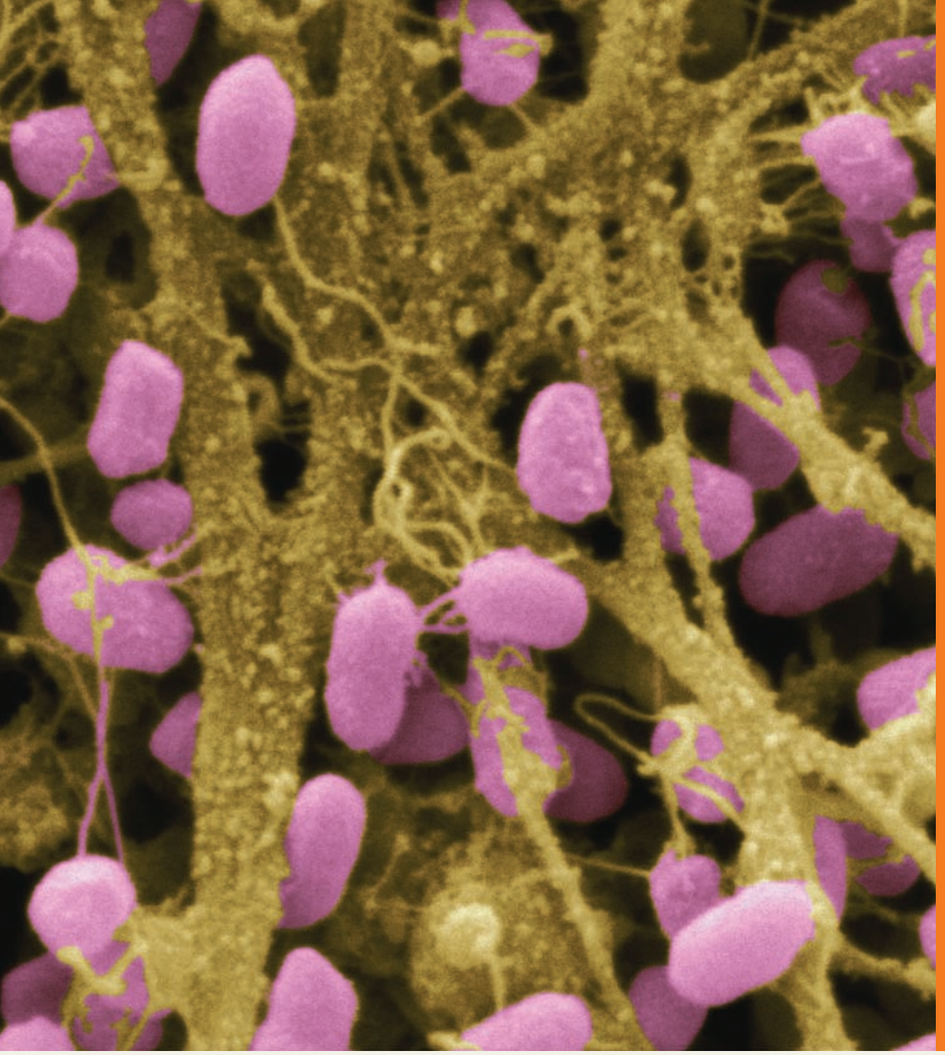


6

Microbial Growth

MasteringMICROBIOLOGY™

Visualize microbiology and check your understanding with a pre-test at www.masteringmicrobiology.com.

When we talk about microbial growth, we are really referring to the *number* of cells, not the *size* of the cells. Microbes that are "growing" are increasing in number, accumulating into *colonies* (groups of cells large enough to be seen without a microscope) of hundreds of thousands of cells or *populations* of billions of cells. Although individual cells approximately double in size during their lifetime, this change is not very significant compared with the size increases observed during the lifetime of plants and animals.

Many bacteria survive and grow slowly in nutrient-poor environments by forming biofilms. The *Serratia marcescens* bacteria in the photo have formed a biofilm on a piece of plastic. Biofilms are frequently sources of health care associated infections such as the one described in the Clinical Case.

Microbial populations can become incredibly large in a very short time. By understanding the conditions necessary for microbial growth, we can determine how to control the growth of microbes that cause diseases and food spoilage. We can also learn how to encourage the growth of helpful microbes and those we wish to study.

In this chapter we will examine the physical and chemical requirements for microbial growth, the various kinds of culture media, bacterial cell division, the phases of microbial growth, and the methods of measuring microbial growth.

The Requirements for Growth

LEARNING OBJECTIVES

- **6-1** Classify microbes into five groups on the basis of preferred temperature range.
- **6-2** Identify how and why the pH of culture media is controlled.
- **6-3** Explain the importance of osmotic pressure to microbial growth.
- **6-4** Name a use for each of the four elements (carbon, nitrogen, sulfur, and phosphorus) needed in large amounts for microbial growth.
- **6-5** Explain how microbes are classified on the basis of oxygen requirements.
- **6-6** Identify ways in which aerobes avoid damage by toxic forms of oxygen.

The requirements for microbial growth can be divided into two main categories: physical and chemical. Physical aspects include temperature, pH, and osmotic pressure. Chemical requirements include sources of carbon, nitrogen, sulfur, phosphorus, oxygen, trace elements, and organic growth factors.

Clinical Case: Glowing in the Dark

Reginald MacGruder, an investigator at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, has a mystery on his hands. Earlier this year, he was involved in the recall of an intravenous heparin solution that was blamed for causing *Pseudomonas fluorescens* bloodstream infections in patients in four different states. It seemed that everything was under control, but now, three months after the recall, 19 patients in two other states develop the same *P. fluorescens* bloodstream infections. It makes no sense to Dr. MacGruder; how could this infection be popping up again so soon after the recall? Could another heparin batch be tainted?

What is *P. fluorescens*? Read on to find out.

154 166 175 177

Physical Requirements

Temperature

Most microorganisms grow well at the temperatures that humans favor. However, certain bacteria are capable of growing at extremes of temperature that would certainly hinder the survival of almost all eukaryotic organisms.

Microorganisms are classified into three primary groups on the basis of their preferred range of temperature: **psychrophiles** (cold-loving microbes), **mesophiles** (moderate-temperature–loving microbes), and **thermophiles** (heat-loving microbes). Most bacteria grow only within a limited range of temperatures, and their maximum and minimum growth temperatures are only about 30°C apart. They grow poorly at the high and low temperature extremes within their range.

Each bacterial species grows at particular minimum, optimum, and maximum temperatures. The **minimum growth temperature** is the lowest temperature at which the species will grow. The **optimum growth temperature** is the temperature at which the species grows best. The **maximum growth temperature** is the highest temperature at which growth is possible. By graphing the growth response over a temperature range, we can see that the optimum growth temperature is usually near the top of the range; above that temperature the rate of growth drops off rapidly (Figure 6.1). This happens presumably because the high temperature has inactivated necessary enzymatic systems of the cell.

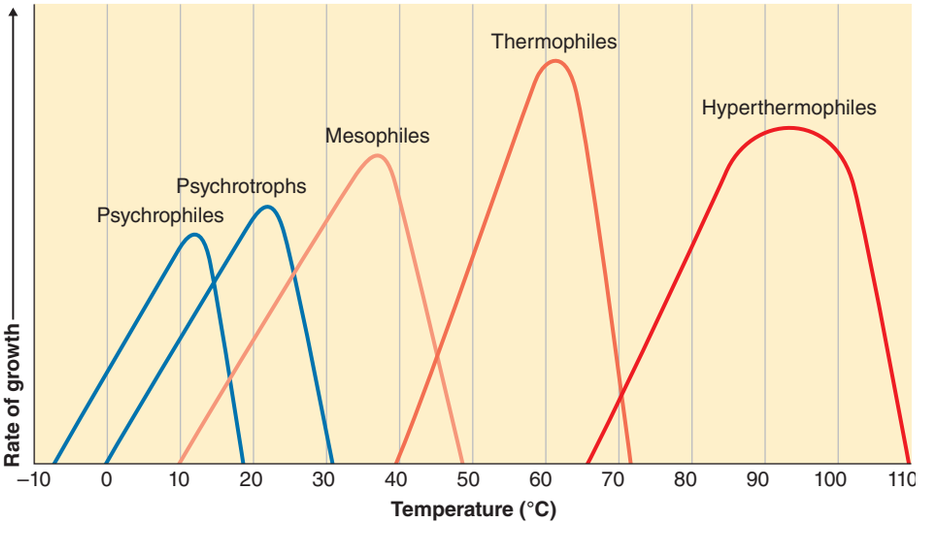


Figure 6.1 Typical growth rates of different types of microorganisms in response to temperature. The peak of the curve represents optimum growth (fastest reproduction). Notice that the reproductive rate drops off very quickly at temperatures only a little above the optimum. At either extreme of the temperature range, the reproductive rate is much lower than the rate at the optimum temperature.

Q Why is it difficult to define *psychrophile, mesophile,* and *thermophile*?

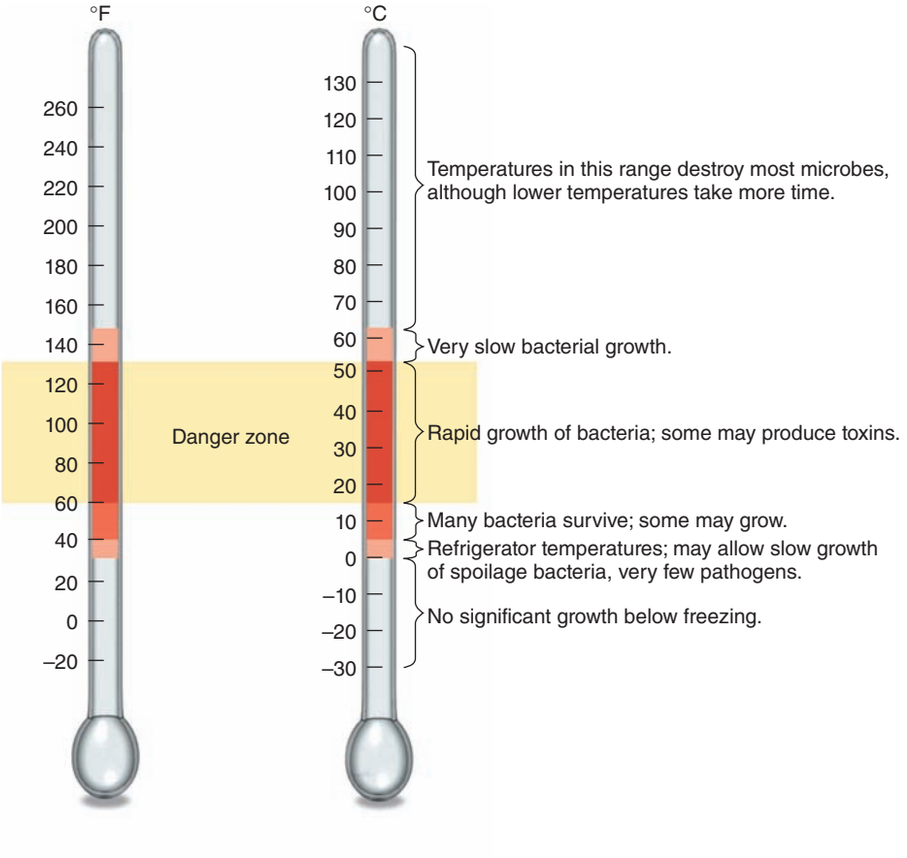


Figure 6.2 Food preservation temperatures. Low temperatures decrease microbial reproduction rates, which is the basic principle of refrigeration. There are always some exceptions to the temperature responses shown here; for example, certain bacteria grow well at temperatures that would kill most bacteria, and a few bacteria can actually grow at temperatures well below freezing.

Q Which bacterium would theoretically be more likely to grow at refrigerator temperatures: a human intestinal pathogen or a soilborne plant pathogen?

The ranges and maximum growth temperatures that define bacteria as psychrophiles, mesophiles, or thermophiles are not rigidly defined. Psychrophiles, for example, were originally considered simply to be organisms capable of growing at 0°C. However, there seem to be two fairly distinct groups capable of growth at that temperature. One group, composed of psychrophiles in the strictest sense, can grow at 0°C but has an optimum growth temperature of about 15°C. Most of these organisms are so sensitive to higher temperatures that they will not even grow in a reasonably warm room (25°C). Found mostly in the oceans' depths or in certain polar regions, such organisms seldom cause problems in food preservation. The other group that can grow at 0°C has higher optimum temperatures, usually 20–30°C and cannot grow above about 40°C. Organisms of this type are much more common than psychrophiles and are the most likely to be encountered in low-temperature food spoilage because they grow fairly well at refrigerator temperatures. We will use the term **psychrotrophs,** which food microbiologists favor, for this group of spoilage microorganisms.

Refrigeration is the most common method of preserving household food supplies. It is based on the principle that microbial reproductive rates decrease at low temperatures. Although microbes usually survive even subfreezing temperatures (they might become entirely dormant), they gradually decline in number. Some species decline faster than others. Psychrotrophs actually do not grow well at low temperatures, except in comparison with other organisms; given time, however, they are able to slowly degrade food. Such spoilage might take the form of mold mycelium, slime on food surfaces, or off-tastes or off-colors in foods. The temperature inside a properly set refrigerator will greatly slow the growth of most spoilage organisms and will entirely prevent the growth of all but a few pathogenic bacteria. **Figure 6.2** illustrates the importance of low temperatures for preventing the growth of spoilage and disease organisms. When large amounts of food must be refrigerated, it is important to keep in mind the slow cooling rate of a large quantity of warm food (**Figure 6.3**).

Mesophiles, with an optimum growth temperature of 25–40°C, are the most common type of microbe. Organisms that have adapted to live in the bodies of animals usually have an optimum temperature close to that of their hosts. The optimum temperature for many pathogenic bacteria is about 37°C, and incubators for clinical cultures are usually set at about this temperature. The mesophiles include most of the common spoilage and disease organisms.

Thermophiles are microorganisms capable of growth at high temperatures. Many of these organisms have an optimum growth temperature of 50–60°C, about the temperature of water from a hot water tap. Such temperatures can also be reached in sunlit soil and in thermal waters such as hot springs. Remarkably, many thermophiles cannot grow at temperatures below

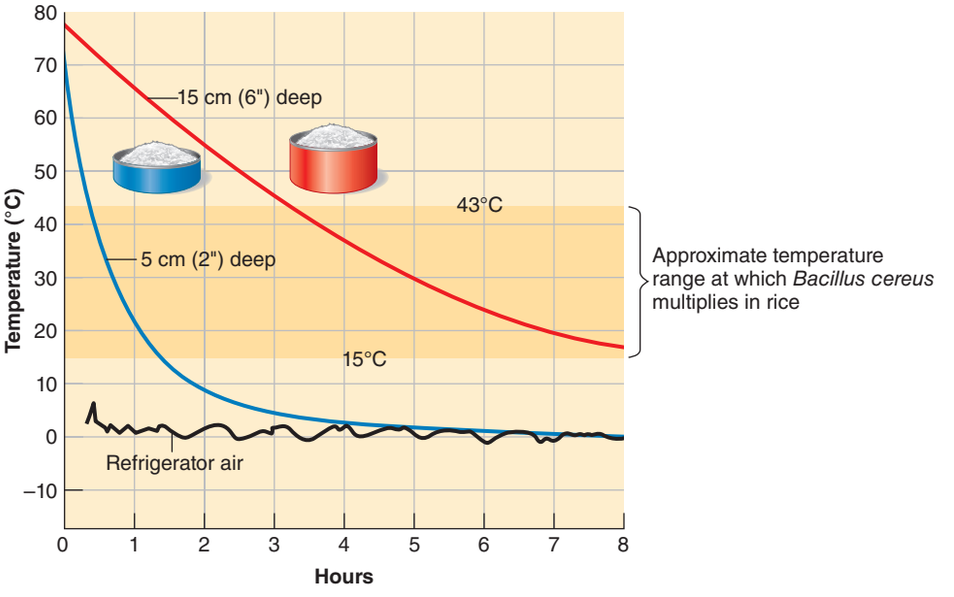


Figure 6.3 The effect of the amount of food on its cooling rate in a refrigerator and its chance of spoilage. Notice that in this example, the pan of rice with a depth of 5 cm (2 in) cooled through the incubation temperature range of the *Bacillus cereus* in about 1 hour, whereas the pan of rice with a depth of 15 cm (6 in) remained in this temperature range for about 5 hours.

Q Given a shallow pan and a deep pot with the same volume, which would cool faster? Why?

about 45°C. Endospores formed by thermophilic bacteria are unusually heat resistant and may survive the usual heat treatment given canned goods. Although elevated storage temperatures may cause surviving endospores to germinate and grow, thereby spoiling the food, these thermophilic bacteria are not considered a public health problem. Thermophiles are important in organic compost piles (see Figure 27.10 on page 782), in which the temperature can rise rapidly to 50–60°C.

Some microbes, members of the Archaea (page 4), have an optimum growth temperature of 80°C or higher. These organisms are called **hyperthermophiles,** or sometimes **extreme thermophiles.** Most of these organisms live in hot springs associated with volcanic activity; sulfur is usually important in their metabolic activity. The known record for bacterial growth and replication at high temperatures is about 121°C near deep-sea hydrothermal vents. See the box on the facing page. The immense pressure in the ocean depths prevents water from boiling even at temperatures well above 100°C.

pH

Recall from Chapter 2 (pages 34–35) that pH refers to the acidity or alkalinity of a solution. Most bacteria grow best in a narrow pH range near neutrality, between pH 6.5 and 7.5. Very few bacteria grow at an acidic pH below about pH 4. This is why a number of foods, such as sauerkraut, pickles, and many cheeses, are preserved from spoilage by acids produced by bacterial fermentation. Nonetheless, some bacteria, called **acidophiles,** are remarkably tolerant of acidity. One type of chemoautotrophic bacteria, which is found in the drainage water from coal mines and oxidizes sulfur to form sulfuric acid, can survive at a pH value of 1. Molds and yeasts will grow over a greater pH range than bacteria will, but the optimum pH of molds and yeasts is generally below that of bacteria, usually about pH 5 to 6. Alkalinity also inhibits microbial growth but is rarely used to preserve foods.

When bacteria are cultured in the laboratory, they often produce acids that eventually interfere with their own growth. To neutralize the acids and maintain the proper pH, chemical buffers are included in the growth medium. The peptones and amino acids in some media act as buffers, and many media also contain phosphate salts. Phosphate salts have the advantage of exhibiting their buffering effect in the pH growth range of most bacteria. They are also nontoxic; in fact, they provide phosphorus, an essential nutrient.

Osmotic Pressure

Microorganisms obtain almost all their nutrients in solution from the surrounding water. Thus, they require water for growth, and their composition is 80–90% water. High osmotic pressures have the effect of removing necessary water from a cell. When a microbial cell is in a solution whose concentration of solutes is higher than in the cell (the environment is *hypertonic* to the cell), the cellular water passes out through the plasma membrane to the high solute concentration. (See the discussion of osmosis in Chapter 4, pages 92–93, and review Figure 4.18 for the three types of solution environments a cell may encounter.) This osmotic loss of water causes **plasmolysis,** or shrinkage of the cell's cytoplasm (Figure 6.4).

The importance of this phenomenon is that the growth of the cell is inhibited as the plasma membrane pulls away from the cell wall. Thus, the addition of salts (or other solutes) to a solution, and the resulting increase in osmotic pressure, can be used to preserve foods. Salted fish, honey, and sweetened condensed milk are preserved largely by this mechanism; the high salt or sugar concentrations draw water out of any microbial cells that are present

Life in the Extreme

Until humans explored the deep-ocean floor, scientists believed that only a few forms of life could survive in that high-pressure, completely dark, oxygen-poor environment. Then, in 1977, *Alvin,* the deep-sea submersible carried two scientists 2600 meters below the surface at the Galápagos Rift (about 350 km northeast of the Galápagos Islands). There, amid the vast expanse of barren basalt rocks, the scientists found unexpectedly rich oases of life. Superheated water from beneath the seafloor rises through fractures in the Earth's crust called vents. Mats of bacteria grow along the sides of the vents, where temperatures exceed 100°C (see the figure).

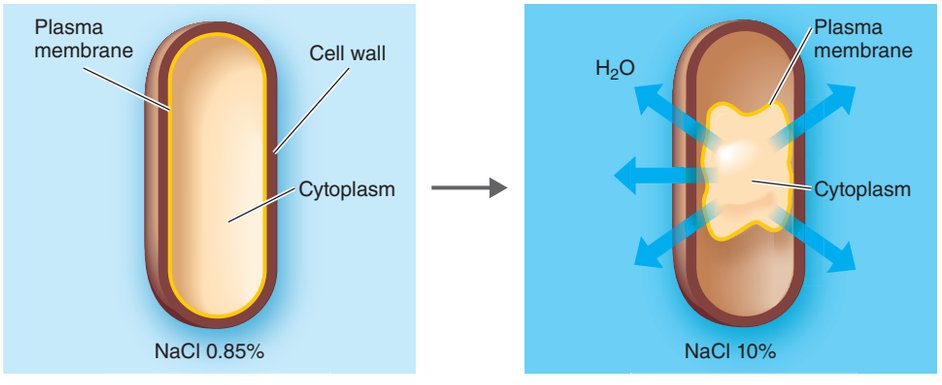
Ecosystem of the Hydrothermal Vents

Life at the surface of the world's oceans depends on photosynthetic organisms, such as plants and algae, which harness the sun's energy to fix carbon dioxide (CO_2) to make carbohydrates. At the deep-ocean floor, where no light penetrates, photosynthesis is not possible. The scientists found that the primary producers at the ocean floor are chemoautotrophic bacteria. Using chemical energy from hydrogen sulfide (H_2S) as a source of energy to fix CO_2, the chemoautotrophs create an environment that supports higher life forms. Hydrothermal vents in the seafloor supply the H_2S and CO_2.

New Products from Hydrothermal Vents

Terrestrial fungi and bacteria have had a major impact on the development of antimicrobial and antitumor compounds since the 1930s. Hydrothermal vents are the next frontier in the hunt for new drugs. In 2010 a peptide produced by *Thermovibrio ammonificans* was shown to induce apoptosis (cell death) and thus potential anticancer activity. Currently, researchers are growing *Pyrococcus furiosus* because it produces alternative fuels, hydrogen gas, and butanol. DNA polymerases (enzymes that synthesize DNA) isolated from two archaea living near deep-sea vents are being used in the polymerase chain reaction (PCR), a technique for making many copies of DNA. In PCR, single-stranded DNA is made by heating a chromosome fragment to 98°C and cooling it so that DNA polymerase can copy each strand. DNA polymerase from *Thermococcus litoralis*, called $Vent_R$, and from *Pyrococcus*, called Deep $Vent_R$, are not denatured at 98°C. These enzymes can be used in automatic thermalcyclers to repeat the heating and cooling cycles, allowing many copies of DNA to be made easily and quickly.

A white microbial biofilm is visible on this deep-sea hydrothermal vent. Water is being emitted through the ocean floor at temperatures above 100°C.



(a) Cell in isotonic solution. Under these conditions, the solute concentration in the cell is equivalent to a solute concentration of 0.85% sodium chloride (NaCl). See Figure 4.18.

(b) Plasmolyzed cell in hypertonic solution. If the concentration of solutes such as NaCl is higher in the surrounding medium than in the cell (the environment is hypertonic), water tends to leave the cell. Growth of the cell is inhibited.

Figure 6.4 **Plasmolysis.**

Q Why is osmotic pressure an important factor in microbial growth?

and thus prevent their growth. These effects of osmotic pressure are roughly related to the *number* of dissolved molecules and ions in a volume of solution.

Some organisms, called **extreme halophiles**, have adapted so well to high salt concentrations that they actually require them for growth. In this case, they may be termed **obligate halophiles.** Organisms from such saline waters as the Dead Sea often require nearly 30% salt, and the inoculating loop (a device for handling bacteria in the laboratory) used to transfer them must first be dipped into a saturated salt solution. More common are **facultative halophiles,** which do not require high salt concentrations but are able to grow at salt concentrations up to 2%, a concentration that inhibits the growth of many other organisms. A few species of facultative halophiles can tolerate even 15% salt.

Most microorganisms, however, must be grown in a medium that is nearly all water. For example, the concentration of agar (a complex polysaccharide isolated from marine algae) used to solidify microbial growth media is usually about 1.5%. If markedly higher concentrations are used, the increased osmotic pressure can inhibit the growth of some bacteria.

If the osmotic pressure is unusually low (the environment is *hypotonic*)—such as in distilled water, for example—water tends to enter the cell rather than leave it. Some microbes that have a relatively weak cell wall may be lysed by such treatment.

CHECK YOUR UNDERSTANDING

- Why are hyperthermophiles that grow at temperatures above 100°C seemingly limited to oceanic depths? **6-1**
- Other than controlling acidity, what is an advantage of using phosphate salts as buffers in growth media? **6-2**
- Why might primitive civilizations have used food preservation techniques that rely on osmotic pressure? **6-3**

Chemical Requirements

Carbon

Besides water, one of the most important requirements for microbial growth is carbon. Carbon is the structural backbone of living matter; it is needed for all the organic compounds that make up a living cell. Half the dry weight of a typical bacterial cell is carbon. Chemoheterotrophs get most of their carbon from the source of their energy—organic materials such as proteins, carbohydrates, and lipids. Chemoautotrophs and photoautotrophs derive their carbon from carbon dioxide.

Nitrogen, Sulfur, and Phosphorus

In addition to carbon, microorganisms need other elements to synthesize cellular material. For example, protein synthesis requires considerable amounts of nitrogen as well as some sulfur. The syntheses of DNA and RNA also require nitrogen and some phosphorus, as does the synthesis of ATP, the molecule so important for the storage and transfer of chemical energy within the cell. Nitrogen makes up about 14% of the dry weight of a bacterial cell, and sulfur and phosphorus together constitute about another 4%.

Organisms use nitrogen primarily to form the amino group of the amino acids of proteins. Many bacteria meet this requirement by decomposing protein-containing material and reincorporating the amino acids into newly synthesized proteins and other nitrogen-containing compounds. Other bacteria use nitrogen from ammonium ions (NH_4^+), which are already in the reduced form and are usually found in organic cellular material. Still other bacteria are able to derive nitrogen from nitrates (compounds that dissociate to give the nitrate ion, NO_3^-, in solution).

Some important bacteria, including many of the photosynthesizing cyanobacteria (page 137), use gaseous nitrogen (N_2) directly from the atmosphere. This process is called **nitrogen fixation.** Some organisms that can use this method are free-living, mostly in the soil, but others live cooperatively in symbiosis with the roots of legumes such as clover, soybeans, alfalfa, beans, and peas. The nitrogen fixed in the symbiosis is used by both the plant and the bacterium (see Chapter 27).

Sulfur is used to synthesize sulfur-containing amino acids and vitamins such as thiamine and biotin. Important natural sources of sulfur include the sulfate ion (SO_4^{2-}), hydrogen sulfide, and the sulfur-containing amino acids.

Phosphorus is essential for the synthesis of nucleic acids and the phospholipids of cell membranes. Among other places, it is also found in the energy bonds of ATP. A source of phosphorus is the phosphate ion (PO_4^{3-}). Potassium, magnesium, and calcium are also elements that microorganisms require, often as cofactors for enzymes (see Chapter 5, pages 114–115).

Trace Elements

Microbes require very small amounts of other mineral elements, such as iron, copper, molybdenum, and zinc; these are referred to as **trace elements.** Most are essential for the functions of certain enzymes, usually as cofactors. Although these elements are sometimes added to a laboratory medium, they are usually assumed to be naturally present in tap water and other components of media. Even most distilled waters contain adequate amounts, but tap water is sometimes specified to ensure that these trace minerals will be present in culture media.

Oxygen

We are accustomed to thinking of molecular oxygen (O_2) as a necessity of life, but it is actually in a sense a poisonous gas. Very little molecular oxygen existed in the atmosphere during most of Earth's history—in fact, it is possible that life could not have arisen had oxygen been present. However, many current forms of life have metabolic systems that require oxygen for aerobic respiration. As we have seen, hydrogen atoms that have been stripped from organic compounds combine with oxygen to form water, as shown in Figure 5.14 (page 127). This process yields a great deal of energy while neutralizing a potentially toxic gas—a very neat solution, all in all.

TABLE 6.1 The Effect of Oxygen on the Growth of Various Types of Bacteria

	a. Obligate Aerobes	b. Facultative Anaerobes	c. Obligate Anaerobes	d. Aerotolerant Anaerobes	e. Microaerophiles
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concentration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

Microbes that use molecular oxygen (aerobes) extract more energy from nutrients than microbes that do not use oxygen (anaerobes). Organisms that require oxygen to live are called **obligate aerobes** (Table 6.1a).

Obligate aerobes are at a disadvantage because oxygen is poorly soluble in the water of their environment. Therefore, many of the aerobic bacteria have developed, or retained, the ability to continue growing in the absence of oxygen. Such organisms are called **facultative anaerobes** (Table 6.1b). In other words, facultative anaerobes can use oxygen when it is present but are able to continue growth by using fermentation or anaerobic respiration when oxygen is not available. However, their efficiency in producing energy decreases in the absence of oxygen. Examples of facultative anaerobes are the familiar *Escherichia coli* that are found in the human intestinal tract. Many yeasts are also facultative anaerobes. Recall from the discussion of anaerobic respiration in Chapter 5 (page 130) that many microbes are able to substitute other electron acceptors, such as nitrate ions, for oxygen, which is something humans are unable to do.

Obligate anaerobes (Table 6.1c) are bacteria that are unable to use molecular oxygen for energy-yielding reactions. In fact, most are harmed by it. The genus *Clostridium* (klôs-tri′dē-um), which contains the species that cause tetanus and botulism, is the most familiar example. These bacteria do use oxygen atoms present in cellular materials; the atoms are usually obtained from water.

Understanding how organisms can be harmed by oxygen requires a brief discussion of the toxic forms of oxygen:

1. **Singlet oxygen** ($^1O_2$) is normal molecular oxygen (O_2) that has been boosted into a higher-energy state and is extremely reactive.
2. **Superoxide radicals** (O_2^-), or **superoxide anions,** are formed in small amounts during the normal respiration of organisms that use oxygen as a final electron acceptor, forming water. In the presence of oxygen, obligate anaerobes also appear to form some superoxide radicals, which are so toxic to cellular components that all organisms attempting to grow in atmospheric oxygen must produce an enzyme, **superoxide dismutase (SOD),** to neutralize them. Their toxicity is caused by their great instability, which leads them to steal an electron from a neighboring molecule, which in turn becomes a radical and steals an electron, and so on. Aerobic bacteria, facultative anaerobes growing aerobically, and aerotolerant anaerobes (discussed shortly) produce SOD, with which they convert the superoxide radical into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2):

$$O_2^- + O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

3. The hydrogen peroxide produced in this reaction contains the **peroxide anion** O_2^{2-} and is also toxic. In Chapter 7 (page 199) we will encounter it as the active principle in the antimicrobial agents hydrogen peroxide and benzoyl peroxide. Because the hydrogen peroxide produced during normal aerobic respiration is toxic, microbes have developed enzymes to neutralize it. The most familiar of these is **catalase,** which converts it into water and oxygen:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Catalase is easily detected by its action on hydrogen peroxide. When a drop of hydrogen peroxide is added to a colony of bacterial cells producing catalase, oxygen bubbles are released. Anyone who has put hydrogen peroxide on a wound will recognize that cells in human tissue also contain catalase. The other enzyme that breaks down hydrogen peroxide is **peroxidase,** which differs from catalase in that its reaction does not produce oxygen:

$$H_2O_2 + 2\ H^+ \longrightarrow 2\ H_2O$$

Another important form of reactive oxygen, **ozone (O_3),** is also discussed on page 199.

4. The **hydroxyl radical** ($OH\cdot$) is another intermediate form of oxygen and probably the most reactive. It is formed in the cellular cytoplasm by ionizing radiation. Most aerobic respiration produces traces of hydroxyl radicals, but they are transient.

These toxic forms of oxygen are an essential component of one of the body's most important defenses against pathogens, phagocytosis (see page 460 and Figure 16.7). In the phagolysosome of the phagocytic cell, ingested pathogens are killed by exposure to singlet oxygen, superoxide radicals, peroxide anions of hydrogen peroxide, and hydroxyl radicals and other oxidative compounds.

Obligate anaerobes usually produce neither superoxide dismutase nor catalase. Because aerobic conditions probably lead to an accumulation of superoxide radicals in their cytoplasm, obligate anaerobes are extremely sensitive to oxygen.

Aerotolerant anaerobes (Table 6.1d) cannot use oxygen for growth, but they tolerate it fairly well. On the surface of a solid medium, they will grow without the use of special techniques (discussed later) required for obligate anaerobes. Many of the aerotolerant bacteria characteristically ferment carbohydrates to lactic acid. As lactic acid accumulates, it inhibits the growth of aerobic competitors and establishes a favorable ecological niche for lactic acid producers. A common example of lactic acid–producing aerotolerant anaerobes is the lactobacilli used in the production of many acidic fermented foods, such as pickles and cheese. In the laboratory, they are handled and grown much like any other bacteria, but they make no use of the oxygen in the air. These bacteria can tolerate oxygen because they possess SOD or an equivalent system that neutralizes the toxic forms of oxygen previously discussed.

A few bacteria are **microaerophiles** (Table 6.1e). They are aerobic; they do require oxygen. However, they grow only in oxygen concentrations lower than those in air. In a test tube of solid nutrient medium, they grow only at a depth where small amounts of oxygen have diffused into the medium; they do not grow near the oxygen-rich surface or below the narrow zone of adequate oxygen. This limited tolerance is probably due to their sensitivity to superoxide radicals and peroxides, which they produce in lethal concentrations under oxygen-rich conditions.

Organic Growth Factors

Essential organic compounds an organism is unable to synthesize are known as **organic growth factors;** they must be directly obtained from the environment. One group of organic growth factors for humans is vitamins. Most vitamins function as coenzymes, the organic cofactors required by certain enzymes in order to function. Many bacteria can synthesize all their own vitamins and do not depend on outside sources. However, some bacteria lack the enzymes needed for the synthesis of certain vitamins, and for them those vitamins are organic growth factors. Other organic growth factors required by some bacteria are amino acids, purines, and pyrimidines.

CHECK YOUR UNDERSTANDING

- If bacterial cells were given a sulfur source containing radioactive sulfur (^{35}S) in their culture media, in what molecules would the ^{35}S be found in the cells? 6-4
- How would one determine whether a microbe is a strict anaerobe? 6-5
- Oxygen is so pervasive in the environment that it would be very difficult for a microbe to always avoid physical contact with it. What, therefore, is the most obvious way for a microbe to avoid damage? 6-6

Biofilms

LEARNING OBJECTIVE

6-7 Describe the formation of biofilms and their potential for causing infection.

In nature, microorganisms seldom live in the isolated single-species colonies that we see on laboratory plates. They more typically live in communities called **biofilms.** This fact was not well appreciated until the development of confocal microscopy (see page 61) made the three-dimensional structure of biofilms more visible. Biofilms reside in a matrix made up primarily of polysaccharides, but also containing DNA and proteins, that is often informally called *slime.* A biofilm also can be considered a *hydrogel,* which is a complex polymer containing many times its dry weight in water. Cell-to-cell chemical communication, or *quorum sensing,* allows bacteria to coordinate their activity and group together into communities that provide benefits not unlike those of multicellular organisms (see the box in Chapter 3, page 56). Therefore, biofilms are not just bacterial

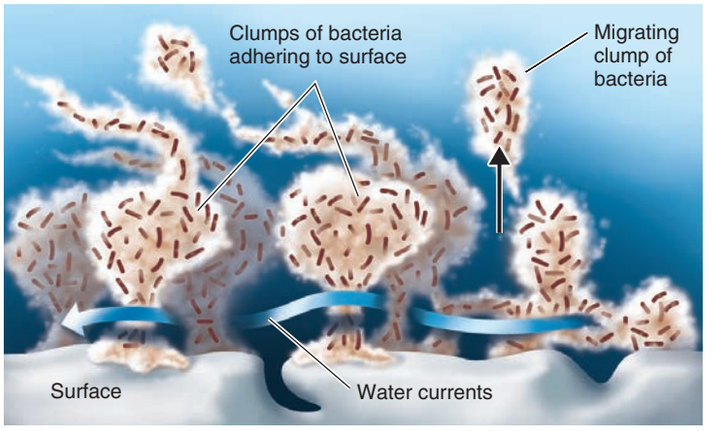
slime layers but biological systems; the bacteria are organized into a coordinated, functional community. Biofilms are usually attached to a surface, such as a rock in a pond, a human tooth (plaque; see Figure 25.3 on page 714), or a mucous membrane. This community might be of a single species or of a diverse group of microorganisms. Biofilms also might take other, more varied forms. The floc that forms in certain types of sewage treatment (see Figure 27.19, page 791) is an example. In fast-flowing streams, the biofilm might be in the form of filamentous streamers. Within a biofilm community, the bacteria are able to share nutrients and are sheltered from harmful factors in the environment, such as desiccation, antibiotics, and the body's immune system. The close proximity of microorganisms within a biofilm might also have the advantage of facilitating the transfer of genetic information by, for example, conjugation.

A biofilm usually begins to form when a free-swimming (*planktonic*) bacterium attaches to a surface. If these bacteria grew in a uniformly thick monolayer, they would become overcrowded, nutrients would not be available in lower depths, and toxic wastes could accumulate. Microorganisms in biofilm communities sometimes avoid these problems by forming pillar-like structures (**Figure 6.5**) with channels between them, through which water can carry incoming nutrients and outgoing wastes. This constitutes a primitive circulatory system. Individual microbes and clumps of slime occasionally leave the established biofilm and move to a new location where the biofilm becomes extended. Such a biofilm is generally composed of a surface layer about 10 µm thick, with pillars that extend up to 200 µm above it.

The microorganisms in biofilms can work cooperatively to carry out complex tasks. For example, the digestive systems of ruminant animals, such as cattle, require many different microbial species to break down cellulose. The microbes in a ruminant's digestive system are located mostly within biofilm communities. Biofilms are also essential elements in the proper functioning of sewage treatment systems, which we will discuss in Chapter 27. They can also, however, be a problem in pipes and tubing, where their accumulations impede circulation.

Biofilms are an important factor in human health. For example, microbes in biofilms are probably 1000 times more resistant to microbicides. Experts at the Centers for Disease Control and Prevention (CDC) estimate that 70% of human bacterial infections involve biofilms. Most nosocomial infections (infections acquired in health care facilities) are probably related to biofilms on medical catheters (see Figure 1.8 on page 18 and Figure 21.3 on page 592). In fact, biofilms form on almost all indwelling medical devices, including mechanical heart valves. Biofilms, which also can include those formed by fungi such as *Candida,* are encountered in many disease conditions, such as infections related to the use of contact lenses, dental caries (see page 713), and infections by pseudomonad bacteria (see page 307).

One approach to preventing biofilm formation is to incorporate antimicrobials into surfaces on which biofilms might form (see page 56). Because the chemical signals that allow quorum sensing are essential to biofilm formation, research is underway to determine the makeup of these chemical signals and perhaps block them. Another approach involves the discovery that lactoferrin (see page 473), which is abundant in many human secretions, can inhibit biofilm formation. Lactoferrin binds iron, especially among the pseudomonads that are responsible for cystic fibrosis biofilms, the cause of the pathology of this hereditary disease. The lack of iron inhibits the surface motility essential for the aggregation of the bacteria into biofilms.



Water currents move, as shown by the blue arrow, among pillars of slime formed by the growth of bacteria attached to solid surfaces. This allows efficient access to nutrients and removal of bacterial waste products. Individual slime-forming bacteria or bacteria in clumps of slime detach and move to new locations. See Figure 1.8.

Figure 6.5 **Biofilms.**

Q Why is the prevention of biofilms important in a health care environment?

Most laboratory methods in microbiology today use organisms being cultured in their planktonic mode. However, microbiologists now predict that there will be an increasing focus on how microorganisms actually live in relation with one another and that this will be considered in industrial and medical research.

CHECK YOUR UNDERSTANDING

✓ Identify a way in which pathogens find it advantageous to form biofilms. **6-7**

Culture Media

LEARNING OBJECTIVES

6-8 Distinguish chemically defined and complex media.

6-9 Justify the use of each of the following: anaerobic techniques, living host cells, candle jars, selective and differential media, enrichment medium.

6-10 Differentiate biosafety levels 1, 2, 3, and 4.

A nutrient material prepared for the growth of microorganisms in a laboratory is called a **culture medium.** Some bacteria can

grow well on just about any culture medium; others require special media, and still others cannot grow on any nonliving medium yet developed. Microbes that are introduced into a culture medium to initiate growth are called an **inoculum.** The microbes that grow and multiply in or on a culture medium are referred to as a **culture.**

Suppose we want to grow a culture of a certain microorganism, perhaps the microbes from a particular clinical specimen. What criteria must the culture medium meet? First, it must contain the right nutrients for the specific microorganism we want to grow. It should also contain sufficient moisture, a properly adjusted pH, and a suitable level of oxygen, perhaps none at all. The medium must initially be **sterile**—that is, it must initially contain no living microorganisms—so that the culture will contain only the microbes (and their offspring) we add to the medium. Finally, the growing culture should be incubated at the proper temperature.

A wide variety of media are available for the growth of microorganisms in the laboratory. Most of these media, which are available from commercial sources, have premixed components and require only the addition of water and then sterilization. Media are constantly being developed or revised for use in the isolation and identification of bacteria that are of interest to researchers in such fields as food, water, and clinical microbiology.

When it is desirable to grow bacteria on a solid medium, a solidifying agent such as agar is added to the medium. A complex polysaccharide derived from a marine alga, **agar** has long been used as a thickener in foods such as jellies and ice cream.

Agar has some very important properties that make it valuable to microbiology, and no satisfactory substitute has ever been found. Few microbes can degrade agar, so it remains solid. Also, agar liquefies at about 100°C (the boiling point of water) and at sea level remains liquid until the temperature drops to about 40°C. For laboratory use, agar is held in water baths at about 50°C. At this temperature, it does not injure most bacteria when it is poured over them (as shown in Figure 6.17a, page 173). Once the agar has solidified, it can be incubated at temperatures approaching 100°C before it again liquefies; this property is particularly useful when thermophilic bacteria are being grown.

Agar media are usually contained in test tubes or *Petri dishes.* The test tubes are called *slants* when their contents are allowed to solidify with the tube held at an angle so that a large surface area for growth is available. When the agar solidifies in a vertical tube, it is called a *deep.* Petri dishes, named for their inventor, are shallow dishes with a lid that nests over the bottom to prevent contamination; when filled, they are called *Petri* (or culture) *plates.*

Chemically Defined Media

To support microbial growth, a medium must provide an energy source, as well as sources of carbon, nitrogen, sulfur, phosphorus, and any organic growth factors the organism is unable to synthesize. A **chemically defined medium** is one whose exact chemical composition is known. For a chemoheterotroph, the chemically defined medium must contain organic growth factors that serve as a source of carbon and energy. For example, as shown in **Table 6.2**, glucose is included in the medium for growing the chemoheterotroph *E. coli.*

TABLE 6.2 A Chemically Defined Medium for Growing a Typical Chemoheterotroph, Such as *Escherichia coli*

Constituent	Amount
Glucose	5.0 g
Ammonium phosphate, monobasic ($NH_4H_2PO_4$)	1.0 g
Sodium chloride (NaCl)	5.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Potassium phosphate, dibasic (K_2HPO_4)	1.0 g
Water	1 liter

As **Table 6.3** shows, many organic growth factors must be provided in the chemically defined medium used to cultivate a species of *Leuconostoc*. Organisms that require many growth factors are described as *fastidious.* Organisms of this type, such as *Lactobacillus* (page 316), are sometimes used in tests that determine the concentration of a particular vitamin in a substance. To perform such a *microbiological assay,* a growth medium is prepared that contains all the growth requirements of the bacterium except the vitamin being assayed. Then the medium, test substance, and bacterium are combined, and the growth of bacteria is measured. This bacterial growth, which is reflected by the amount of lactic acid produced, will be proportional to the amount of vitamin in the test substance. The more lactic acid, the more the *Lactobacillus* cells have been able to grow, so the more vitamin is present.

Complex Media

Chemically defined media are usually reserved for laboratory experimental work or for the growth of autotrophic bacteria. Most heterotrophic bacteria and fungi, such as you would work with in an introductory lab course, are routinely grown on **complex media** made up of nutrients including extracts from yeasts, meat, or plants, or digests of proteins from these and other sources. The exact chemical composition varies slightly from batch to batch. **Table 6.4** shows one widely used recipe.

In complex media, the energy, carbon, nitrogen, and sulfur requirements of the growing microorganisms are provided primarily by protein. Protein is a large, relatively insoluble molecule that a minority of microorganisms can

TABLE 6.3 Defined Culture Medium for *Leuconostoc mesenteroides*

Carbon and Energy
Glucose, 25 g
Salts
NH_4Cl, 3.0 g K_2HPO_4*, 0.6 g KH_2PO_4*, 0.6 g $MgSO_4$, 0.1 g
Amino Acids, 100–200 µg each
Alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine
Purines and Pyrimidines, 10 mg of each
Adenine, guanine, uracil, xanthine
Vitamins, 0.01–1 mg each
Biotin, folate, nicotinic acid, pyridoxal, pyridoxamine, pyridoxine, riboflavin, thiamine, pantothenate, *p*-aminobenzoic acid
Trace Elements, 2–10 µg each
Fe, Co, Mn, Zn, Cu, Ni, Mo
Buffer, pH 7
Sodium acetate, 25 g
Distilled Water, 1,000 ml
*Also serves as buffer.

TABLE 6.4 Composition of Nutrient Agar, a Complex Medium for the Growth of Heterotrophic Bacteria

Constituent	Amount
Peptone (partially digested protein)	5.0 g
Beef extract	3.0 g
Sodium chloride	8.0 g
Agar	15.0 g
Water	1 liter

utilize directly, but a partial digestion by acids or enzymes reduces protein to shorter chains of amino acids called *peptones*. These small, soluble fragments can be digested by most bacteria.

Vitamins and other organic growth factors are provided by meat extracts or yeast extracts. The soluble vitamins and minerals from the meats or yeasts are dissolved in the extracting water, which is then evaporated so that these factors are concentrated. (These extracts also supplement the organic nitrogen and carbon compounds.) Yeast extracts are particularly rich in the B vitamins. If a complex medium is in liquid form, it is called **nutrient broth.** When agar is added, it is called **nutrient agar.** (This terminology can be confusing; just remember that agar itself is not a nutrient.)

Anaerobic Growth Media and Methods

The cultivation of anaerobic bacteria poses a special problem. Because anaerobes might be killed by exposure to oxygen, special media called **reducing media** must be used. These media contain ingredients, such as sodium thioglycolate, that chemically combine with dissolved oxygen and deplete the oxygen in the culture medium. To routinely grow and maintain pure cultures of obligate anaerobes, microbiologists use reducing media stored in ordinary, tightly capped test tubes. These media are heated shortly before use to drive off absorbed oxygen.

When the culture must be grown in Petri plates to observe individual colonies, several methods are available. Laboratories that work with relatively few culture plates at a time can use systems that can incubate the microorganisms in sealed boxes and jars in which the oxygen is chemically removed after the culture plates have been introduced and the container sealed. Some systems require that water be added to an envelope of chemicals before the container is closed, as shown in Figure 6.6, and require a catalyst. The chemicals produce hydrogen and carbon dioxide (about 4–10%) and remove the oxygen in the container by combining it, in the presence of the catalyst, with hydrogen to form water. In another commercially available system, the envelope of chemicals (the active ingredient is ascorbic acid) is simply opened to expose it to oxygen in the container's atmosphere. No water or catalyst is needed. The atmosphere in such containers usually has less than 5% oxygen, about 18% CO_2, and no hydrogen. In a recently introduced system, each individual Petri plate (OxyPlate) becomes an anaerobic chamber. The medium in the plate contains an enzyme, oxyrase, which combines oxygen with hydrogen, removing oxygen as water is formed.

Laboratories that have a large volume of work with anaerobes often use an anaerobic chamber, such as that shown in Figure 6.7. The chamber is filled with inert gases (typically about 85% N_2, 10% H_2, and 5% CO_2) and is equipped with air locks to introduce cultures and materials.

Special Culture Techniques

Many bacteria have never been successfully grown on artificial laboratory media. *Mycobacterium leprae,* the leprosy bacillus, is now usually grown in armadillos, which have a relatively low body temperature that matches the requirements of the microbe.

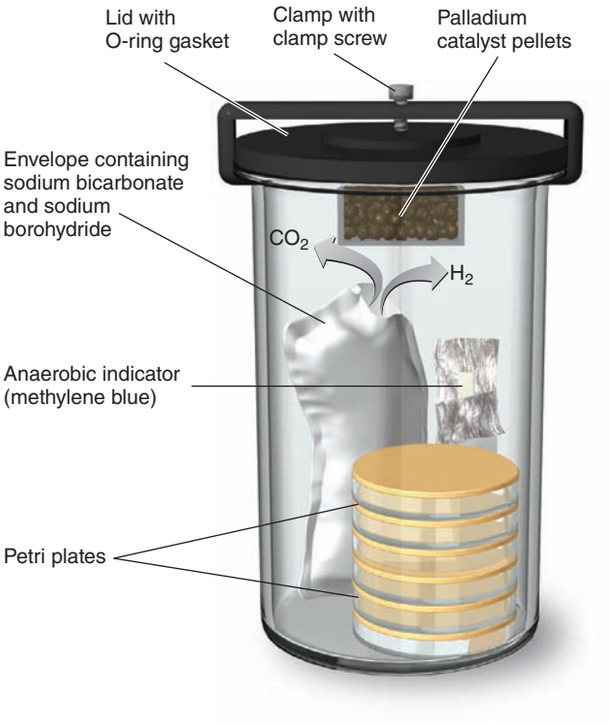


Figure 6.6 A jar for cultivating anaerobic bacteria on Petri plates. When water is mixed with the chemical packet containing sodium bicarbonate and sodium borohydride, hydrogen and carbon dioxide are generated. Reacting on the surface of a palladium catalyst in a screened reaction chamber, which may also be incorporated into the chemical packet, the hydrogen and atmospheric oxygen in the jar combine to form water. The oxygen is thus removed. Also in the jar is an anaerobic indicator containing methylene blue, which is blue when oxidized and turns colorless when the oxygen is removed (as shown here).

Q What is the technical name for bacteria that require a higher-than-atmospheric-concentration of CO_2 for growth?

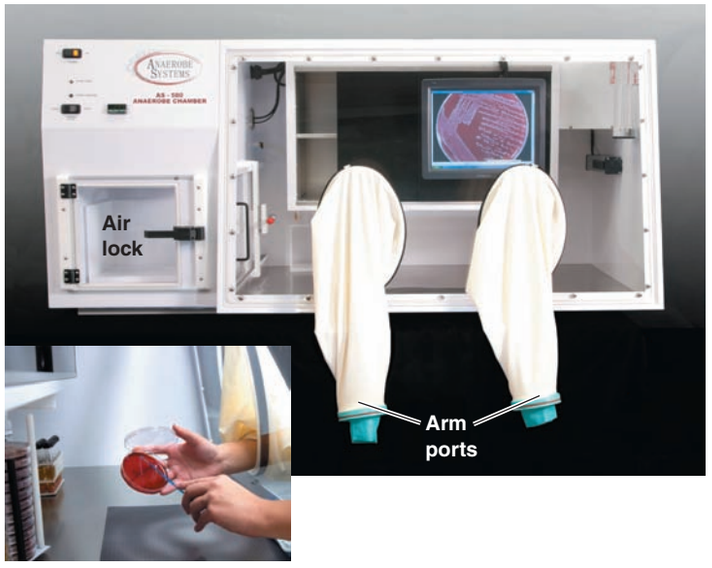


Figure 6.7 An anaerobic chamber. Materials are introduced through the small doors in the air-lock chamber at the left. The operator works through arm ports in airtight sleeves. The airtight sleeves extend into the cabinet when it is in use. This unit also features an internal camera and monitor.

Q In what way would an anaerobic chamber resemble the Space Laboratory orbiting in the vacuum of space?

Another example is the syphilis spirochete, although certain nonpathogenic strains of this microbe have been grown on laboratory media. With few exceptions, the obligate intracellular bacteria, such as the rickettsias and the chlamydias, do not grow on artificial media. Like viruses, they can reproduce only in a living host cell. See the discussion of cell culture, page 379.

Many clinical laboratories have special *carbon dioxide incubators* in which to grow aerobic bacteria that require concentrations of CO_2 higher or lower than that found in the atmosphere. Desired CO_2 levels are maintained by electronic controls. High CO_2 levels are also obtained with simple *candle jars.* Cultures are placed in a large sealed jar containing a lighted candle, which consumes oxygen. The candle stops burning when the air in the jar has a lowered concentration of oxygen (at about 17% O_2, still adequate for the growth of aerobic bacteria). An elevated concentration of CO_2 (about 3%) is also present. Microbes that grow better at high CO_2 concentrations are called **capnophiles.** The low-oxygen, high-CO_2 conditions resemble those found in the intestinal tract, respiratory tract, and other body tissues where pathogenic bacteria grow.

Candle jars are still used occasionally, but more often commercially available chemical packets are used to generate carbon dioxide atmospheres in containers. When only one or two Petri plates of cultures are to be incubated, clinical laboratory investigators often use small plastic bags with self-contained chemical gas generators that are activated by crushing the packet or moistening it with a few milliliters of water. These packets are sometimes specially designed to provide precise concentrations of carbon dioxide (usually higher than can be obtained in candle jars) and oxygen for culturing organisms such as the microaerophilic *Campylobacter* bacteria (page 313).

Some microorganisms are so dangerous that they can be handled only under extraordinary systems of containment called *biosafety level 4 (BSL-4).* Level 4 labs are popularly known as "the hot zone." Only a handful of such labs exists in the United States. The lab is a sealed environment within a larger building and has an atmosphere under negative pressure, so that aerosols containing pathogens will not escape. Both intake and exhaust air is filtered through high-efficiency particulate air filters (see HEPA filters, page 188); the exhaust air is filtered twice. All waste materials leaving the lab are rendered noninfectious. The personnel wear "space suits" that are connected to an air supply (**Figure 6.8**).

Figure 6.8 Technicians in a biosafety level 4 (BSL-4) laboratory. Personnel working in a BSL-4 facility wear a "space suit" that is connected to an outside air supply.

Q If a technician were working with pathogenic prions, how would material leaving the lab be rendered noninfectious? (*Hint*: See Chapter 7.)

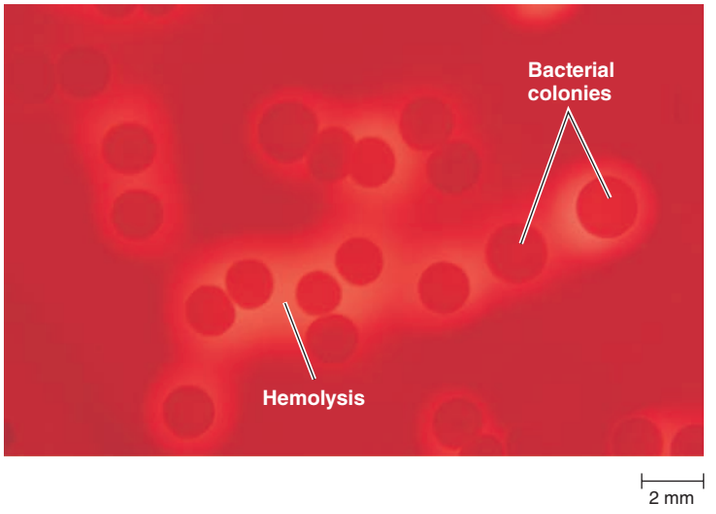


Figure 6.9 Blood agar, a differential medium containing red blood cells. The bacteria have lysed the red blood cells (beta-hemolysis), causing the clear areas around the colonies.

Q Of what value are hemolysins to pathogens?

Less dangerous organisms are handled at lower levels of biosafety. For example, a basic microbiology teaching laboratory would be BSL-1. Organisms that present a moderate risk of infection can be handled at BSL-2 levels, that is, on open laboratory benchtops with appropriate gloves, lab coats, or possibly face and eye protection. BSL-3 labs are intended for highly infectious airborne pathogens such as the tuberculosis agent. Biological safety cabinets similar in appearance to the anaerobic chamber shown in Figure 6.7 are used. The laboratory itself should be negatively pressurized and equipped with air filters to prevent release of the pathogen from the laboratory.

Selective and Differential Media

In clinical and public health microbiology, it is frequently necessary to detect the presence of specific microorganisms associated with disease or poor sanitation. For this task, selective and differential media are used. **Selective media** are designed to suppress the growth of unwanted bacteria and encourage the growth of the desired microbes. For example, bismuth sulfite agar is one medium used to isolate the typhoid bacterium, the gram-negative *Salmonella typhi* (tī′fē), from feces. Bismuth sulfite inhibits gram-positive bacteria and most gram-negative intestinal bacteria (other than *S. typhi*), as well. Sabouraud's dextrose agar, which has a pH of 5.6, is used to isolate fungi that outgrow most bacteria at this pH.

Differential media make it easier to distinguish colonies of the desired organism from other colonies growing on the same plate. Similarly, pure cultures of microorganisms have identifiable reactions with differential media in tubes or plates. Blood agar (which contains red blood cells) is a medium that microbiologists often use to identify bacterial species that destroy red blood cells. These species, such as *Streptococcus pyogenes* (pī-äj′en-ēz), the bacterium that causes strep throat, show a clear ring around their colonies (beta-hemolysis, page 317) where they have lysed the surrounding blood cells (**Figure 6.9**).

Sometimes, selective and differential characteristics are combined in a single medium. Suppose we want to isolate the common bacterium *Staphylococcus aureus,* found in the nasal passages. This organism has a tolerance for high concentrations of sodium chloride; it can also ferment the carbohydrate mannitol to form acid. Mannitol salt agar contains 7.5% sodium chloride, which will discourage the growth of competing organisms and thus *select for* (favor the growth of) *S. aureus.* This salty medium also contains a pH indicator that changes color if the mannitol in the medium is fermented to acid; the mannitol-fermenting colonies of *S. aureus* are thus *differentiated from* colonies of bacteria that do not ferment mannitol. Bacteria that grow at the high salt concentration *and* ferment mannitol to acid can be readily identified by the color change (**Figure 6.10**). These are probably colonies of *S. aureus,* and their identification can be confirmed by additional tests. The use of differential media to identify toxin-producing *E. coli* is discussed in Chapter 5, page 136.

Enrichment Culture

Because bacteria present in small numbers can be missed, especially if other bacteria are present in much larger numbers, it is sometimes necessary to use an **enrichment culture.**

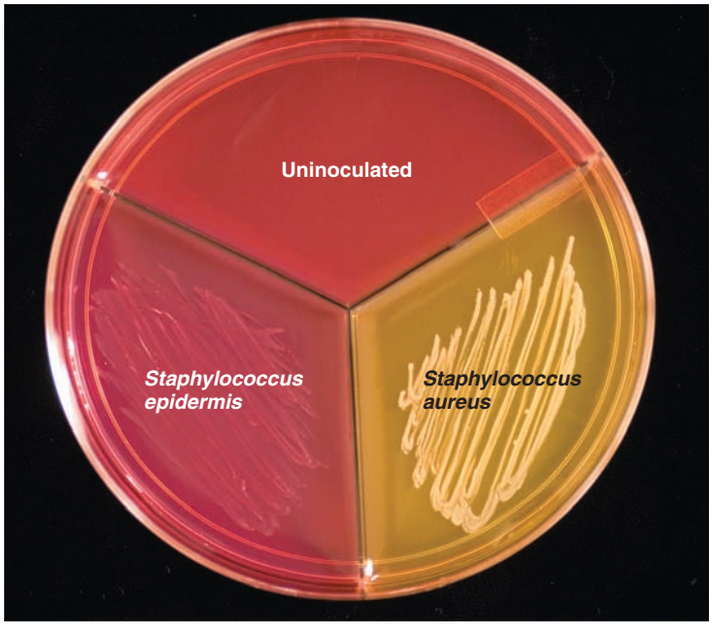


Figure 6.10 Differential medium. This medium is mannitol salt agar, and bacteria capable of fermenting the mannitol in the medium to acid (*Staphylococcus aureus*) cause the medium to change color to yellow. This **differentiates** between bacteria that can ferment mannitol and those that cannot. Actually, this medium is also *selective* because the high salt concentration prevents the growth of most bacteria but not *Staphlylococcus* spp.

Q Are bacteria capable of growing at a high osmotic pressure likely to be capable of growing in the mucus found in nostrils?

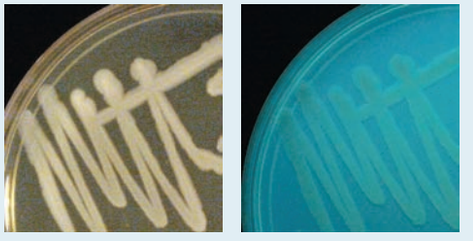
This is often the case for soil or fecal samples. The medium (enrichment medium) for an enrichment culture is usually liquid and provides nutrients and environmental conditions that favor the growth of a particular microbe but not others. In this sense, it is also a selective medium, but it is designed to increase very small numbers of the desired type of organism to detectable levels.

Suppose we want to isolate from a soil sample a microbe that can grow on phenol and is present in much smaller numbers than other species. If the soil sample is placed in a liquid enrichment medium in which phenol is the only source of carbon and energy, microbes unable to metabolize phenol will not grow. The culture medium is allowed to incubate for a few days, and then a small amount of it is transferred into another flask of the same medium. After a series of such transfers, the surviving population will consist of bacteria capable of metabolizing phenol. The bacteria are given time to grow in the medium between transfers; this is the enrichment stage. (See the box in Chapter 28 page 808.) Any nutrients in the original inoculum are rapidly diluted out with the successive transfers. When the last dilution is streaked onto a solid medium of the same composition, only those colonies of organisms capable of using phenol should grow. A remarkable aspect of this particular technique is that phenol is normally lethal to most bacteria.

Table 6.5 summarizes the purposes of the main types of culture media.

Clinical Case

P. fluorescens is an aerobic, gram-negative rod that grows best between 25°C and 30°C and grows poorly at the standard hospital microbiology incubation temperatures (35°C to 37°C). The bacteria are so named because they produce a pigment that fluoresces under ultraviolet light. While reviewing the facts of the latest outbreak, Dr. MacGruder learns that the most recent patients were last exposed to the contaminated heparin 84 to 421 days before onset of their infections. On-site investigations confirmed that the patients' clinics are no longer using the recalled heparin and had, in fact, returned all unused inventory. Concluding that these patients did not develop infections during the previous outbreak, Dr. MacGruder must look for a new source of infection. The patients all have indwelling venous catheters: tubes that are inserted into a vein for long-term delivery of concentrated solutions, such as anticancer drugs. Dr. MacGruder orders cultures of the new heparin being used, but the results do not recover any organisms. He then orders blood and catheter cultures from each of the patients.



Illuminated with white light | Illuminated with ultraviolet light

The organism cultured from both the patients' blood and their catheters is shown in the figure. What organism is it?

154 **166** 175 177

CHECK YOUR UNDERSTANDING

- Could humans exist on chemically defined media, at least under laboratory conditions? **6-8**
- Could Louis Pasteur, in the 1800s, have grown rabies viruses in cell culture instead of in living animals? **6-9**
- What BSL is your laboratory? **6-10**

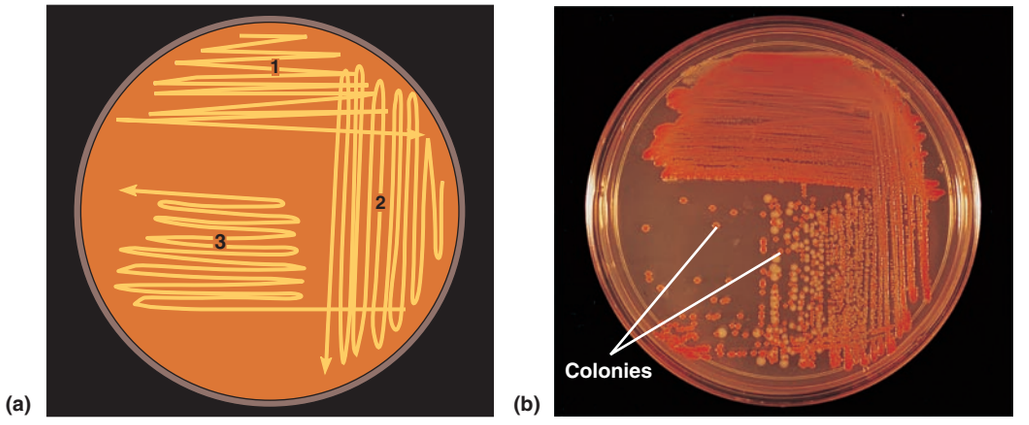


Figure 6.11 The streak plate method for isolating pure bacterial cultures. (**a**) Arrows indicate the direction of streaking. Streak series 1 is made from the original bacterial culture. The incoculating loop is sterilized following each streak series. In series 2 and 3, the loop picks up bacteria from the previous series, diluting the number of cells each time. There are numerous variants of such patterns. (**b**) In series 3 of this example, notice that well-isolated colonies of bacteria of two different types, red and yellow, have been obtained.

Q Is a colony formed as a result of streaking a plate always derived from a single bacterium? Why or why not?

Obtaining Pure Cultures

LEARNING OBJECTIVES

6-11 Define *colony*.

6-12 Describe how pure cultures can be isolated by using the streak plate method.

Most infectious materials, such as pus, sputum, and urine, contain several different kinds of bacteria; so do samples of soil, water, or food. If these materials are plated out onto the surface of a solid medium, colonies will form that are exact copies of the original organism. A visible **colony** theoretically arises from a single spore or vegetative cell or from a group of the same microorganisms attached to one another in clumps or chains. Estimates are that only about 1% of bacteria in ecosystems produce colonies by conventional culture methods. Microbial colonies often have a distinctive appearance that distinguishes one microbe from another (see Figure 6.10). The bacteria must be distributed widely enough so that the colonies are visibly separated from each other.

Most bacteriological work requires pure cultures, or clones, of bacteria. The isolation method most commonly used to get pure cultures is the **streak plate method** (Figure 6.11). A sterile inoculating loop is dipped into a mixed culture that contains more than one type of microbe and is streaked in a pattern over the surface of the nutrient medium. As the pattern is traced, bacteria are rubbed off the loop onto the medium. The last cells to be rubbed off the loop are far enough apart to grow into isolated colonies. These colonies can be picked up with an inoculating loop and transferred to a test tube of nutrient medium to form a pure culture containing only one type of bacterium.

The streak plate method works well when the organism to be isolated is present in large numbers relative to the total population. However, when the microbe to be isolated is present only in very small numbers, its numbers must be greatly increased by selective enrichment before it can be isolated with the streak plate method.

TABLE 6.5 Culture Media

Type	Purpose
Chemically Defined	Growth of chemoautotrophs and photoautotrophs; microbiological assays
Complex	Growth of most chemoheterotrophic organisms
Reducing	Growth of obligate anaerobes
Selective	Suppression of unwanted microbes; encouraging desired microbes
Differential	Differentiation of colonies of desired microbes from others
Enrichment	Similar to selective media but designed to increase numbers of desired microbes to detectable levels

CHECK YOUR UNDERSTANDING

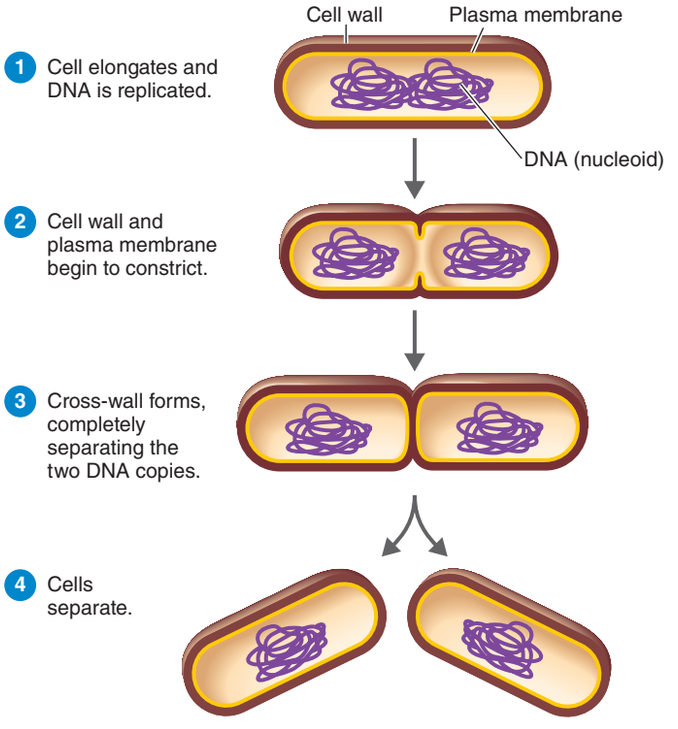
- Can you think of any reason why a colony does not grow to an infinite size, or at least fill the confines of the Petri plate? 6-11
- Could a pure culture of bacteria be obtained by the streak plate method if there were only one desired microbe in a bacterial suspension of billions? 6-12

Preserving Bacterial Cultures

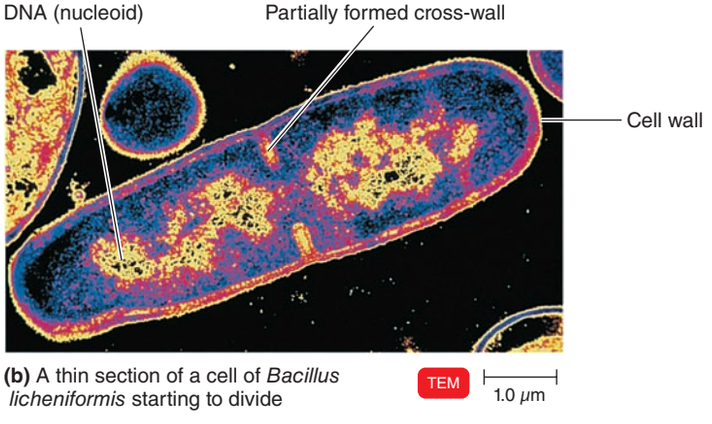
LEARNING OBJECTIVE

6-13 Explain how microorganisms are preserved by deep-freezing and lyophilization (freeze-drying).

Refrigeration can be used for the short-term storage of bacterial cultures. Two common methods of preserving microbial cultures



(a) A diagram of the sequence of cell division



(b) A thin section of a cell of *Bacillus licheniformis* starting to divide

Figure 6.12 **Binary fission in bacteria.**

Q In what way is budding different from binary fission?

for long periods are deep-freezing and lyophilization. **Deep-freezing** is a process in which a pure culture of microbes is placed in a suspending liquid and quick-frozen at temperatures ranging from –50°C to –95°C. The culture can usually be thawed and cultured even several years later. During **lyophilization (freeze-drying),** a suspension of microbes is quickly frozen at temperatures ranging from –54°C to –72°C, and the water is removed by a high vacuum (sublimation). While under vacuum, the container is sealed by melting the glass with a high-temperature torch. The remaining powderlike residue that contains the surviving microbes can be stored for years. The organisms can be revived at any time by hydration with a suitable liquid nutrient medium.

CHECK YOUR UNDERSTANDING

If the Space Station in Earth orbit suddenly ruptured, the humans on board would die instantly from cold and the vacuum of space. Would all the bacteria in the capsule also be killed? 6-13

The Growth of Bacterial Cultures

LEARNING OBJECTIVES

6-14 Define *bacterial growth,* including *binary fission.*

6-15 Compare the phases of microbial growth, and describe their relation to generation time.

6-16 Explain four direct methods of measuring cell growth.

6-17 Differentiate direct and indirect methods of measuring cell growth.

6-18 Explain three indirect methods of measuring cell growth.

Being able to represent graphically the enormous populations resulting from the growth of bacterial cultures is an essential part of microbiology. It is also necessary to be able to determine microbial numbers, either directly, by counting, or indirectly, by measuring their metabolic activity.

Bacterial Division

As we mentioned at the beginning of the chapter, bacterial growth refers to an increase in bacterial numbers, not an increase in the size of the individual cells. Bacteria normally reproduce by **binary fission** (Figure 6.12).

A few bacterial species reproduce by **budding;** they form a small initial outgrowth (a bud) that enlarges until its size approaches that of the parent cell, and then it separates. Some filamentous bacteria (certain actinomycetes) reproduce by producing chains of conidiospores carried externally at the tips of the filaments. A few filamentous species simply fragment, and the fragments initiate the growth of new cells. MM **Animations** Binary Fission; Bacterial Growth: Overview

Generation Time

For purposes of calculating the generation time of bacteria, we will consider only reproduction by binary fission, which is by far the most common method. As you can see in Figure 6.13, one cell's division produces two cells, two cells' divisions produce four cells, and so on. When the number of cells in each generation is expressed as a power of 2, the exponent tells the number of doublings (generations) that have occurred.

The time required for a cell to divide (and its population to double) is called the **generation time.** It varies considerably among organisms and with environmental conditions, such as temperature. Most bacteria have a generation time of 1 to 3 hours; others require more than 24 hours per generation. (The math required to calculate generation times is

Numbers of Cells	Numbers Expressed as a Power of 2	Visual Representation of Numbers
1	2^0	●
2	2^1	●●
4	2^2	●●●●
8	2^3	●●●●●●●●
16	2^4	●●●●●●●●●●●●●●●●
32	2^5	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●

(a) Visual representation of increase in bacterial number over five generations. The number of bacteria doubles in each generation. The superscript indicates the generation; that is, $2^5 = 5$ generations.

Generation Number	Number of Cells	Log_{10} of Number of Cells
0	$2^0 = 1$	0
5	$2^5 = 32$	1.51
10	$2^{10} = 1{,}024$	3.01
15	$2^{15} = 32{,}768$	4.52
16	$2^{16} = 65{,}536$	4.82
17	$2^{17} = 131{,}072$	5.12
18	$2^{18} = 262{,}144$	5.42
19	$2^{19} = 524{,}288$	5.72
20	$2^{20} = 1{,}048{,}576$	6.02

(b) Conversion of the number of cells in a population into the logarithmic expression of this number. To arrive at the numbers in the center column, use the y^x key on your calculator. Enter 2 on the calculator; press y^x; enter 5; then press the = sign. The calculator will show the number 32. Thus, the fifth-generation population of bacteria will total 32 cells. To arrive at the numbers in the right-hand column, use the log key on your calculator. Enter the number 32; then press the log key. The calculator will show, rounded off, that the $\log_{10}$ of 32 is 1.51.

Figure 6.13 **Cell division.**

Q If a single bacterium reproduced every 30 minutes, how many would there be in 2 hours?

presented in Appendix B.) If binary fission continues unchecked, an enormous number of cells will be produced. If a doubling occurred every 20 minutes—which is the case for *E. coli* under favorable conditions—after 20 generations a single initial cell would increase to over 1 million cells. This would require a little less than 7 hours. In 30 generations, or 10 hours, the population would be 1 billion, and in 24 hours it would be a number trailed by 21 zeros. It is difficult to graph population changes of such enormous magnitude by using arithmetic numbers. This is why logarithmic scales are generally used to graph bacterial growth. Understanding logarithmic representations of bacterial populations requires some use of mathematics and is necessary for anyone studying microbiology. (See Appendix B.)

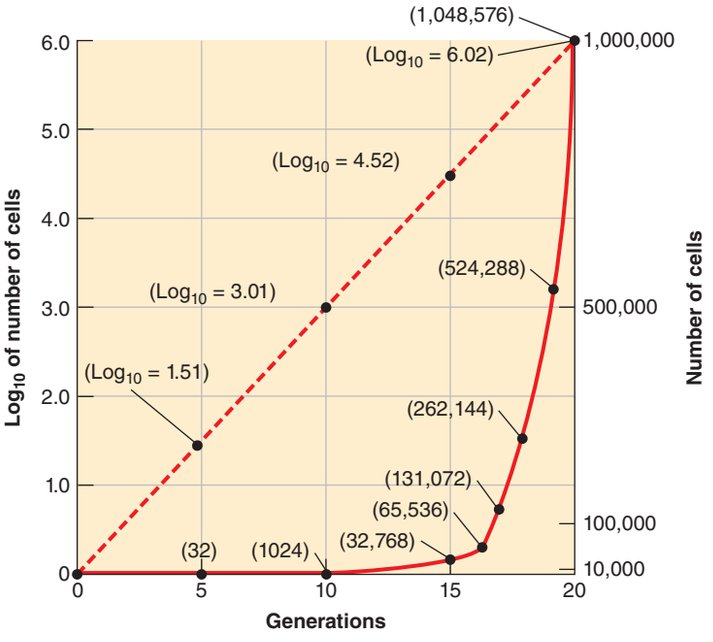


Figure 6.14 **A growth curve for an exponentially increasing population, plotted logarithmically (dashed line) and arithmetically (solid line).** For demonstration purposes, this graph has been drawn so that the arithmetic and logarithmic curves intersect at 1 million cells. This figure demonstrates why it is necessary to graph changes in the immense numbers of bacterial populations by logarithmic plots rather than by arithmetic numbers. For example, note that at ten generations the line representing arithmetic numbers has not even perceptibly left the baseline, whereas the logarithmic plot point for the tenth generation (3.01) is halfway up the graph.

Q If the arithmetic numbers (solid line) were plotted for two more generations, would the line still be on the page?

Logarithmic Representation of Bacterial Populations

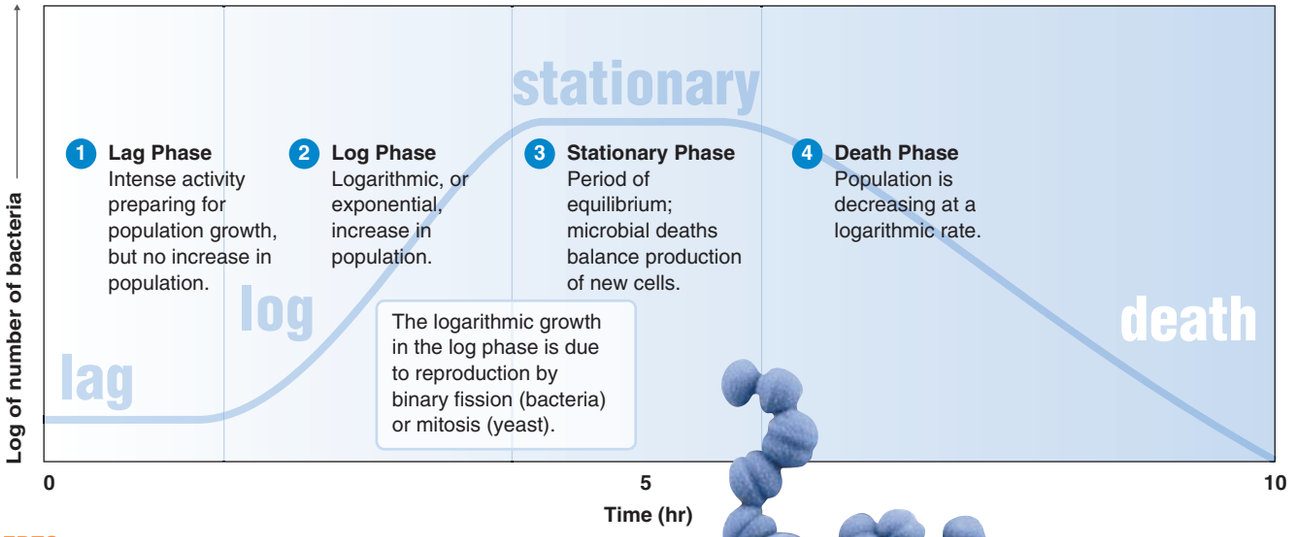
To illustrate the difference between logarithmic and arithmetic graphing of bacterial populations, let's express 20 bacterial generations both logarithmically and arithmetically. In five generations (2^5), there would be 32 cells; in ten generations (2^{10}), there would be 1024 cells, and so on. (If your calculator has a y^x key and a log key, you can duplicate the numbers in the third column of Figure 6.13.)

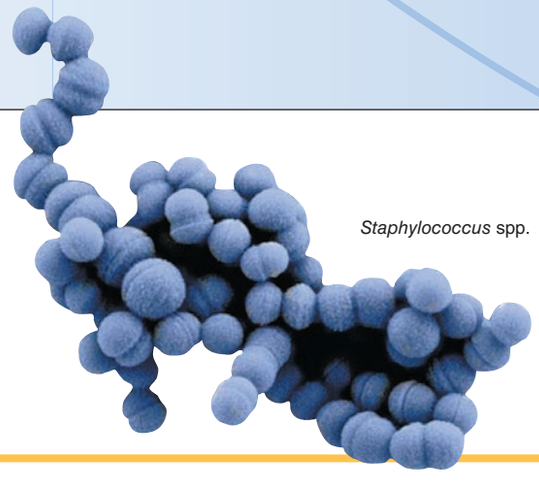
In **Figure 6.14**, notice that the arithmetically plotted line (solid) does not clearly show the population changes in the early stages of the growth curve at this scale. In fact, the first ten generations do not even appear to leave the baseline. Furthermore, another one or two arithmetic generations graphed to the same scale would greatly increase the height of the graph and take the line off the page.

The dashed line in Figure 6.14 shows how these plotting problems can be avoided by graphing the $\log_{10}$ of the population numbers. The $\log_{10}$ of the population is plotted at 5, 10, 15, and 20 generations. Notice that a straight line is formed and that a

FOUNDATION FIGURE **6.15**

Understanding the Bacterial Growth Curve





Staphylococcus spp.

KEY CONCEPTS

- Bacterial populations follow a sequential series of growth phases: the lag, log, stationary, and death phases.
- Knowledge of the bacterial growth curve is critical to understanding population dynamics and population control in the course of infectious diseases, in food preservation and spoilage, as well as in industrial microbiology processes, such as ethanol production.

thousand times this population (1,000,000,000, or $\log_{10}$ 9.0) could be accommodated in relatively little extra space. However, this advantage is obtained at the cost of distorting our "common sense" perception of the actual situation. We are not accustomed to thinking in logarithmic relationships, but it is necessary for a proper understanding of graphs of microbial populations.

CHECK YOUR UNDERSTANDING

Can a complex organism, such as a beetle, divide by binary fission? **6-14**

Phases of Growth

When a few bacteria are inoculated into a liquid growth medium and the population is counted at intervals, it is possible to plot a **bacterial growth curve** that shows the growth of cells over time (**Figure 6.15**). There are four basic phases of growth: the lag, log, stationary, and death phases. **Animation** Bacterial Growth Curve

The Lag Phase

For a while, the number of cells changes very little because the cells do not immediately reproduce in a new medium. This period of little or no cell division is called the **lag phase,** and it can last for 1 hour or several days. During this time, however, the cells are not dormant. The microbial population is undergoing a period of intense metabolic activity involving, in particular, synthesis of enzymes and various molecules. (The situation is analogous to a factory being equipped to produce automobiles; there is considerable tooling-up activity but no immediate increase in the automobile population.)

The Log Phase

Eventually, the cells begin to divide and enter a period of growth, or logarithmic increase, called the **log phase,** or **exponential growth phase.** Cellular reproduction is most active during this period, and generation time reaches a constant minimum. Because the generation time is constant, a logarithmic plot of growth during the log phase is a straight line. The log phase is the time when cells are most active metabolically and is preferred for industrial purposes where, for example, a product needs to be produced efficiently.

The Stationary Phase

If exponential growth continued unchecked, startlingly large numbers of cells could arise. For example, a single bacterium

(at a weight of 9.5×10^{-13} g per cell) dividing every 20 minutes for only 25.5 hours can theoretically produce a population equivalent in weight to that of an 80,000-ton aircraft carrier. In reality, this does not happen. Eventually, the growth rate slows, the number of microbial deaths balances the number of new cells, and the population stabilizes. This period of equilibrium is called the **stationary phase.**

What causes exponential growth to stop is not always clear. The exhaustion of nutrients, accumulation of waste products, and harmful changes in pH may all play a role.

The Death Phase

The number of deaths eventually exceeds the number of new cells formed, and the population enters the **death phase,** or **logarithmic decline phase.** This phase continues until the population is diminished to a tiny fraction of the number of cells in the previous phase or until the population dies out entirely. Some species pass through the entire series of phases in only a few days; others retain some surviving cells almost indefinitely. Microbial death will be discussed further in Chapter 7.

CHECK YOUR UNDERSTANDING

- If two mice started a family within a fixed enclosure, with a fixed food supply, would the population curve be the same as a bacterial growth curve? **6-15**

Direct Measurement of Microbial Growth

The growth of microbial populations can be measured in a number of ways. Some methods measure cell numbers; other methods measure the population's total mass, which is often directly proportional to cell numbers. Population numbers are usually recorded as the number of cells in a milliliter of liquid or in a gram of solid material. Because bacterial populations are usually very large, most methods of counting them are based on direct or indirect counts of very small samples; calculations then determine the size of the total population. Assume, for example, that a millionth of a milliliter (10^{-6} ml) of sour milk is found to contain 70 bacterial cells. Then there must be 70 times 1 million, or 70 million, cells per milliliter.

However, it is not practical to measure out a millionth of a milliliter of liquid or a millionth of a gram of food. Therefore, the procedure is done indirectly, in a series of dilutions. For example, if we add 1 ml of milk to 99 ml of water, each milliliter of this dilution now has one-hundredth as many bacteria as each milliliter of the original sample had. By making a series of such dilutions, we can readily estimate the number of bacteria in our original sample. To count microbial populations in solid foods (such as hamburger), an homogenate of one part food to nine parts water is finely ground in a food blender. Samples of this initial one-tenth dilution can then be transferred with a pipette for further dilutions or cell counts.

Plate Counts

The most frequently used method of measuring bacterial populations is the **plate count.** An important advantage of this method is that it measures the number of viable cells. One disadvantage may be that it takes some time, usually 24 hours or more, for visible colonies to form. This can be a serious problem in some applications, such as quality control of milk, when it is not possible to hold a particular lot for this length of time.

Plate counts assume that each live bacterium grows and divides to produce a single colony. This is not always true, because bacteria frequently grow linked in chains or as clumps (see Figure 4.1, page 77). Therefore, a colony often results, not from a single bacterium, but from short segments of a chain or from a bacterial clump. To reflect this reality, plate counts are often reported as **colony-forming units (CFU).**

When a plate count is performed, it is important that only a limited number of colonies develop in the plate. When too many colonies are present, some cells are overcrowded and do not develop; these conditions cause inaccuracies in the count. The U.S. Food and Drug Administration convention is to count only plates with 25 to 250 colonies, but many microbiologists prefer plates with 30 to 300 colonies. To ensure that some colony counts will be within this range, the original inoculum is diluted several times in a process called **serial dilution** (**Figure 6.16**).

Serial Dilutions Let's say, for example, that a milk sample has 10,000 bacteria per milliliter. If 1 ml of this sample were plated out, there would theoretically be 10,000 colonies formed in the Petri plate of medium. Obviously, this would not produce a countable plate. If 1 ml of this sample were transferred to a tube containing 9 ml of sterile water, each milliliter of fluid in this tube would now contain 1000 bacteria. If 1 ml of this sample were inoculated into a Petri plate, there would still be too many potential colonies to count on a plate. Therefore, another serial dilution could be made. One milliliter containing 1000 bacteria would be transferred to a second tube of 9 ml of water. Each milliliter of this tube would now contain only 100 bacteria, and if 1 ml of the contents of this tube were plated out, potentially 100 colonies would be formed—an easily countable number.

Pour Plates and Spread Plates A plate count is done by either the pour plate method or the spread plate method. The **pour plate method** follows the procedure shown in **Figure 6.17a**. Either 1.0 ml or 0.1 ml of dilutions of the bacterial suspension is introduced into a Petri dish. The nutrient medium, in which the agar is kept liquid by holding it in a water bath at about 50°C, is poured over the sample, which is then mixed into the medium by gentle agitation of the plate. When the agar solidifies, the plate is incubated. With the pour plate technique, colonies will grow within the nutrient agar (from cells suspended in the nutrient medium as the agar solidifies) as well as on the surface of the agar plate.

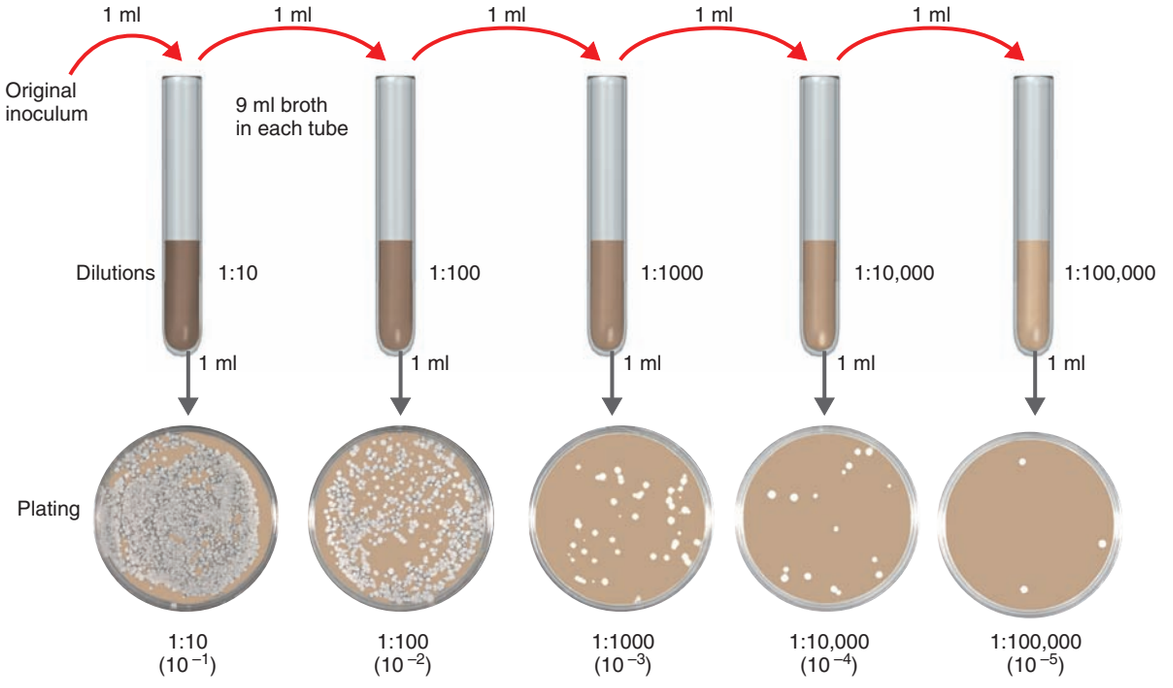


Figure 6.16 Serial dilutions and plate counts. In serial dilutions, the original inoculum is diluted in a series of dilution tubes. In our example, each succeeding dilution tube will have only one-tenth the number of microbial cells as the preceding tube. Then, samples of the dilution are used to inoculate Petri plates, on which colonies grow and can be counted. This count is then used to estimate the number of bacteria in the original sample.

Q Why were the dilutions of 1:10,000 and 1:100,000 not counted? Theoretically, how many colonies should appear on the 1:100 plate?

This technique has some drawbacks because some relatively heat-sensitive microorganisms may be damaged by the melted agar and will therefore be unable to form colonies. Also, when certain differential media are used, the distinctive appearance of the colony on the surface is essential for diagnostic purposes. Colonies that form beneath the surface of a pour plate are not satisfactory for such tests. To avoid these problems, the **spread plate method** is frequently used instead (Figure 6.17b). A 0.1-ml inoculum is added to the surface of a prepoured, solidified agar medium. The inoculum is then spread uniformly over the surface of the medium with a specially shaped, sterilized glass or metal rod. This method positions all the colonies on the surface and avoids contact between the cells and melted agar.

Filtration

When the quantity of bacteria is very small, as in lakes or relatively pure streams, bacteria can be counted by **filtration** methods (Figure 6.18). In this technique, at least 100 ml of water are passed through a thin membrane filter whose pores are too small to allow bacteria to pass. Thus, the bacteria are filtered out and retained on the surface of the filter. This filter is then transferred to a Petri dish containing a pad soaked in liquid nutrient medium, where colonies arise from the bacteria on the filter's surface. This method is applied frequently to detection and enumeration of coliform bacteria, which are indicators of fecal contamination of food or water (see Chapter 27). The colonies formed by these bacteria are distinctive when a differential nutrient medium is used. (The colonies shown in Figure 6.18b are examples of coliforms.)

The Most Probable Number (MPN) Method

Another method for determining the number of bacteria in a sample is the **most probable number (MPN) method,** illustrated in Figure 6.19. This statistical estimating technique is based on the fact that the greater the number of bacteria in a sample, the more dilution is needed to reduce the density to the point at which no bacteria are left to grow in the tubes in a dilution series. The MPN method is most useful when the

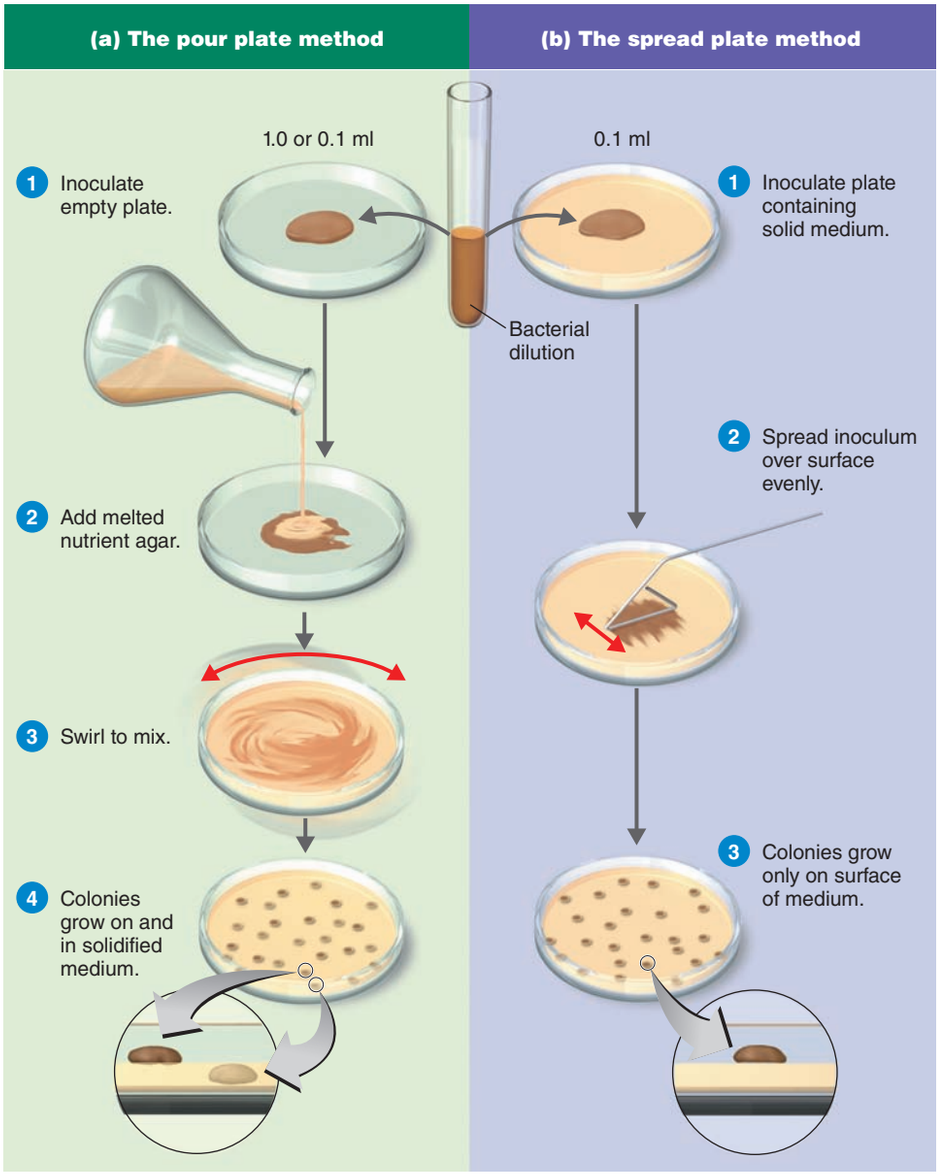


Figure 6.17 Methods of preparing plates for plate counts. (**a**) The pour plate method. (**b**) The spread plate method.

In what instances would the pour plate method be more appropriate than the spread plate method?

microbes being counted will not grow on solid media (such as the chemoautotrophic nitrifying bacteria). It is also useful when the growth of bacteria in a liquid differential medium is used to identify the microbes (such as coliform bacteria, which selectively ferment lactose to acid, in water testing). The MPN is only a statement that there is a 95% chance that the bacterial population falls within a certain range and that the MPN is statistically the most probable number.

Direct Microscopic Count

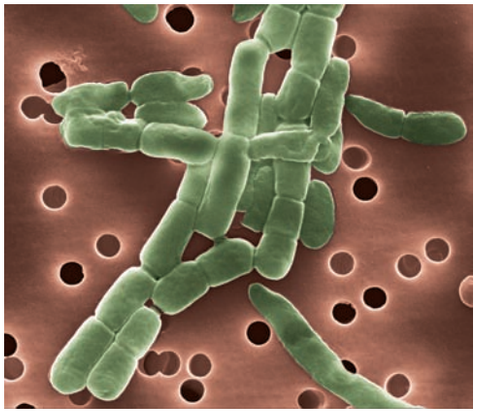
In the method known as the **direct microscopic count,** a measured volume of a bacterial suspension is placed within a defined area on a microscope slide. Because of time considerations, this method is often used to count the number of bacteria in milk. A 0.01-ml sample is spread over a marked square centimeter of slide, stain is added so that the bacteria can be seen, and the sample is viewed under the oil immersion objective lens. The area of the viewing field of this objective can be determined. Once the number of bacteria has been counted in several different fields, the average number of bacteria per viewing field can be calculated. From these data, the number of bacteria in the square centimeter over which the sample was spread can also be calculated. Because this area on the slide contained 0.01 ml of sample, the number of bacteria in each milliliter of the suspension is the number of bacteria in the sample times 100.

A specially designed slide called a *Petroff-Hausser cell counter* is also used in direct microscopic counts (Figure 6.20).

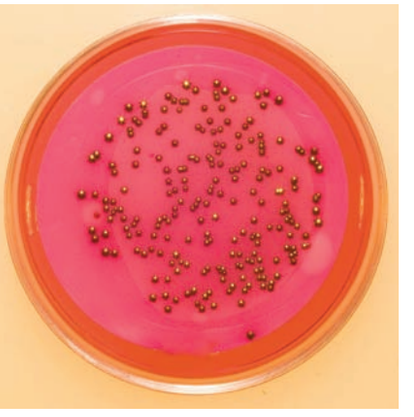
Motile bacteria are difficult to count by this method, and, as happens with other microscopic methods, dead cells are about

Figure 6.18 **Counting bacteria by filtration.**

Q Could you make a pour plate in the usual Petri dish with a 10-ml inoculum? Why or why not?



(a) The bacterial populations in bodies of water can be determined by passing a sample through a membrane filter. Here, the bacteria in a 100 ml water sample have been sieved out onto the surface of a membrane filter. These bacteria form visible colonies when placed on the surface of a suitable medium.



(b) A membrane filter with bacteria on its surface, as described in (a), has been placed on Endo agar. This medium is selective for gram-negative bacteria; lactose fermenters, such as the coliforms, form distinctive colonies. There are 214 colonies visible, so we would record 214 bacteria per 100 ml in the water sample.

Volume of Inoculum for Each Set of Five Tubes	Tubes of Nutrient Medium (Sets of Five Tubes)	Number of Positive Tubes in Set
10 ml		5
1 ml		3
0.1 ml		1

(a) Most probable number (MPN) dilution series. In this example, there are three sets of tubes and five tubes in each set. Each tube in the first set of five tubes receives 10 ml of the inoculum, such as a sample of water. Each tube in the second set of five tubes receives 1 ml of the sample, and the third set, 0.1 ml each. There were enough bacteria in the sample so that all five tubes in the first set showed bacterial growth and were recorded as positive. In the second set, which received only one-tenth as much inoculum, only three tubes were positive. In the third set, which received one-hundredth as much inoculum, only one tube was positive.

Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits	
		Lower	Upper
4-2-0	22	6.8	50
4-2-1	26	9.8	70
4-3-0	27	9.9	70
4-3-1	33	10	70
4-4-0	34	14	100
5-0-0	23	6.8	70
5-0-1	31	10	70
5-0-2	43	14	100
5-1-0	33	10	100
5-1-1	46	14	120
5-1-2	63	22	150
5-2-0	49	15	150
5-2-1	70	22	170
5-2-2	94	34	230
5-3-0	79	22	220
5-3-1	110	34	250
5-3-2	140	52	400

(b) MPN table. MPN tables enable us to calculate for a sample the microbial numbers that are statistically likely to lead to such a result. The number of positive tubes is recorded for each set: in the shaded example, 5, 3, and 1. If we look up this combination in an MPN table, we find that the MPN index per 100 ml is 110. Statistically, this means that 95% of the water samples that give this result contain 34–250 bacteria, with 110 being the most probable number.

Figure 6.19 **The most probable number (MPN) method.**

Q Under what circumstances is the MPN method used to determine the number of bacteria in a sample?

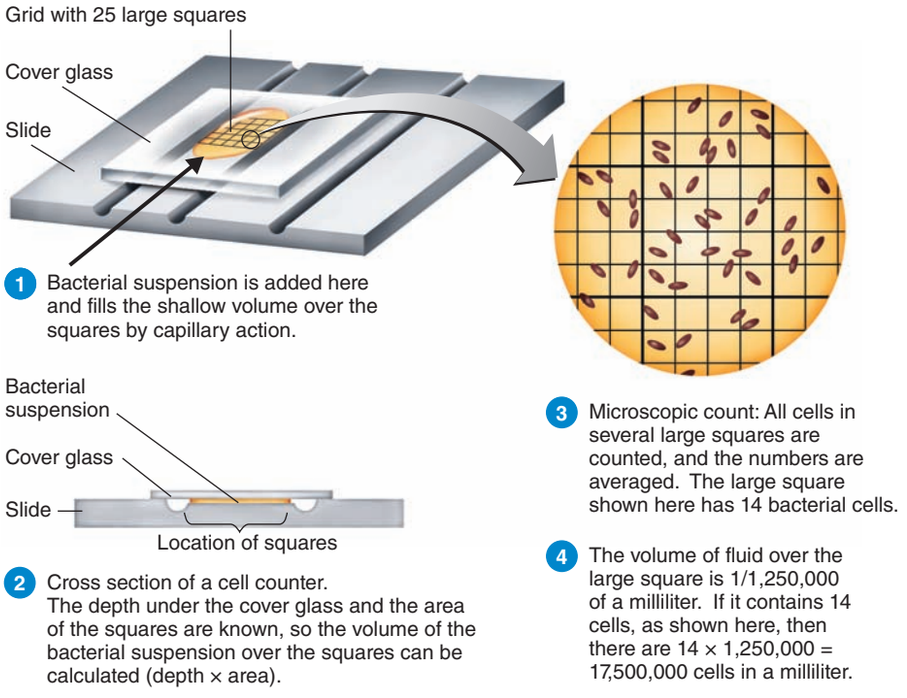


Figure 6.20 Direct microscopic count of bacteria with a Petroff-Hausser cell counter. The average number of cells within a large square multiplied by a factor of 1,250,000 gives the number of bacteria per milliliter.

Q This type of counting, despite its obvious disadvantages, is often used in estimating the bacterial population in dairy products. Why?

as likely to be counted as live ones. In addition to these disadvantages, a rather high concentration of cells is required to be countable—about 10 million bacteria per milliliter. The chief advantage of microscopic counts is that no incubation time is required, and they are usually reserved for applications in which time is the primary consideration. This advantage also holds for *electronic cell counters,* sometimes known as *Coulter counters,* which automatically count the number of cells in a measured volume of liquid. These instruments are used in some research laboratories and hospitals.

Clinical Case

The bacteria in the blood and catheter cultures fluoresce under ultraviolet light. The results from the culture show that *P. fluorescens* is present in the blood of 15 patients, in 17 catheters, and in the blood and catheters of four patients. The bacteria survived even after the heparin recall. Dr. MacGruder would like to have some idea how many bacteria are colonizing a patient's catheter. Because the amount of nutrients in a patient's catheter is minimal, he concludes that the bacteria grow slowly. He does some calculations based on the assumption that five *Pseudomonas* cells, with a generation time of 35 hours, may have been originally introduced into the catheters.

Approximately how many cells would there be after a month?

154 166 **175** 177

CHECK YOUR UNDERSTANDING

Why is it difficult to measure realistically the growth of a filamentous mold isolate by the plate count method? 6-16

Estimating Bacterial Numbers by Indirect Methods

It is not always necessary to count microbial cells to estimate their numbers. In science and industry, microbial numbers and activity are determined by some of the following indirect means as well.

Turbidity

For some types of experimental work, estimating **turbidity** is a practical way of monitoring bacterial growth. As bacteria multiply in a liquid medium, the medium becomes turbid, or cloudy with cells.

The instrument used to measure turbidity is a *spectrophotometer* (or colorimeter). In the spectrophotometer, a beam of light is transmitted through a bacterial suspension to a light-sensitive detector (Figure 6.21). As bacterial numbers increase, less light will reach the detector. This change of light will register on the instrument's scale as the *percentage of transmission.* Also printed on the instrument's scale is a logarithmic expression called the *absorbance* (sometimes called *optical density,* or *OD,* which is calculated as Abs = 2 − log of % transmittance). The absorbance is used to plot bacterial growth. When the bacteria are in logarithmic growth or decline, a graph of absorbance versus time will form an approximately straight line. If absorbance readings are matched with plate counts of the same culture, this correlation can be used in future estimations of bacterial numbers obtained by measuring turbidity.

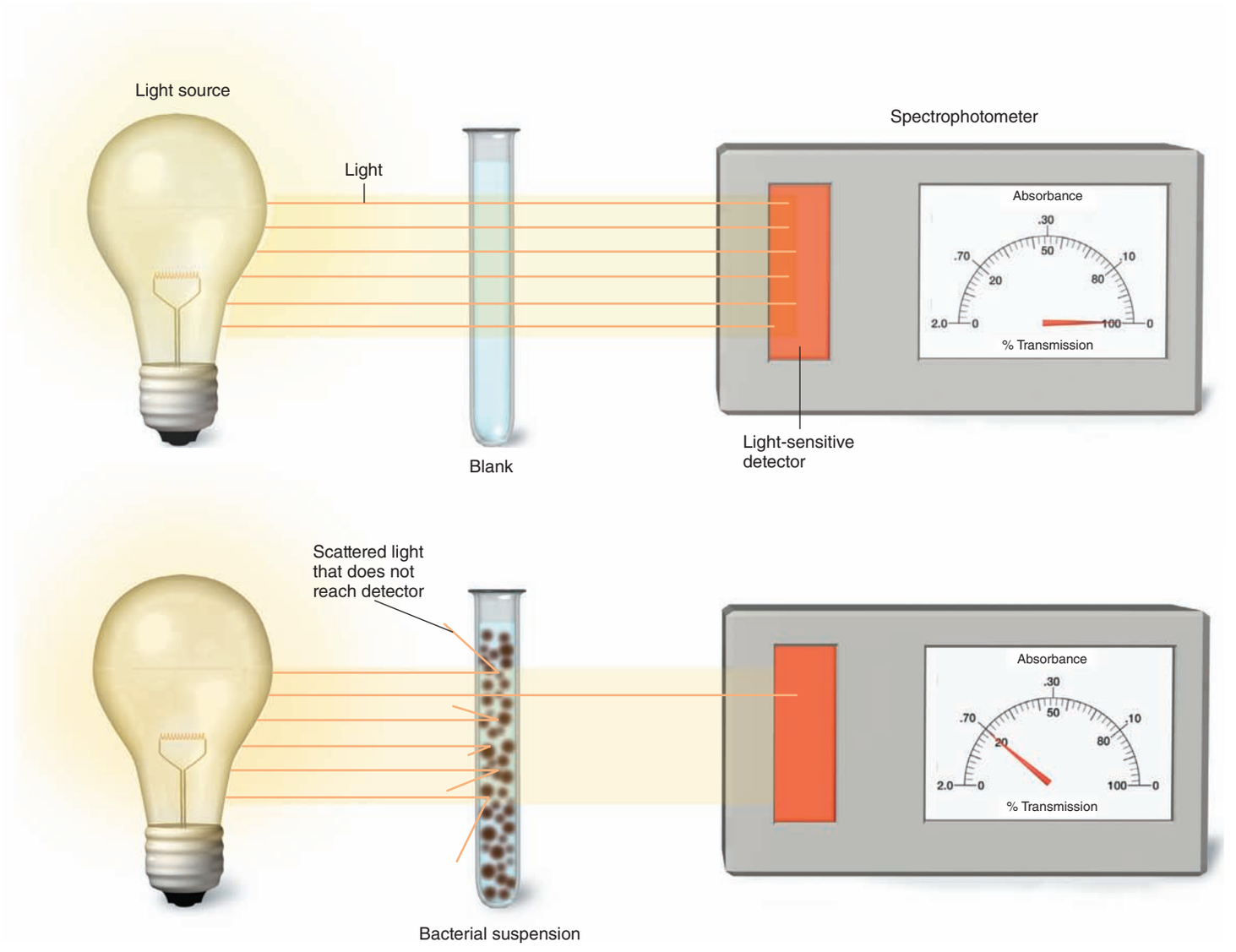


Figure 6.21 Turbidity estimation of bacterial numbers. The amount of light striking the light-sensitive detector on the spectrophotometer is inversely proportional to the number of bacteria under standardized conditions. The less light transmitted, the more bacteria in the sample. The turbidity of the sample could be reported as either 20% transmittance or 0.7 absorbance. Readings in absorbance are a logarithmic function and are sometimes useful in plotting data.

Q Why is turbidity more useful in measuring contamination of liquids by large numbers, rather than small numbers, of bacteria?

More than a million cells per milliliter must be present for the first traces of turbidity to be visible. About 10 million to 100 million cells per milliliter are needed to make a suspension turbid enough to be read on a spectrophotometer. Therefore, turbidity is not a useful measure of contamination of liquids by relatively small numbers of bacteria.

Metabolic Activity

Another indirect way to estimate bacterial numbers is to measure a population's *metabolic activity*. This method assumes that the amount of a certain metabolic product, such as acid or CO_2, is in direct proportion to the number of bacteria present. An example of a practical application of a metabolic test is the microbiological assay in which acid production is used to determine amounts of vitamins.

Dry Weight

For filamentous bacteria and molds, the usual measuring methods are less satisfactory. A plate count would not measure this increase in filamentous mass. In plate counts of actinomycetes (see Figure 11.20, page 320) and molds, it is mostly the number of asexual spores that is counted instead. This is not a good measure of growth. One of the better ways to measure

the growth of filamentous organisms is by *dry weight*. In this procedure, the fungus is removed from the growth medium, filtered to remove extraneous material, and dried in a desiccator. It is then weighed. For bacteria, the same basic procedure is followed.

CHECK YOUR UNDERSTANDING

- Direct methods usually require an incubation time for a colony. Why is this not always feasible for analyzing foods? **6-17**
- If there is no good method for analyzing a product for its vitamin content, what is a feasible method of determining the vitamin content? **6-18**

* * *

You now have a basic understanding of the requirements for, and measurements of, microbial growth. In Chapter 7, we will look at how this growth is controlled in laboratories, hospitals, industry, and our homes.

Clinical Case Resolved

Biofilms are dense accumulations of cells. Five cells might go through 20 generations in a month, producing 7.79×10^6 cells. Now Dr. MacGruder knows that the *P. fluorescens* bacteria are present in the patients' indwelling catheters. He orders the catheters to be replaced and has the CDC examine the used catheters with scanning electron microscopy. They discover that the *P. fluorescens* colonized the inside of the catheters by forming biofilms. In his report to the CDC, Dr. MacGruder explains that the *P. fluorescens* bacteria may have entered the bloodstreams of these patients at the same time as the first outbreak, but not in sufficient quantities to cause symptoms at that time. Biofilm formation enabled the bacteria to persist in the patients' catheters. He notes that previous electron microscopy studies indicate that nearly all indwelling vascular catheters become colonized by microorganisms that are embedded in a biofilm layer and that heparin has been reported to stimulate biofilm formation. Dr. MacGruder concludes that the bacteria in the biofilm were dislodged by subsequent uncontaminated intravenous solutions and released into the bloodstream, finally causing infections months after initial colonization.

154 166 175 177

Study Outline

MasteringMICROBIOLOGY™

Test your understanding with quizzes, microbe review, and a chapter post-test at www.masteringmicrobiology.com.

The Requirements for Growth (pp. 154–160)

1. The growth of a population is an increase in the number of cells.
2. The requirements for microbial growth are both physical and chemical.

Physical Requirements (pp. 154–158)

3. On the basis of preferred temperature ranges, microbes are classified as psychrophiles (cold-loving), mesophiles (moderate-temperature–loving), and thermophiles (heat-loving).
4. The minimum growth temperature is the lowest temperature at which a species will grow, the optimum growth temperature is the temperature at which it grows best, and the maximum growth temperature is the highest temperature at which growth is possible.
5. Most bacteria grow best at a pH value between 6.5 and 7.5.
6. In a hypertonic solution, most microbes undergo plasmolysis; halophiles can tolerate high salt concentrations.

Chemical Requirements (pp. 158–160)

7. All organisms require a carbon source; chemoheterotrophs use an organic molecule, and autotrophs typically use carbon dioxide.
8. Nitrogen is needed for protein and nucleic acid synthesis. Nitrogen can be obtained from the decomposition of proteins or from NH_4^+ or NO_3^-; a few bacteria are capable of nitrogen (N_2) fixation.
9. On the basis of oxygen requirements, organisms are classified as obligate aerobes, facultative anaerobes, obligate anaerobes, aerotolerant anaerobes, and microaerophiles.
10. Aerobes, facultative anaerobes, and aerotolerant anaerobes must have the enzymes superoxide dismutase ($2\ O_2^{\cdot-} + 2\ H^+ \longrightarrow O_2 + H_2O_2$) and either catalase ($2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$) or peroxidase ($H_2O_2 + 2\ H^+ \longrightarrow 2\ H_2O$).
11. Other chemicals required for microbial growth include sulfur, phosphorus, trace elements, and, for some microorganisms, organic growth factors.

Biofilms (pp. 160–161)

1. Microbes adhere to surfaces and accumulate as biofilms on solid surfaces in contact with water.
2. Biofilms form on teeth, contact lenses, and catheters.
3. Microbes in biofilms are more resistant to antibiotics than are free-swimming microbes.

Culture Media (pp. 161–166)

1. A culture medium is any material prepared for the growth of bacteria in a laboratory.
2. Microbes that grow and multiply in or on a culture medium are known as a culture.
3. Agar is a common solidifying agent for a culture medium.

Chemically Defined Media (p. 162)

4. A chemically defined medium is one in which the exact chemical composition is known.

Complex Media (pp. 162–163)

5. A complex medium is one in which the exact chemical composition varies slightly from batch to batch.

Anaerobic Growth Media and Methods (p. 163)

6. Reducing media chemically remove molecular oxygen (O_2) that might interfere with the growth of anaerobes.
7. Petri plates can be incubated in an anaerobic jar, anaerobic chamber, or OxyPlate.

Special Culture Techniques (pp. 163–165)

8. Some parasitic and fastidious bacteria must be cultured in living animals or in cell cultures.
9. CO_2 incubators or candle jars are used to grow bacteria that require an increased CO_2 concentration.
10. Procedures and equipment to minimize exposure to pathogenic microorganisms are designated as biosafety levels 1 through 4.

Selective and Differential Media (p. 165)

11. By inhibiting unwanted organisms with salts, dyes, or other chemicals, selective media allow growth of only the desired microbes.
12. Differential media are used to distinguish different organisms.

Enrichment Culture (pp. 165–166)

13. An enrichment culture is used to encourage the growth of a particular microorganism in a mixed culture.

Obtaining Pure Cultures (p. 167)

1. A colony is a visible mass of microbial cells that theoretically arose from one cell.
2. Pure cultures are usually obtained by the streak plate method.

Preserving Bacterial Cultures (pp. 167–168)

1. Microbes can be preserved for long periods of time by deep-freezing or lyophilization (freeze-drying).

The Growth of Bacterial Cultures (pp. 168–177)

Bacterial Division (p. 168)

1. The normal reproductive method of bacteria is binary fission, in which a single cell divides into two identical cells.
2. Some bacteria reproduce by budding, aerial spore formation, or fragmentation.

Generation Time (pp. 168–169)

3. The time required for a cell to divide or a population to double is known as the generation time.

Logarithmic Representation of Bacterial Populations (pp. 169–170)

4. Bacterial division occurs according to a logarithmic progression (two cells, four cells, eight cells, and so on).

Phases of Growth (pp. 170–171)

5. During the lag phase, there is little or no change in the number of cells, but metabolic activity is high.
6. During the log phase, the bacteria multiply at the fastest rate possible under the conditions provided.
7. During the stationary phase, there is an equilibrium between cell division and death.
8. During the death phase, the number of deaths exceeds the number of new cells formed.

Direct Measurement of Microbial Growth (pp. 171–175)

9. A heterotrophic plate count reflects the number of viable microbes and assumes that each bacterium grows into a single colony; plate counts are reported as number of colony-forming units (CFU).
10. A plate count may be done by either the pour plate method or the spread plate method.
11. In filtration, bacteria are retained on the surface of a membrane filter and then transferred to a culture medium to grow and subsequently be counted.
12. The most probable number (MPN) method can be used for microbes that will grow in a liquid medium; it is a statistical estimation.
13. In a direct microscopic count, the microbes in a measured volume of a bacterial suspension are counted with the use of a specially designed slide.

Estimating Bacterial Numbers by Indirect Methods (pp. 175–177)

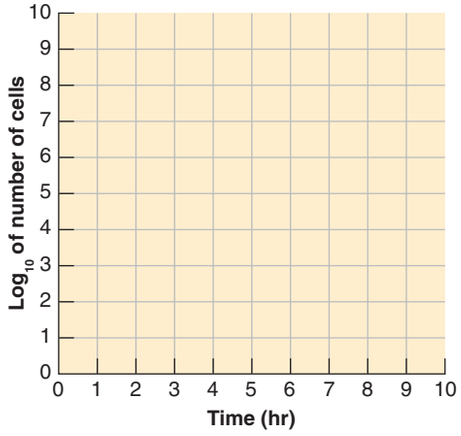
14. A spectrophotometer is used to determine turbidity by measuring the amount of light that passes through a suspension of cells.
15. An indirect way of estimating bacterial numbers is measuring the metabolic activity of the population (for example, acid production or oxygen consumption).
16. For filamentous organisms such as fungi, measuring dry weight is a convenient method of growth measurement.

Study Questions

Answers to the Review and Multiple Choice questions can be found by turning to the Answers tab at the back of the textbook.

Review

1. Describe binary fission.
2. Macronutrients (needed in relatively large amounts) are often listed as CHONPS. What does each of these letters indicate, and why are they needed by the cell?
3. Define and explain the importance of each of the following:
   - **a.** catalase
   - **b.** hydrogen peroxide
   - **c.** peroxidase
   - **d.** superoxide radical
   - **e.** superoxide dismutase
4. Seven methods of measuring microbial growth were explained in this chapter. Categorize each as either a direct or an indirect method.
5. By deep-freezing, bacteria can be stored without harm for extended periods. Why do refrigeration and freezing preserve foods?
6. A pastry chef accidentally inoculated a cream pie with six *S. aureus* cells. If *S. aureus* has a generation time of 60 minutes, how many cells would be in the cream pie after 7 hours?
7. Nitrogen and phosphorus added to beaches following an oil spill encourage the growth of natural oil-degrading bacteria. Explain why the bacteria do not grow if nitrogen and phosphorus are not added.
8. Differentiate complex and chemically defined media.
9. DRAW IT Draw the following growth curves for *E. coli,* starting with 100 cells with a generation time of 30 minutes at 35°C, 60 minutes at 20°C, and 3 hours at 5°C.
   - **a.** The cells are incubated for 5 hours at 35°C.
   - **b.** After 5 hours, the temperature is changed to 20°C for 2 hours.
   - **c.** After 5 hours at 35°C, the temperature is changed to 5°C for 2 hours followed by 35°C for 5 hours.



10. NAME IT A prokaryotic cell hitched a ride to Earth on a space shuttle from some unknown planet. The organism is a psychrophile, an obligate halophile, and an obligate aerobe. Based on the characteristics of the microbe, describe the planet.

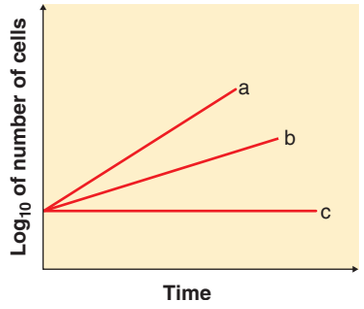
Multiple Choice

Use the following information to answer questions 1 and 2. Two culture media were inoculated with four different bacteria. After incubation, the following results were obtained:

Organism	Medium 1	Medium 2
Escherichia coli	Red colonies	No growth
Staphylococcus aureus	No growth	Growth
Staphylococcus epidermidis	No growth	Growth
Salmonella enterica	Colorless colonies	No growth

1. Medium 1 is
   - **a.** selective.
   - **b.** differential.
   - **c.** both selective and differential.
2. Medium 2 is
   - **a.** selective.
   - **b.** differential.
   - **c.** both selective and differential.

Use the following graph to answer questions 3 and 4.

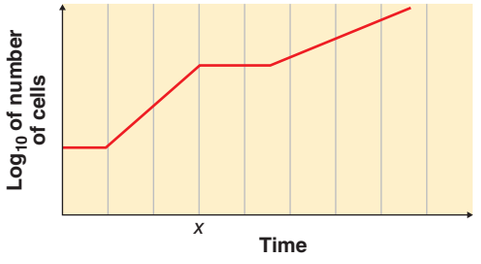


3. Which of the lines best depicts the log phase of a thermophile incubated at room temperature?
4. Which of the lines best depicts the log phase of *Listeria monocytogenes* growing in a human?
5. Assume you inoculated 100 facultatively anaerobic cells onto nutrient agar and incubated the plate aerobically. You then inoculated 100 cells of the same species onto nutrient agar and incubated the second plate anaerobically. After incubation for 24 hours, you should have
   - **a.** more colonies on the aerobic plate.
   - **b.** more colonies on the anaerobic plate.
   - **c.** the same number of colonies on both plates.
6. The term *trace elements* refers to
   - **a.** the elements CHONPS.
   - **b.** vitamins.
   - **c.** nitrogen, phosphorus, and sulfur.
   - **d.** small mineral requirements.
   - **e.** toxic substances.

7. Which one of the following temperatures would most likely kill a mesophile?
   a. −50°C
   b. 0°C
   c. 9°C
   d. 37°C
   e. 60°C
8. Which of the following is *not* a characteristic of biofilms?
   a. antibiotic resistance
   b. hydrogel
   c. iron deficiency
   d. quorum sensing
9. Which of the following types of media would *not* be used to culture aerobes?
   a. selective media
   b. reducing media
   c. enrichment media
   d. differential media
   e. complex media
10. An organism that has peroxidase and superoxide dismutase but lacks catalase is most likely an
    a. aerobe.
    b. aerotolerant anaerobe.
    c. obligate anaerobe.

Critical Thinking

1. *E. coli* was incubated with aeration in a nutrient medium containing two carbon sources, and the following growth curve was made from this culture.
   a. Explain what happened at the time marked *x*.
   b. Which substrate provided "better" growth conditions for the bacteria? How can you tell?



2. *Clostridium* and *Streptococcus* are both catalase-negative. *Streptococcus* grows by fermentation. Why is *Clostridium* killed by oxygen, whereas *Streptococcus* is not?
3. Most laboratory media contain a fermentable carbohydrate and peptone because the majority of bacteria require carbon, nitrogen, and energy sources in these forms. How are these three needs met by glucose–minimal salts medium? (*Hint:* See Table 6.2.)
4. Flask A contains yeast cells in glucose–minimal salts broth incubated at 30°C with aeration. Flask B contains yeast cells in glucose–minimal salts broth incubated at 30°C in an anaerobic jar. The yeasts are facultative anaerobes.
   a. Which culture produced more ATP?
   b. Which culture produced more alcohol?
   c. Which culture had the shorter generation time?
   d. Which culture had the greater cell mass?
   e. Which culture had the higher absorbance?

Clinical Applications

1. Assume that after washing your hands, you leave ten bacterial cells on a new bar of soap. You then decide to do a plate count of the soap after it was left in the soap dish for 24 hours. You dilute 1 g of the soap 1:10^6 and plate it on heterotrophic plate count agar. After 24 hours of incubation, there are 168 colonies. How many bacteria were on the soap? How did they get there?
2. Heat lamps are commonly used to maintain foods at about 50°C for as long as 12 hours in cafeteria serving lines. The following experiment was conducted to determine whether this practice poses a potential health hazard.

   Beef cubes were surface-inoculated with 500,000 bacterial cells and incubated at 43–53°C to establish temperature limits for bacterial growth. The following results were obtained from heterotrophic plate counts performed on beef cubes at 6 and 12 hours after inoculation:

		Bacteria per Gram of Beef After	
	Temp. (°C)	**6 hr**	**12 hr**
Staphylococcus aureus	43	140,000,000	740,000,000
	51	810,000	59,000
	53	650	300
Salmonella typhimurium	43	3,200,000	10,000,000
	51	950,000	83,000
	53	1,200	300
Clostridium perfringens	43	1,200,000	3,600,000
	51	120,000	3,800
	53	300	300

   Draw the growth curves for each organism. What holding temperature would you recommend? Assuming that cooking kills bacteria in foods, how could these bacteria contaminate the cooked foods? What disease does each organism cause? (*Hint:* See Chapter 25.)
3. The number of bacteria in saliva samples was determined by collecting the saliva, making serial dilutions, and inoculating nutrient agar by the pour plate method. The plates were incubated aerobically for 48 hours at 37°C.

	Bacteria per ml Saliva	
	Before Using Mouthwash	**After Using Mouthwash**
Mouthwash 1	13.1×10^6	10.9×10^6
Mouthwash 2	11.7×10^6	14.2×10^5
Mouthwash 3	9.3×10^5	7.7×10^5

   What can you conclude from these data? Did all the bacteria present in each saliva sample grow?