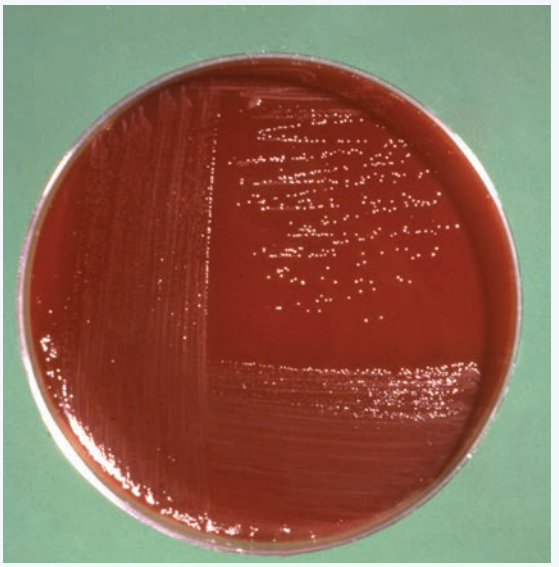
Most of the best-characterized microbial species are **mesophiles** (*meso* = "middle"), which thrive at the middle temperature range of 10°C to 45°C. This includes the pathogens that grow in warm-blooded animals, including humans, as well as those species found in aquatic and soil environments in temperate and tropical regions of the world. *E. coli* is a typical mesophile. The vast majority of microbes discussed in this text are mesophiles.

Oxygen. The growth of many microbes depends on a plentiful supply of oxygen gas, and in this respect, such **obligate aerobes** must use the gas as a final electron acceptor to make

CLINICAL CASE 5

An Outbreak of Food Poisoning Caused by *Campylobacter jejuni*

1. On August 15, a cook began his day by cutting up raw chickens to be roasted for dinner.
2. He also cut up lettuce, tomatoes, cucumbers, and other salad ingredients on the same countertop. The countertop surface where he worked was unusually small.
3. For lunch, the cook prepared sandwiches on the same countertop. Most were garnished with lettuce.
4. Restaurant patrons enjoyed sandwiches for lunch and roasted chicken for dinner. Many patrons also had a portion of salad with their meal. None of the foods had been refrigerated.
5. During the next 3 days, 14 people experienced stomach cramps, nausea, and vomiting.
6. Public health officials learned that all the affected patrons had eaten salad with lunch or dinner. *Campylobacter jejuni,* a bacterial pathogen of the intestines, was isolated from their stools (see figure).



Campylobacter jejuni agar culture.

Courtesy of Sheila Mitchell/CDC.

7. On inspection, microbiologists concluded that the chicken was probably contaminated with *C. jejuni.* However, the microbiologists suspected the cooked chicken was not the cause of the illness. Rather, *C. jejuni* from the raw chicken was the source.

Questions:

(Answers can be found in **Appendix E**.)

A. Why would the cooked chicken not be the source for the illness?

B. Why was the raw chicken identified as the source?

C. How, in fact, did the patrons become ill?

For additional information see http://www.cdc.gov/mmwr/preview/mmwrhtml/00051427.htm.

cellular energy (ATP). Other species, such as *Treponema pallidum,* the agent of syphilis, are termed **microaerophiles** because they survive in environments where the concentration of oxygen is relatively low. In the body, certain microaerophiles cause disease of the oral cavity, urinary tract, and gastrointestinal tract. Conditions can be established in the laboratory to study these microbes (FIGURE 5.10A).

The **anaerobes**, by contrast, are microbes that do not or cannot use oxygen. Some are **aerotolerant**, meaning they are insensitive to oxygen. Many bacterial and archaeal species, as well as a few fungal and protistan species, are **obligate anaerobes**, which are inhibited or killed if oxygen is present. This means they need other ways to make ATP. Some anaerobic bacterial species use sulfur in their metabolic activities instead of oxygen, and therefore they produce hydrogen sulfide (H_2S) rather than water (H_2O) as a waste product of their metabolism. Others we have already encountered, such as the ruminant archaeal organisms that produce methane as the byproduct of the energy conversions. In

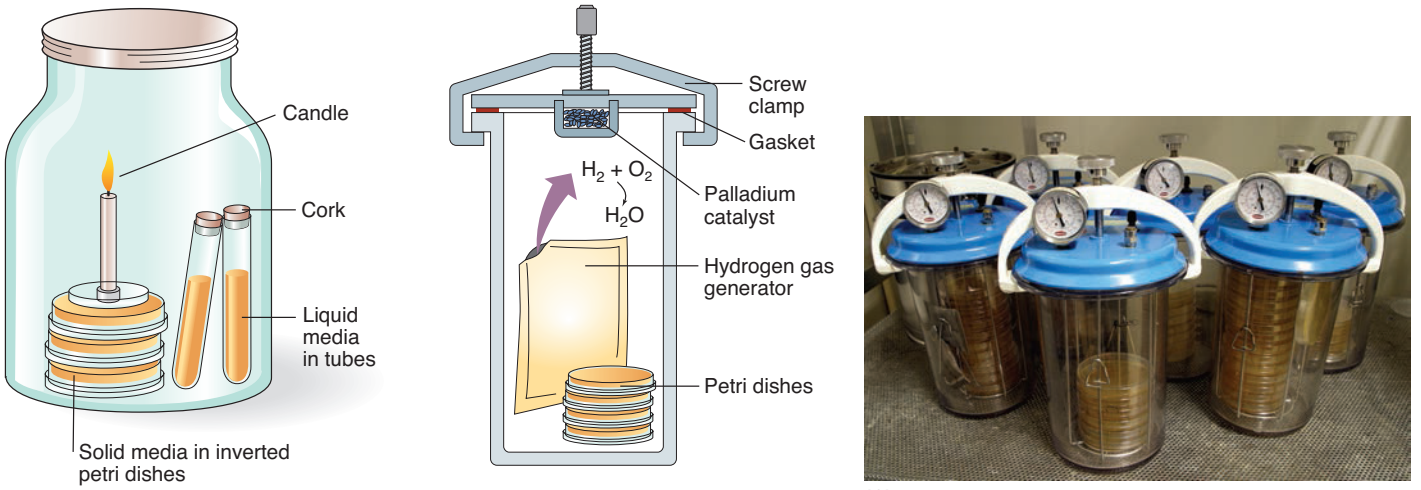


FIGURE 5.10 Bacterial Cultivation in Different Gas Environments. Two types of cultivation methods are shown for bacterial species growing poorly in an oxygen-rich environment. **(A)** A candle jar, in which microaerophilic bacterial species grow in an atmosphere where the oxygen is reduced by the burning candle. **(B, C)** An anaerobic jar, in which hydrogen is released from a generator and then combines with oxygen through a palladium catalyst to form water and creates an anaerobic environment. **In which jar would a facultative anaerobe grow?**

(**C**) © Scott Coutts/Alamy.

fact, life originated on Earth in an anaerobic environment consisting of methane and other gases. **MicroFocus 5.3** recounts the events leading to an oxygen-rich atmosphere.

Some anaerobic bacterial species cause disease in humans. For example, the *Clostridium* species mentioned above causing tetanus and gas gangrene multiply in the dead, anaerobic tissue of a wound and produce toxins causing tissue damage. Botulism, caused by another species of *Clostridium* multiplies in the oxygen-free environment of an improperly sealed can of food, where it produces the lethal toxin of botulism.

Among the most widely used methods to establish anaerobic conditions in the laboratory is the GasPak system, in which hydrogen reacts with oxygen in the presence of a catalyst to form water, thereby creating an oxygen-free atmosphere (FIGURE 5.10B, C).

Many microbes are neither strictly aerobic nor anaerobic but rather can grow in either the presence of or a reduced concentration of oxygen. This group includes *E. coli,* many staphylococci and streptococci, members of the genus *Bacillus,* and the fungal yeasts. Thus, we often refer to these organisms as **facultative anaerobes** because they can grow best in the presence of oxygen gas but will switch to anaerobic metabolism when oxygen gas is absent.

A common way to test an organism's oxygen sensitivity is to use a **thioglycollate broth**, which binds free oxygen so that only fresh oxygen entering at the top of the tube would be available (FIGURE 5.11).

Finally, there are bacterial species said to be **capnophilic** (*capno* = "smoke"); they require an atmosphere low in oxygen but rich in carbon dioxide gas. Members of the genera *Neisseria* and *Streptococcus* are capnophiles.

pH. The cytoplasm of most microorganisms has a pH near 7.0. This means the majority of species are **neutrophiles**, growing optimally near neutral pH but having a growth range that covers 2 to 3 pH units (1,000-fold change in H^+ concentration). However, some bacterial species, such as *Vibrio cholerae,* can tolerate acidic conditions as low as pH 2.0 and alkaline conditions as high as pH 9.5.

Acid-tolerant bacteria called **acidophiles** grow best at pHs below 5 and are valuable in the food and dairy industries. For example, certain species of *Lactobacillus* and *Streptococcus* produce the acid that converts milk to buttermilk and cream to sour cream. These species pose no threat to good health even when consumed in large amounts. The "active cultures" in a cup of yogurt also are acidophilic bacterial species. **Extreme acidophiles**, preferring pHs of 1 to 2,

Bacteria: © NIAID

MICROFOCUS 5.3: EVOLUTION

"It's Not Toxic to Us!"

It's hard to think of oxygen as a poisonous gas considering how many organisms need it to survive. Yet billions of years ago, oxygen was extremely toxic. One whiff by an organism and a cascade of highly destructive cellular reactions was set into motion. Death followed quickly.

Difficult to believe? Not if you realize that the ancient members of the Bacteria and Archaea relied on anaerobic chemistry for their energy needs. The atmosphere was full of methane, carbon dioxide, and other gases that they could use to generate energy. But no oxygen. And it stayed that way for around 1 billion years.

Then, about 3 billion years ago, along came the cyanobacteria with their ability to perform photosynthesis. Floating on the surface of the oceans, the cyanobacteria trapped sunlight and converted it to chemical energy in carbohydrates; the process was photosynthesis. But there was a downside to photosynthesis: oxygen was a waste product of the process—and it was deadly because the oxygen radicals (O_2^-, $OH^\bullet$) produced could disrupt cellular metabolism by "tearing away" electrons from other molecules.

Over the next few hundred million years, oxygen gas remained scarce. Then, about 2.4 billion years ago, there was a sudden and dramatic increase in oxygen gas. Unable to cope with the toxic conditions, enormous numbers of microbial species became extinct. Others "escaped" to oxygen-free environments, such as lake and deep ocean sediments where they still exist today. The cyanobacteria survived in the open oceans because they evolved the enzymes to safely tuck away oxygen atoms in a nontoxic form—that form was water.

Among the survivors of these first communities were gigantic, shallow-water colonies called "stromatolites." In fact, these rock-like looking structures are still found in a few places on Earth, such as Shark Bay off the western coast of Australia (see figure). These structures formed from ocean sediments and calcium carbonate that became trapped in the microbial community as a biofilm. The top few inches in the crown of a stromatolite contain the oxygen-evolving, photosynthetic cyanobacteria, while below the crown are other bacterial species that can also tolerate oxygen and sunlight. Buried yet beneath these organisms are other bacterial species surviving the anaerobic, dark niche of the stromatolite interior where neither oxygen nor sunlight can reach. A couple of billion years would pass before one particularly well-known species of oxygen-breathing creature evolved: *Homo sapiens.*

Stromatolites, Shark Bay, Western Australia.

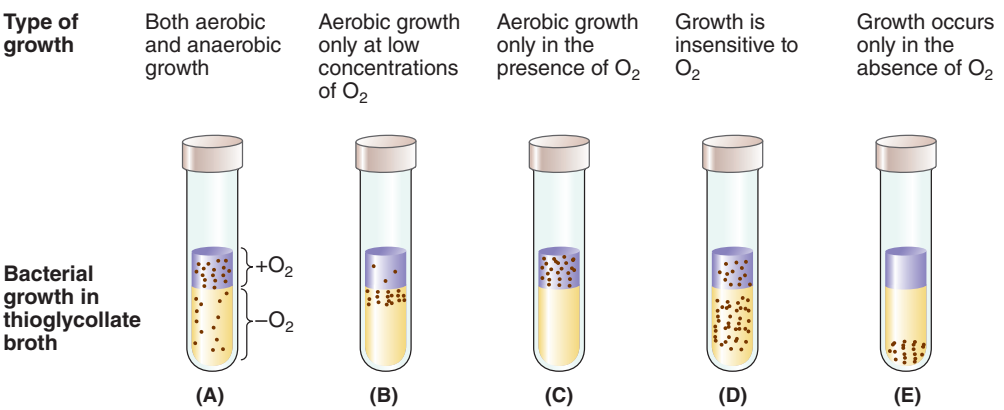


FIGURE 5.11 The Effect of Oxygen on Microbial Growth. Each tube contains a thioglycollate broth into which was inoculated a different bacterial species. **» Identify the O_2 requirement in each thioglycollate tube based on the growth density [example: (A) represents facultative anaerobe species].**

are found among the Archaea. At the opposite extreme are the **alkaliphiles**, which grow best at pHs above 9.

The majority of known bacterial species, however, do not grow well under acidic conditions. Thus, the acidic environment of the stomach helps deter disease, while providing a natural barrier to the organs beyond. In addition, you may have noted certain acidic foods such as lemons, oranges, and other citrus fruits as well as tomatoes and many vegetables are hardly ever contaminated by bacterial growth. However, such damaged produce may be subject to fungal growth because many fungi are acidophiles and grow well at a pH of 5 or lower.

Other Factors. Further environmental factors can influence the growth of microbial cells. Psychrophiles in deep ocean waters and sediments are under extremely high hydrostatic pressure. In some deep marine trenches the hydrostatic pressure can be as high as 16,000 pounds per square inch (psi) and only some extremophiles may be adapted to the pressure. Such **barophiles** (*baro* = "pressure") in fact will rupture quite quickly at normal atmospheric pressures (14.7 psi).

Hydrostatic pressure: The pressure exerted by the weight of water.

Other microbes have adapted to saline or highly saline environments such as the Great Salt Lake in Utah, the Dead Sea, and in evaporation ponds. These **halophiles** (*halo* = "salt") are characterized by their need for hypersaline conditions for growth. They include species, such as *Vibrio cholerae,* that grow optimally at 2% to 5% NaCl and other genera that grow optimally at 5% to 20% NaCl. **Extreme halophiles**, like *Halobacterium salinarium* and the eukaryotic green alga *Dunaliella salina,* grow optimally at 20% to 30% NaCl. In contrast, species like *E. coli* are **nonhalophiles** because they grow optimally at less than 2% NaCl and genera, such as *Staphylococcus*, are **halotolerant** because they can grow in saline (up to 8% NaCl) as well as nonsaline environments.

The hypersaline conditions can be deadly to microbial cells because water will be lost to the hypertonic external medium. To prevent the loss of cellular water, halophiles accumulate high cytoplasmic concentrations of solutes, such as sugars, amino acids, or ions, to balance out the osmotic difference.

The fact that some microbial life can survive under extreme environmental conditions is clear from reading **MicroFocus 5.4**.

FIGURE 5.12 summarizes the physical factors influencing microbial growth.

CONCEPT AND REASONING CHECKS 2

Bacteria: © MMD

a. In a broth tube, describe the status of the bacterial cell population in each phase of the growth curve.
b. Explain how the trigger to persister cell survival is different from the trigger to endospore formation.
c. Identify what would be extremophile-type conditions for each of the physical factors described in this section.

CHAPTER CHALLENGE B

You have now studied many of the major physical factors (temperature, oxygen, pH, and salt) that influence microbial growth and reproduction and you have heard about many that can survive these physical extremes (see Table 5.1).

Question B:
Could a microbe survive the physical extremes on Mars? What kind of earthly microbe might this Martian microbe be like? Here are some useful facts about Mars.

Surface temperature: Estimated to be from a warm 27°C (81°F) to –143°C (–225°F) at the winter polar caps.

Atmosphere: 95% carbon dioxide, but also present are nitrogen (2.7%), argon (1.6%), and oxygen (0.13%; 21% on Earth) gases; mean surface pressure is much lower than Earth's. UV radiation is 3× Earth's (note: there are earthly bacterial species able to survive 5,000× the dose that would kill human cells).

Soil: Existence of water ice confirmed; soil pH = about 7.7; several chemicals found that could serve as nutrients for life forms, including magnesium, potassium, and chloride.

Salt: Dark, finger-like features could be the flow of salty water perhaps equivalent in salinity to Earth's oceans.

Answers can be found in **Appendix F**.

Bacteria: © NIAID

MICROFOCUS 5.4: Environmental Microbiology

Drilling for Microbes

There are some 400 known subglacial lakes beneath the Antarctic ice sheet. One of these, Lake Vostok, lies at a depth of more than 3 kilometers below Vostok Station, a Russian research outpost some 1,300 kilometers southeast of the South Pole. Many scientists believe Lake Vostok, which is estimated to be 260 kilometers long and 48 kilometers across at its widest point, similar in area to Lake Ontario, has been isolated from the atmosphere for 15 million years. Most interesting from a microbiological perspective is the presence of liquid water, which means there is the possibility of microbial life existing within the ancient lake.

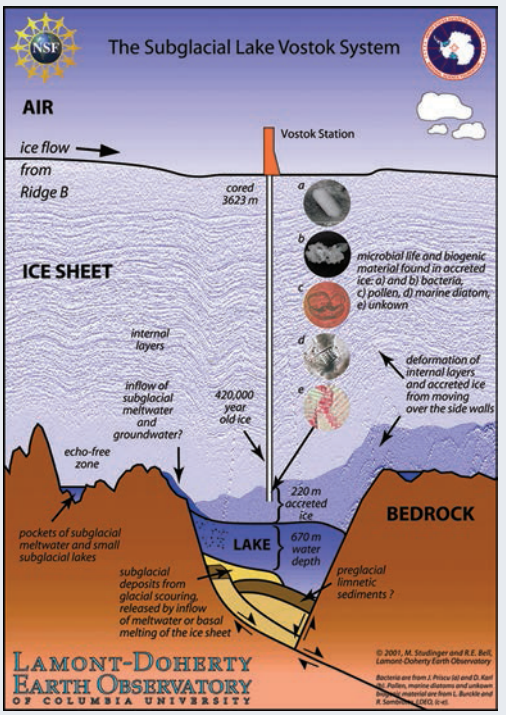
For more than two decades and 57 Antarctic expeditions, Russian scientists have been slowly and carefully drilling down through the ice toward the lake to examine the water contents. Then, on Sunday, February 5, 2012, just a day before the expedition would end its season, they hit the lake! However, when the drill contacted the lake, the lake water, being under such great pressure from the ice above, shot up the bore hole and froze. At this writing, the scientists have not reported if the frozen water contains any microbes.

Meanwhile, an American research group has collected ice cores from frozen water at the bottom (underside) of the glacier ice covering Lake Vostok. These cores should represent a record of the lake surface water that froze at the glacier interface, and correspond to what is in the liquid at the surface of the lake.

In 2013, the group reported they discovered a diverse microbial population in these cores. DNA sequence analyses from four cores identified more than 3,500 unique sequences, which presumably equates to that number of species or strains. About 95% of the sequences were associated with bacterial species (two additional sequences were related with archaeal species) and 5% liked with eukaryotic species, especially the fungi. Therefore, Lake Vostok itself might contain a complex web of organisms that have evolved and developed over the tens of millions of years of its existence.

Note: An American team in 2012 reported the discovery of diverse bacterial life in another Antarctic lake (Lake Vida) that lies under just 27 meters of ice. This highly saline lake with a water temperature of –13°C was distinct from that in other saline lakes in the same area. And yet another American team in 2013 reported preliminary evidence for microbial life growing in culture dishes from samples of water taken from Lake Whillans that lies 800 meters below the ice sheet.

Should we be surprised with these discoveries? Perhaps not as we know microbes will eke out an existence anywhere an energy source is available.



Courtesy of Michael Studinger/Lamont-Doherty Earth Observatory.

KEY CONCEPT 5.3

Bacteria: © NIAID

Culture Media Are Used to Grow Microbes and Measure Their Growth

Microorganisms can be grown (cultured) in a liquid medium (*pl.* media) or on a solid medium. Liquid media, called **broths**, are contained in tubes and bottles, and consist of the growth nutrients dissolved in water. After sterilization and specimen inoculation, the broth will eventually become **turbid** or cloudy as the microbial population grows in the container.

Solid media consist of the liquid media to which has been added a solidifying agent called **agar**. Agar, a polysaccharide derived from red seaweed, contains no nutrients, and like gelatin,

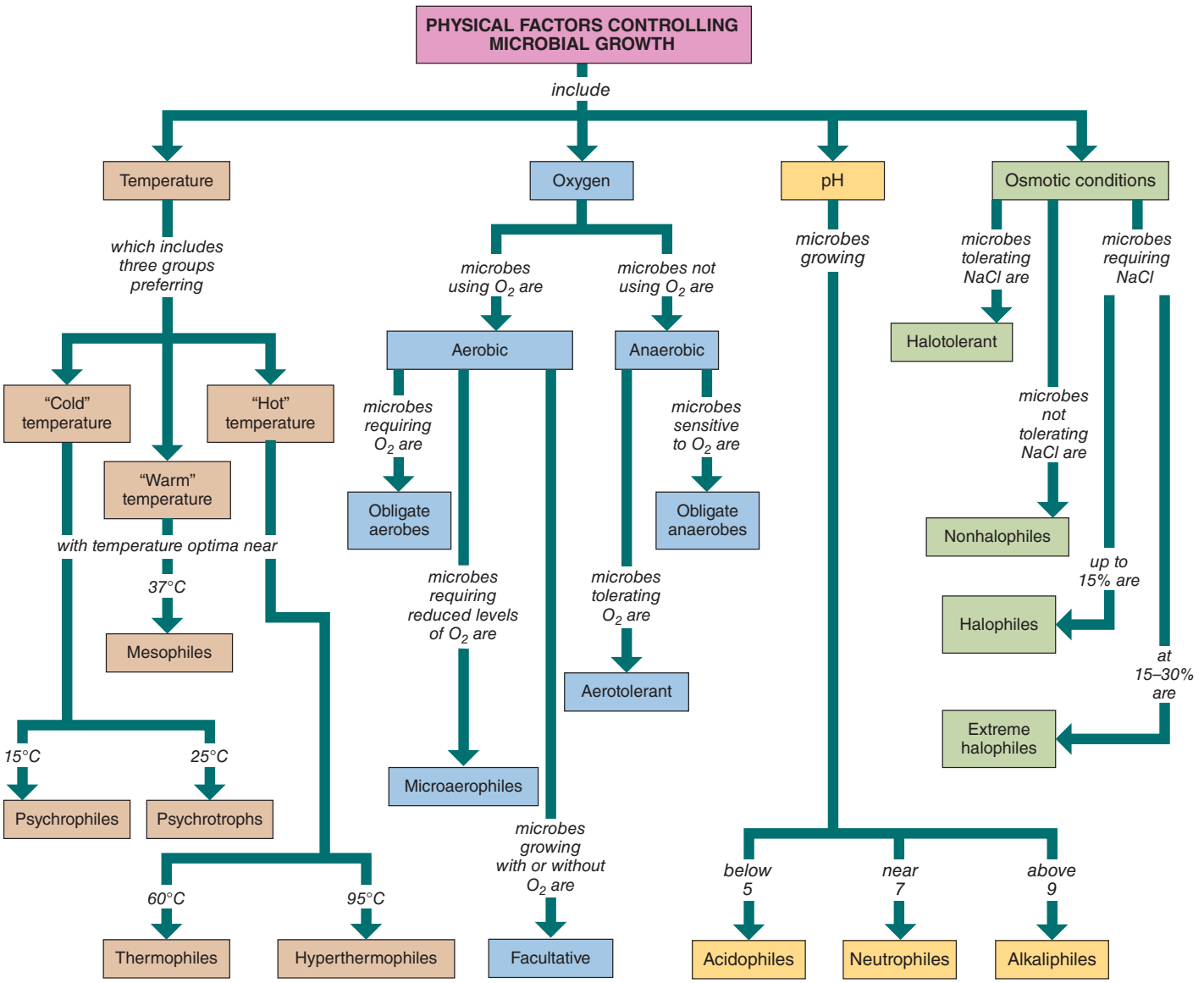


FIGURE 5.12 Types of Microbes Based on Physical Factors. This concept map shows four major physical factors controlling microbial growth. ***Escherichia coli* is a mesophilic, facultative, nonhalophilic neutrophile. What would be the makeup of the environment where the organism would optimally grow?**

melts when heated and then solidifies when cooled. After sterilization of the nutrient agar medium, it is poured into a culture (Petri) plate or tube where it will solidify on cooling. After specimen inoculation, the specimen will grow as colonies (bacterial organisms and yeasts) or as filaments (molds) on the surface of the agar.

Culture Media Are of Two Basic Types

Since the time of Pasteur and Koch, microbiologists have been growing bacterial and other microbial species in artificial media; that is, in nutrients designed to mimic the natural environment. Today, different culture media recipes are used in the clinical microbiology lab (CML) and research lab because species vary in their nutritional requirements and no single medium will grow all microorganisms.

For the isolation and identification of most microorganisms, two types of culture media are commonly used. A chemically undefined medium, called a **complex medium**, contains nutrients in which the exact components or their quantity is not completely known. Such media typically contain animal or plant digests

TABLE 5.3 Composition of a Complex and a Synthetic Growth Medium

Ingredient	Nutrient Supplied	Amount
A. Complex Agar Medium		
Peptone	Amino acids, peptides	5.0 g
Beef extract	Vitamins, minerals, other nutrients	3.0 g
Sodium chloride (NaCl)	Sodium and chloride ions	8.0 g
Agar		15.0 g
Water		1.0 liter
B. Chemically Defined Broth Medium		
Glucose	Simple sugar	5.0 g
Ammonium phosphate ($(NH_4)_2HPO_4$)	Nitrogen, phosphate	1.0 g
Sodium chloride (NaCl)	Sodium and chloride ions	5.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	Magnesium ions, sulphur	0.2 g
Potassium phosphate (K_2HPO_4)	Potassium ions, phosphate	1.0 g
Water		1.0 liter

(e.g., beef extract, soybean extract) or yeast extracts of an undefined nature (TABLE 5.3A). Complex media are commonly used in the teaching laboratory because most of the organisms used will grow in these nutrient-rich conditions.

The second type of medium is a **chemically defined medium**. In this medium, the precise chemical composition and amount of all components are known (TABLE 5.3B). Chemically defined media are useful when trying to determine an organism's specific growth requirements.

Culture Media Can Be Modified to Select For or Differentiate Between Microbial Species

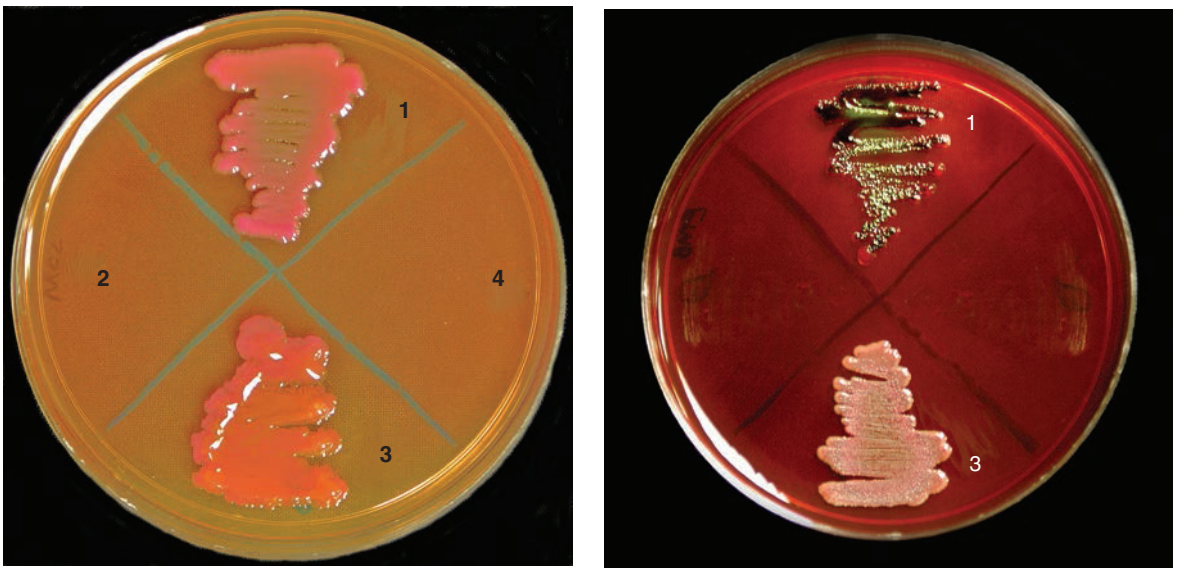
In the CML, the basic ingredients of growth media can be modified in one of three ways to provide fast and critical information about a pathogen causing an infection or disease (TABLE 5.4).

A **selective medium** contains ingredients to inhibit the growth of certain microbes in a mixture while allowing (selecting for) the growth of others. The basic growth medium may contain extra salt (NaCl) or a dye to inhibit the growth of intolerant or sensitive organisms but permit the growth of those species or pathogens one wants to isolate.

Another modification to a basic growth medium is the addition of one or more ingredients that allow the observer to differentiate between very similar species growing in or on a culture medium based on specific biochemical or physiological properties. This **differential medium** contains specific chemicals to indicate which species possess and which lack a particular biochemical process. Such indicators make it easy to distinguish visually colonies of one organism from colonies of other similar organisms on the same culture plate. Look at FIGURE 5.13 and see if you can determine which medium was used in each example.

TABLE 5.4 A Comparison of Special Culture Media

Name	Components	Uses	Examples
Selective medium	Growth stimulants Growth inhibitors	Selecting certain microbes out of a mixture	Mannitol salt agar for staphylococci
Differential medium	Dyes, growth stimulants, growth inhibitors	Distinguishing different microbes in a mixture	MacConkey agar for gram-negative bacteria
Enriched medium	Growth stimulants	Cultivating fastidious microbes	Blood agar for streptococci; chocolate agar for *Neisseria* species



(A) (B)

FIGURE 5.13 Special Media Formulations. (A) Four different bacterial species (1–4), two gram-positive and two gram-negative, were streaked onto separate sections of a MacConkey agar plate and allowed to incubate for 48 hours. MacConkey agar only supports the growth of gram-negative species. **(B)** Because the two gram-negative species cannot be visually distinguished from one another, they were then streaked into an eosin methylene blue agar (EMB) plate and incubated for 48 hours. EMB allows one to distinguish between human enteric bacteria, where *Escherichia coli* produces a green metallic sheen while other enteric species produce a pink color. **Which special medium is selective and which is differential? Explain your reasoning.**

Courtesy of Dr. Jeffrey Pommerville.

Fastidious: Having special nutritional requirements.

MicroInquiry 5 presents another example using these two approaches to identify and differentiate between similar bacterial species.

Although many microorganisms grow well in nutrient broth and nutrient agar, certain so-called **fastidious** organisms may require an **enriched medium** containing extra vitamins, amino acids, or other nutrients to promote growth. For example, the causative agent of gonorrhea, *Neisseria gonorrhoeae,* requires the addition of powdered hemoglobin. The Centers for Disease Control and Prevention (CDC) often is involved with particularly vexing identifications of such fastidious species, as MicroFocus 5.5 details.

Identification of Nongrowing Microbes. Many bacterial and archaeal species are impossible to cultivate in any artificial culture medium yet devised. In fact, less than 2% of the species in nature can be cultured. Consequently, it is impossible to estimate accurately microbial diversity in an environment based solely on culturability. Such "uncultured" organisms are said to be in a **viable but noncultured** (**VBNC**) state. Procedures for identifying VBNC organisms include direct microscopic examination (see below) and, most commonly, amplification of diagnostic gene sequences or 16S rRNA gene sequences.

Why do these organisms remain uncultured? Microbiologists believe part of the reason may be due to their presence in a "foreign" environment. These species have adapted to their own familiar and specific environment; an artificial medium is not their usual home. Therefore, these species go into a type of dormancy state and do not divide; in other words, they are viable, but cannot be cultured. Investigating the Microbial World 5 examines this anomaly. Studies on VBNC bacterial and archaeal species present a vast and as yet unexplored field (they are often referred to as "microbial dark matter"), which is important not only for detection of human pathogens, but also to reveal the tremendous diversity in the microbial world.

MICROINQUIRY 5

Identification of a Bacterial Species

It often is necessary to identify a bacterial species or be able to tell the difference between similar-looking species in a mixture. In microbial ecology, it might be necessary to isolate certain naturally growing species from others in a mixture. In the clinical and public health setting, microbes might be pathogens associated with disease or poor sanitation. In addition, some may be resistant to standard antibiotics normally used to treat an infection. In all these cases, identification can be accomplished by modifying the composition of a complex or chemically defined growth medium. Let's go through two scenarios.

- Suppose you are an undergraduate student in a marine microbiology course. On a field trip, you collect some seawater samples and, now back in the lab, you want to grow only photosynthetic marine microbes.

  How would you select for photosynthetic microbes? First, you know the photosynthetic organisms manufacture their own food, so their energy source will be sunlight and not the organic compounds typically found in culture media (see Table 5.3). So, you would need to use a chemically-defined medium but leave out the glucose. Also, knowing the salt is typically found in ocean waters, you would want to add it to the medium. You would then inoculate a sample of the collected material into a broth tube, place the tube in the light, and incubate for one week at a temperature typical of where the organisms were collected.

5a. What would you expect to find in the broth tube after one week's incubation?

What you have used in this scenario is a selective medium; that is, one that will encourage the growth of photosynthetic microbes (light and sea salt) and suppress the growth of nonphotosynthetic microorganisms (no carbon = no energy source). So, only marine photosynthetic microbes should be present.

- As an infectious disease officer in a local hospital, you routinely swab critical care areas to determine if there are any antibiotic-resistant bacteria present. You are especially concerned about methicillin-resistant *Staphylococcus aureus* (MRSA), as it frequently can cause disease outbreaks in a hospital setting. One swab you put in a broth tube showed turbidity after 48 hours.

5b. Knowing *Staphylococcus* species are halotolerant, how could you devise an agar medium to visually determine if any of the growth is due to *Staphylococcus aureus*?

Again, a selective medium would be used. It would be prepared by adding 7.5% salt to a complex agar medium. A sample from the broth tube would be streaked on the plate and incubated at 37°C for 48 hours.

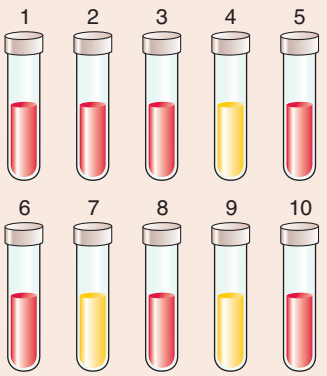
5c. What would you expect to find on the agar plate after 48 hours?

Your selective medium contained 10 discrete colonies. You do a Gram stain and discover all the colonies contain clusters of purple spheres; they are gram-positive. However, other species of *Staphylococcus* do not cause disease. One is *S. epidermidis,* a common skin bacterium. A Gram stain therefore is of no use to differentiate *S. aureus* from *S. epidermidis.*

5d. Knowing that only *S. aureus* will produce acid in the presence of the sugar mannitol, how could you design a differential broth medium to determine if any of the colonies are *S. aureus*? (*Hint:* phenol red is a pH indicator; it is red at neutral pH and yellow at acid pH).

You can identify each bacterial species by taking a complex broth medium, such as nutrient broth, and adding salt and mannitol (mannitol salt broth) and phenol red. Next, you inoculate a sample of each colony into a separate tube. You inoculate the 10 tubes and incubate them for 48 hours at 37°C.

5e. The broth tubes are shown below. What do the results signify? Which tubes contain which species of *Staphylococcus*?



Results from differential broth tubes.

This method is an example of a differential medium because it allowed you to visually differentiate or distinguish between two very similar bacterial species. Knowing which colonies on the original selective medium plate are *S. aureus,* you need to determine which, if any, are resistant to the antibiotic methicillin.

5f. How could you design an agar medium to identify any MRSA colonies?

5g. If the plates are devoid of growth, what can you conclude?

Again, you have used a selective medium; the addition of methicillin will permit the growth of any MRSA bacteria and suppress the growth of staphylococci susceptible to methicillin.

Answers can be found in **Appendix E**.

Bacteria: © NIAID

MICROFOCUS 5.5: Public Health

"Enriching" Koch's Postulates

On July 21–23, 1976, some 5,000 Legionnaires attended the Bicentennial Convention of the American Legion in Philadelphia, PA. About 600 of the Legionnaires stayed at the Bellevue Stratford Hotel. As the meeting was ending, several Legionnaires who stayed at the hotel complained of flu-like symptoms. Four days after the convention ended, an Air Force veteran who had stayed at the hotel died. He would be the first of 34 Legionnaires over several weeks to succumb to a lethal pneumonia, which became known as Legionnaires' disease.

As with any new disease, epidemiological studies look for the source of the disease. The Centers for Disease Control and Prevention (CDC) had an easy time tracing the source back to the Bellevue Stratford Hotel. Epidemiological studies also try to identify the causative agent. Using Koch's postulates, CDC staff collected tissues from lung biopsies and sputum samples. However, no microbes could be detected on slides of stained material. By December 1976, they were no closer to identifying the infectious agent.

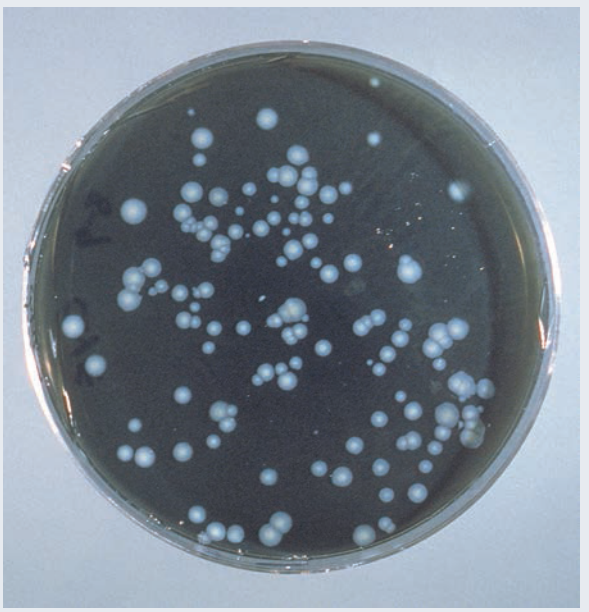
How can you verify Koch's postulates if you have no infectious agent? It was almost like being back in the times of Pasteur and Koch. Why was this bacterial species so difficult to culture on bacteriological media? Perhaps it was a virus.

After trying 17 different culture media formulations, the infectious agent was finally cultured. It turns out it was a bacterial species, but one with fastidious growth requirements. The initial agar medium contained a beef infusion, amino acids, and starch. When this medium was enriched with 1% hemoglobin and 1% isovitalex, small, barely visible colonies were seen after five days of incubation at 37°C. Investigators then realized the hemoglobin was supplying iron to the bacterium and the isovitalex was a source of the amino acid cysteine. Using these two chemicals in pure form, along with charcoal to absorb bacterial waste, a pH of 6.9, and an atmosphere of 2.5% CO_2, bacterial growth was significantly enhanced (see figure). From a microscope examination of these cultures, a gram-negative rod was confirmed and the organism was appropriately named *Legionella pneumophila.*

With an enriched medium to pure culture the organism, susceptible animals (guinea pigs) could be injected as required by Koch's postulates. *L. pneumophila* then was recovered from infected guinea pigs, verifying the organism as the causative agent of Legionnaires' disease.

Today, we know *L. pneumophila* is found in many aquatic environments, both natural and artificial. At the Bellevue Stratford Hotel, epidemiological studies indicated guests were exposed to *L. pneumophila* as a fine aerosol emanating from the air-conditioning system. Through some type of leak, the organism gained access to the system from the water cooling towers.

Koch's postulates are still useful—it's just hard sometimes to satisfy the postulates without an isolated pathogen.



Colonies of *L. pneumophila* on an enriched medium.

Courtesy of Dr. Jim Feeley/CDC.

Bacteria: © NIAID

INVESTIGATING THE MICROBIAL WORLD 5

The Great Plate Count Anomaly

About 98% of bacterial species from the environment cannot be grown using known culture media.

- **OBSERVATION:** Take a sediment sample from an environmental water source, mix it with saline solution (or water), and wait for the sediment to settle. Now take a drop from the liquid and place it on a culture dish with nutrient agar (or any type of complex medium). Place another drop on a slide and add stain. On the stained slide, you will undoubtedly be able to count hundreds of cells and find dozens of different bacterial morphologies. On the plate, if you are lucky, maybe one or two colonies will appear in a few days. The majority of the cells will not grow even though they are inundated in nutrients. This is the so-called "great plate count anomaly." Standard laboratory culture techniques fail to support the growth of these viable but noncultured (VBNC) species residing in environmental sediments.
- **QUESTION:** ***Why won't 98% of the bacterial species grow in laboratory culture media?***
- **HYPOTHESIS:** VBNCs need some essential "nutrient" from their neighbor species in the natural environment. If so, then growing the VBNCs in their natural environment will supply the needed nutrient and the uncultured should grow.
- **EXPERIMENTAL DESIGN:** A diffusion apparatus is designed to sandwich a microbial sediment sample in agar between two semipermeable membranes that allow for the free diffusion of "nutrients" and waste products through the chamber (see figure).
- **EXPERIMENT:** Sediment from a freshwater pond sample is mixed with agar and placed within the diffusion chamber. The chamber is then placed back in its natural environment. Another pond sediment sample from the same environment is cultured on standard agar culture medium in a culture dish. Both the diffusion chamber culture and the lab culture are left undisturbed for 4 weeks. After the incubation period, the colonies isolated on the culture plate and on the agar in the diffusion chamber (but not the biofilm growing on the semipermeable membrane) are identified. Phylum identification is carried out by sequencing each isolate's 16S ribosomal RNA gene.
- **RESULTS:** See Table.
- **CONCLUSIONS:**

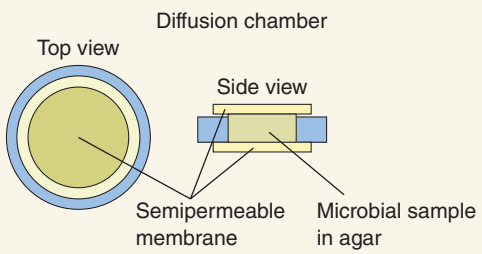
  QUESTION 1: ***Was the hypothesis supported? Explain using the table.***

  QUESTION 2: ***Are the majority of the isolates representative of gram-positive or gram-negative organisms? Explain.***

  QUESTION 3: ***What might have been the result if the biofilm organisms growing on the membranes were included in the analysis?***

Answers can be found in **Appendix E**.

- **FURTHER QUESTION:** What is it that the previously uncultured colonies in the diffusion chamber recognize? New evidence suggests one "nutrient" is iron, which is needed for ATP generation and other biochemical processes. To grow, the uncultured must "capture" the iron (presumably from their neighbors) in the bound form that is otherwise unavailable in a culture dish "environment."



Adapted from: Bollman, A., Lewis, K., and Epstein, S. S. (2007) *Appl. Environ. Microbiol.* 73(20): 6386–6390.

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TABLE Phylogenetic Strains Obtained by Culture Dish and Diffusion-Chamber Methods

Phylum	Number of Strains Isolated By: Culture dish only	Diffusion chamber only	Both methods	Total number of isolated strains
Alphaproteobacteria	20	36	6	62
Betaproteobacteria	3	63	3	69
Gammaproteobacteria	2	4	1	7
Deltaproteobacteria		1		1
Bacteroidetes	5	6	1	12
Spirochaetes		4		4
Firmicutes	5	1		6
Actinobacteria	5	1		6
Total	40	116	11	167

Population Measurements Are Made Using Pure Cultures

Aseptic technique: The practice of transferring microorganisms to a sterile culture medium without introducing other contaminating organisms.

Subculturing: The process of transferring bacterial cells from one culture medium to another.

Microorganisms rarely occur in nature as a single species as Pasteur discovered in trying to prove his germ theory of disease. Rather, they are mixed with other species, in a so-called "mixed culture"—most often as a **biofilm**. Therefore, to study a species, microbiologists and laboratory technologists must use a **pure culture**—that is, a population consisting of only one species. It was Koch's ability to pure culture pathogens that provided the proof for the germ theory.

If one has a mixed broth culture of bacterial species, how can the organisms be isolated as pure colonies? Two established methods are available. The first is the **pour-plate method**. Here, diluted samples of the mixed culture in molten nutrient agar tubes are each poured into a sterile Petri plate and allowed to harden. During a 24 hour to 48 hour incubation, the cells divide to form discrete colonies on and within the agar (FIGURE 5.14).

A second, more commonly used technique, called the **streak-plate method**, uses a single plate of nutrient agar (FIGURE 5.15A-D). An inoculum from a mixed culture is removed with a sterile loop using aseptic technique, and a series of streaks is made on the surface of one area of the plate. The loop is flamed, touched to the first area, and a second series is made in a second area. Similarly, streaks are made in the third and fourth areas, thereby spreading out the individual cells so they grow as separated colonies. After a 24 hour to 48 hour incubation, discrete colonies will be present on the plate (FIGURE 5.15E).

In both methods, each colony is a pure culture because the colony is derived from an original single cell that underwent numerous binary fissions. The researcher, medical technologist, or student can select samples of the colonies for further testing and subculturing.

Population Growth Can Be Measured in Several Ways

Microbial growth in a culture medium can be measured by direct and indirect methods.

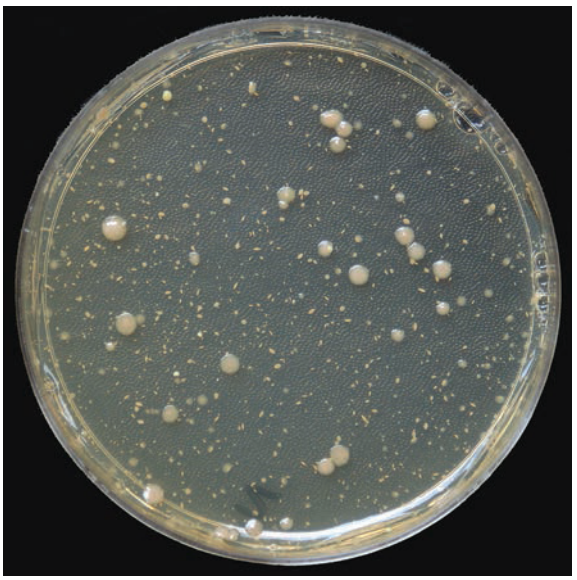


FIGURE 5.14 **A Pour Plate.** The dispersed bacterial cells grow as individual, discrete colonies. **By looking at this plate, how would you know the original broth culture was a mixture of bacterial species?**

Courtesy of Dr. Jeffrey Pommerville.

Direct Counting Methods. There are a number of ways to directly measure cell numbers. Scientists may wish to perform a **direct microscopic count** using a known volume of the liquid sample that has been placed on a specially designed counting chamber (FIGURE 5.16). However, this procedure will count both live and dead cells.

In the **most probable number test**, microbial samples diluted 10× and 100× are added to a set of lactose broth tubes and the presence or absence of gas formed in fermentation gives a rough statistical estimation of the cell number, referred to as the most probable number. This technique has been used for measuring water quality.

In the **standard plate count procedure**, a sample of a broth culture is placed in a sterile Petri dish and melted nutrient agar is added (pour-plate method) (FIGURE 5.17). The assumption is that each cell will undergo

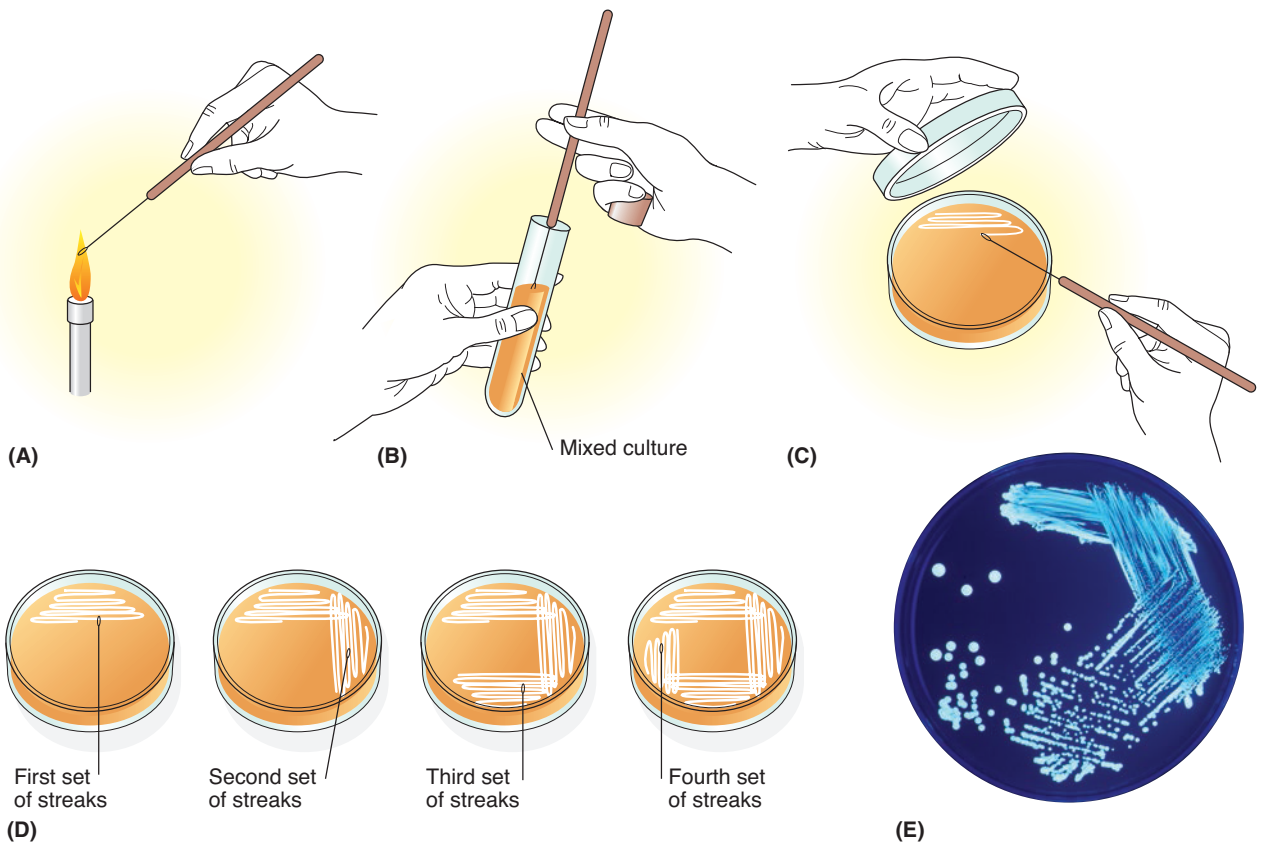


FIGURE 5.15 **The Streak-Plate Method. (A)** A loop is sterilized, **(B)** a sample of cells is obtained from a mixed culture, and **(C)** streaked near one sector of the agar plate. **(D)** Successive streaks are performed, and the plate is incubated. **(E)** Well-isolated and defined colonies illustrate a successful isolation. **Justify the need to streak a mixed sample over four areas on a culture plate.**

Courtesy of James Gathany/CDC.

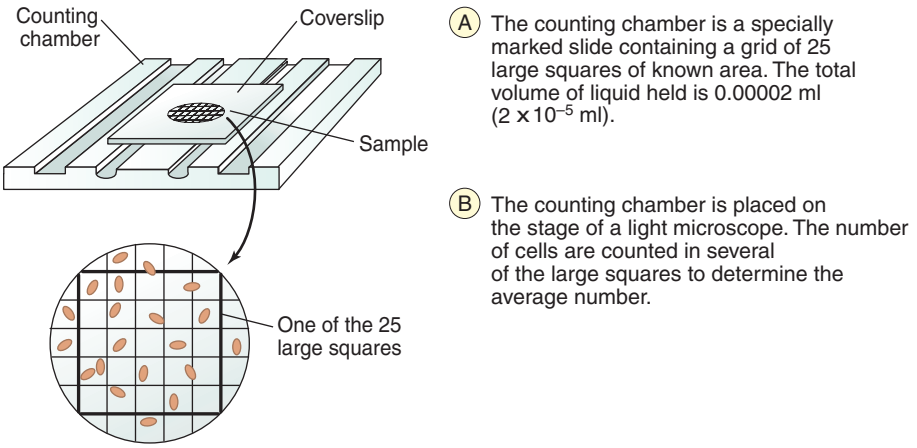


FIGURE 5.16 Direct Microscopic Count. This procedure can be used to estimate the total number of live and dead cells in a culture sample. **Suppose the average number of cells per large square was 14. Calculate the number of cells in a 10 ml sample.**

multiple rounds of cell division to produce separate colonies on the plate. Because two or more cells could clump together on a plate and grow as a single colony, the standard plate count is expressed as the number of **colony-forming units** (**CFUs**). After incubation, the number of CFUs will be used to estimate the number of viable cells originally plated.

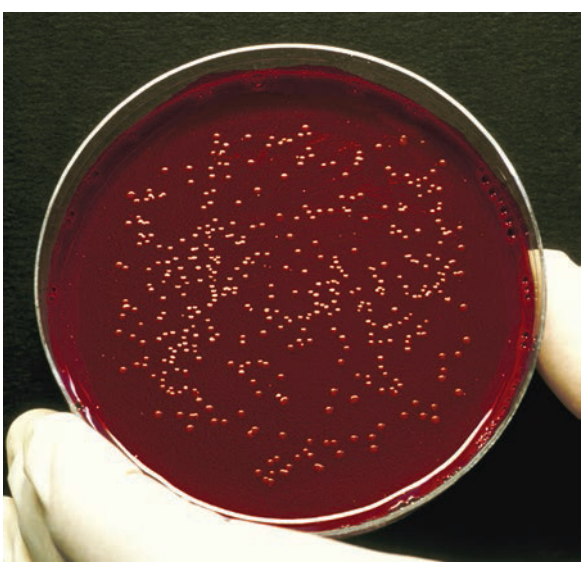


FIGURE 5.17 The Standard Plate Count. Individual bacterial colonies have grown on this blood agar plate. Each colony represents a colony-forming unit (CFU). **If a 0.1 ml sample of a 10^4 dilution contained 250 colonies, how many bacterial cells were in 10 ml of the original broth culture?**

Indirect Measurement Methods. One of the simplest indirect methods of population measurement is to determine the cloudiness, or **turbidity**, in a broth culture using a spectrophotometer. This instrument detects the amount of light scattered by a suspension of cells placed in the spectrophotometer such that the amount of light scatter (optical density, OD) is a function of the cell number; therefore, the more cells present, the more light is scattered or absorbed, resulting in a higher absorbance reading on the spectrophotometer (FIGURE 5.18). A standard curve can be generated to serve as a measure of cell numbers. However, because more than 10 million cells are needed to make a reading on the spectrophotometer, turbidity is not a useful way to study the growth of small populations of bacterial cells.

In conclusion, we examined the major physical factors and nutrient media formulations affecting the rate at which microbial populations grow. In particular, the generation time for a bacterial species is dependent on the physical factors (temperature, oxygen need, etc.) and nutrients available in the environment, be it the soil, a laboratory culture dish, or a human host. In addition, many pathogenic bacteria are fastidious at least in a laboratory setting, so that if they are taken from different parts of the body, the clinical microbiology lab may have to use different growth media for isolation and identification. This brings up an interesting

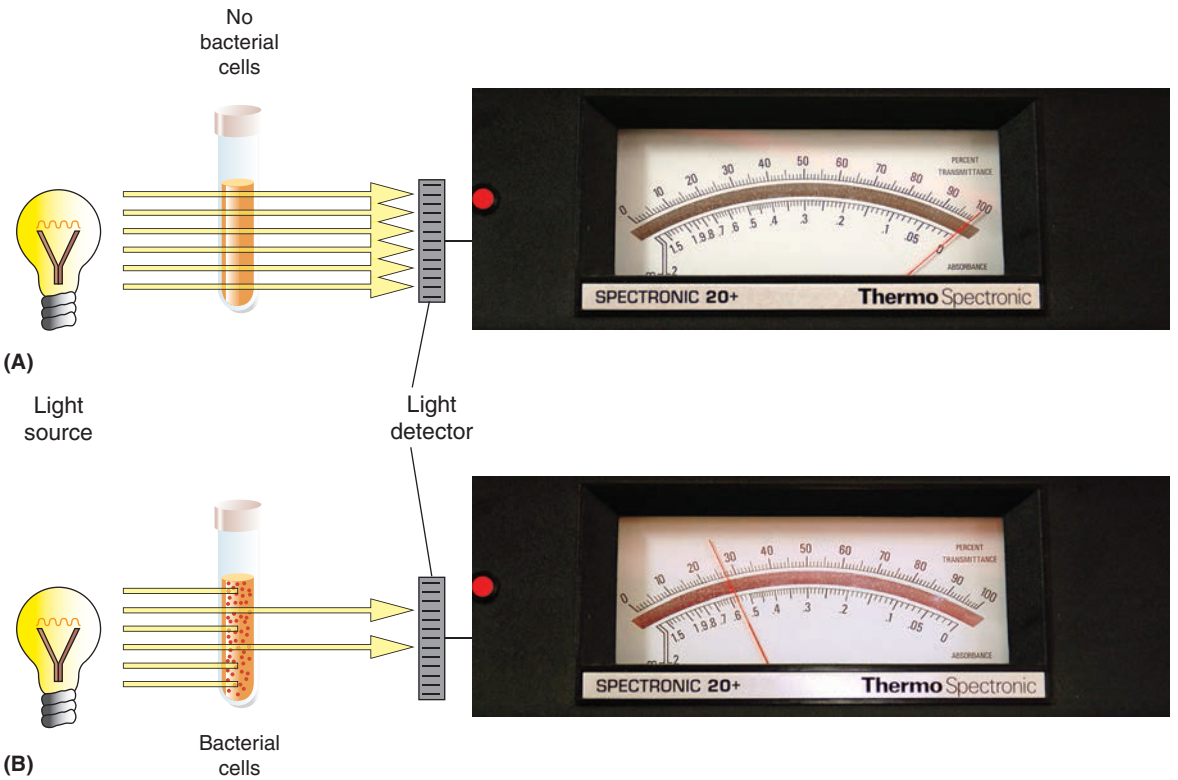
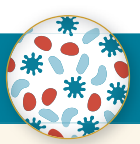


FIGURE 5.18 Using Turbidity to Measure Population Growth. (A) As light passes through a sterile broth tube in the spectrophotometer, the instrument is standardized at 0 absorbance. **(B)** As a bacterial population in a broth tube grows, the cells will scatter more of the light, which on the spectrophotometer is detected as an increase in absorbance.

Why do turbidity measurements represent an indirect method to measure population growth?

Courtesy of Dr. Jeffrey Pommerville.



CHAPTER CHALLENGE C

On November 13, 1971, *Mariner 9* went into orbit around the Red Planet. Before its arrival, we thought Mars was a dead world. Then *Mariner 9* sent back pictures of a landscape where it appeared water once flowed. But the craft found no signs of biological material in the Martian soil. As more spacecraft have gone to Mars, the evidence for life on the Red Planet has seesawed—yet some are hopeful that microbial life may exist near the surface or deeper underground.

Question C:

As a result, several missions to Mars are being planned by American, European, Russian, and Chinese space agencies. As an exomicrobiologist (one who looks and searches for microbial life beyond Earth), what types of experiments would you design (based on this chapter) to see if microbial life does exist on Mars? The spacecraft will not return to Earth, so the experiments need to be completed on the Red Planet.

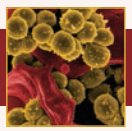
Answers can be found in **Appendix F**.

CONCEPT AND REASONING CHECKS 3

Bacteria: © NIAID

a. **Compare and contrast complex and chemically defined media.**
b. **Explain how microbiologists have figured that some 98% of the microbial world has not been "seen."**
c. **Why might it be more difficult to isolate a colony from a pour-plate than from a streak-plate culture?**
d. **Distinguish between direct and indirect methods to measure population growth.**

problem when considering the diagnosis and treatment of an infectious disease. Such pathogens, including *Treponema pallidum*, have to be identified from actual clinical samples using direct microscopic observation. So, one has to wonder how many human diseases may go undiagnosed today because they are VBNC? In fact, microbiologists have argued whether members of the Archaea can cause human disease. None are currently known to cause disease, yet we know from microscopic observation that archaeal organisms are present in the human body. Could some of them be VBNC pathogens?



SUMMARY OF KEY CONCEPTS

Bacteria: © NIAID

5.1 Microbial Reproduction Is Part of the Cell Cycle

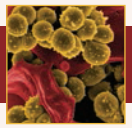
1. The bacterial **cell cycle** involves cell growth, DNA replication, and **binary fission** to produce genetically identical daughter cells. (Fig. 5.2A)
2. Binary fissions occur at intervals called the **generation time**, which may be as short as 20 minutes. (Fig. 5.3)

5.2 Microbial Growth Progresses Through Distinct Phases

3. The dynamics of the **bacterial growth curve** show how a cell population grows logarithmically, reaches a certain peak and levels off, and then may decline. (Fig. 5.4)
4. Dormancy is a response to potential or actual environmental change. (Figs. 5.6, 5.8)
5. Temperature, oxygen, pH, and salt concentration are physical factors that influence microbial growth. Away from the optimal condition, growth slows within a set range. (Figs. 5.9, 5.11)

5.3 Culture Media Are Used to Grow Microbes and Measure Their Growth

6. **Complex** and **chemically defined media** contain the nutrients for microbial growth.
7. Growth media can be modified to select for a desired microbial species, to differentiate between two similar species, or to enrich for species requiring special nutrients. (Fig. 5.13)
8. **Pure cultures** can be produced from a mixed culture by the **pour-plate method** or the **streak-plate method**. In both cases, discrete colonies can be identified that represent only one microbial species. (Figs. 5.14, 5.15)
9. Microbial growth can be measured by **direct microscopic count**, the **most probable number test**, and the **standard plate count** procedure. Indirect methods often use **turbidity** measurements. (Fig. 5.18)



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CHAPTER SELF-TEST

For **STEPS A–D**, answers to questions and problems can be found in **Appendix D**.

STEP A: REVIEW OF FACTS AND TERMS

Multiple Choice

Read each question carefully, then select the ***one*** answer that best fits the question or statement.

1. Which one of the following statements does NOT apply to bacterial reproduction?
   A. A fission ring apparatus is present.
   B. Septum formation occurs.
   C. A spindle apparatus is used.
   D. Symmetrical cell division occurs.
2. If a bacterial cell in a broth tube has a generation time of 40 minutes, how many cells will there be after 6 hours of optimal growth?
   A. 18
   B. 64
   C. 128
   D. 512
3. A bacterial species generation time would be determined during the _____ phase.
   A. decline
   B. lag
   C. log
   D. stationary
4. Which one of the following is NOT an example of dormancy?
   A. Log phase
   B. Endospore formation
   C. VBNCs
   D. Persister cells
5. A microbe that is a microaerophilic mesophile would grow optimally at _____ and _____.
   A. high O_2; 30°C
   B. low O_2; 20°C
   C. no O_2; 30°C
   D. low O_2; 37°C
6. If the carbon source in a growth medium is beef extract, the medium must be an example of a/an _____ medium.
   A. complex
   B. chemically defined
   C. enriched
   D. differential
7. A _____ medium would involve the addition of the antibiotic streptomycin to identify streptomycin-resistant bacteria.
   A. differential
   B. selective
   C. thioglycollate
   D. VBNC
8. Which one of the following is NOT part of the streak-plate method?
   A. Making four sets of streaks on a plate
   B. Diluting a mixed culture in molten agar
   C. Using a mixed culture
   D. Using a sterilized loop
9. Direct methods to measure bacterial growth would include all the following except _____.
   A. total bacterial count
   B. direct microscopic count
   C. turbidity measurements
   D. most probable number

True–False

Each of the following statements is true (T) or false (F). If the statement is false, substitute a word or phrase for the underlined word or phrase to make the statement true.

10. _____ Endospores are produced by some gram-negative bacterial species.
11. _____ Obligate aerobes use oxygen gas as a final electron acceptor in energy production.
12. _____ The most common growth medium used in the teaching laboratory is a complex medium.
13. _____ The majority of bacterial and archaeal organisms can be cultured in growth media.
14. _____ In attempting to culture a fastidious bacterial pathogen, a differential medium would be used.
15. _____ Acidophiles grow best at pHs greater than 9.
16. _____ Mesophiles have their optimal growth near 37°C.
17. _____ Bacterial and archaeal cells lack a mitotic spindle to separate chromosomes.
18. _____ The fastest doubling time would be found in the lag phase of a bacterial growth curve.
19. _____ If *E. coli* cells are placed in distilled water, they will burst.
20. _____ Halophiles would dominate in marine environments.

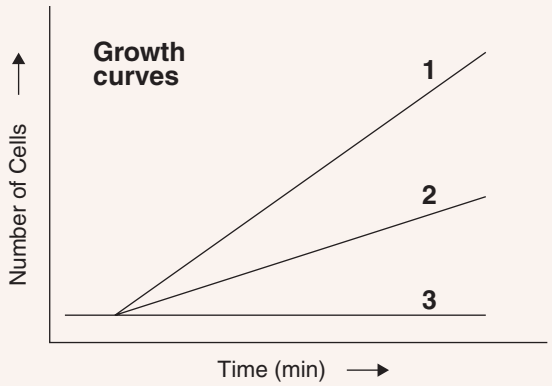
STEP B: CONCEPT REVIEW

21. Describe the three phases of a bacterial **cell cycle**. (**Key Concept 1**)
22. Compare the events of each phase of a **bacterial growth curve**. (**Key Concept 2**)
23. Explain the importance of bacterial **dormancy**. (**Key Concept 2**)
24. Identify the four major physical factors governing microbial growth, and describe how microorganisms have adapted to these physical environments. (**Key Concept 2**)
25. Explain how **selective** and **differential media** are each constructed. (**Key Concept 3**)
26. Explain the procedures used in the **pour-plate** and **streak-plate methods**. (**Key Concept 3**)
27. Construct a concept map for **Growth Measurements** using the following terms. (**Key Concept 3**)

CO_2 production	Indirect measurement methods
Cell mass	Metabolic activity
Colony-forming unit	Most probable number test
Direct-counting method	Oxygen uptake
Direct microscopic count	Standard plate count
	Turbidity

STEP C: APPLICATIONS AND PROBLEMS

28. Use the log phase growth curves (1, 2, or 3) below to answer each of the following questions (a–c).



_____a. Which curve (1, 2, or 3) best represents the growth curve for a mesophile incubated at 60°C?

_____b. Which curve (1, 2, or 3) best represents a nonhalophile growing in 5% salt?

_____c. Which curve (1, 2, or 3) best represents an acidophile growing at pH 4?

29. Consumers are advised to avoid stuffing a turkey the night before cooking, even though the turkey is refrigerated. A homemaker questions this advice and points out that the bacterial species of human disease grow mainly at warm temperatures, not in the refrigerator. What explanation might you offer to counter this argument?

30. Public health officials found the water in a Midwestern town was contaminated with sewage bacteria. The officials suggested homeowners boil their water for a couple of minutes before drinking it. (a) Would this treatment sterilize the water? Why? (b) Is it important for the water to be sterile? Explain.

STEP D: QUESTIONS FOR THOUGHT AND DISCUSSION

31. To prevent decay by bacterial species and to display the mummified remains of ancient peoples, museum officials place the mummies in glass cases where oxygen has been replaced with nitrogen gas. Why do you think nitrogen is used?

32. Extremophiles are of interest to industrial corporations, who see these organisms as important sources of enzymes that function at temperatures of 100°C and pH levels of 10 (the enzymes have been dubbed “extremozymes”). What practical uses can you foresee for these enzymes?

33. During the filming of the 1997 movie *Titanic,* researchers discovered at least 20 different bacterial and archaeal species literally consuming the ship, especially a rather large piece of the midsection. What type of environmental conditions are these bacterial and archaeal species subjected to at the wreck’s depth of 12,600 feet?

34. Every year news media report cases of skin and lung infections in people sitting in hot tubs. How might such infections occur in hot tubs?