Microbial Growth and Its Control

5

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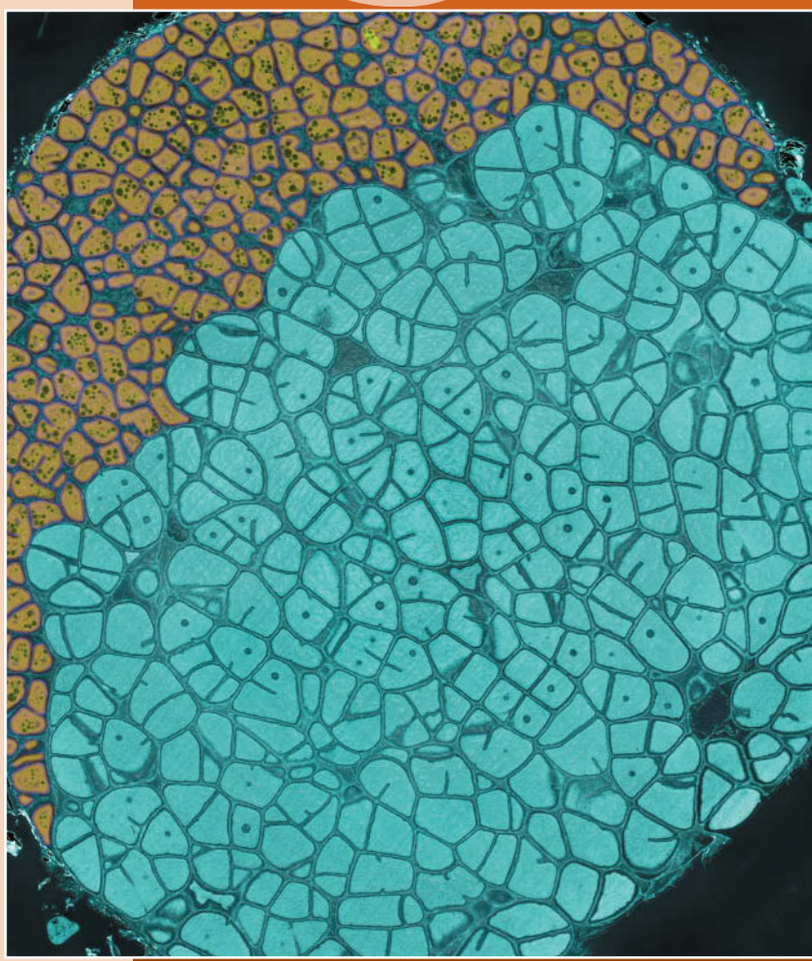
Picking Apart a Microbial Consortium

In nature, certain metabolic processes are carried out by microbes that team up to get the job done, a cozy arrangement called a consortium. Such is the case with the oxidation of methane (CH_4) linked to the reduction of sulfate (SO_4^{2-}) in anoxic marine sediments. The overall reaction ($CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$) is exergonic and the small amount of energy released is shared between two distinct microbes. The methane oxidizer in the consortium is a species of *Archaea* nicknamed ANME (for *an*aerobic *me*thanotroph, blue in photo), and its sulfate-reducing partner is a species of *Bacteria* (brown in photo). The consortium is thought to play a key role in the carbon cycle as a major methane sink, and thus a detailed picture of how it works is important to our understanding of the global carbon economy, climate change, and marine biogeochemistry.

Researchers have tried for years to separate the consortium into its components but always found that methane oxidation required both organisms. However, some researchers hypothesized that it might be possible to replace the sulfate reducer with an artificial electron acceptor and that this might unlock the consortium and allow the methanotroph to grow in pure culture. Using an electron acceptor called AQDS, the scientists discovered that they could turn off sulfate reduction in the consortium while maintaining CH_4 oxidation. During this process, the methanotroph used electrons from CH_4 to reduce AQDS rather than passing them on to its sulfate-reducing partner. Several other electron acceptors known to support anaerobic respiration also sustained methane oxidation, giving hope that ANME may eventually be obtained in pure culture.

The ability to grow a microbe in pure culture is the "gold standard" for the study of its physiology, biochemistry, regulation, and several other aspects of its biology. In the case of the ANME–sulfate reducer consortium, several physiologies were active at once, and resolving these many reactions proved to be a major scientific challenge. However, if further work shows that ANME can be removed from the consortium and grown in pure culture, detailed aspects of its biology can be studied that were not possible when the organism was tightly coupled to its partner in the consortium (photo).

Source: Scheller, S., H. Yu, G.L. Chadwick, S.E. McGlynn, and V.J. Orphan. 2016. Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. *Science 351:* 703–706.



I • Cell Division and Population Growth

In previous chapters we discussed cell structure and function (Chapter 2) and the principles of microbial nutrition and metabolism (Chapter 3). In Chapter 4 we learned the genetic processes that encode the structures and metabolic activities of cells. Here we begin a new unit whose focus is microbial growth and its regulation.

In Chapter 5 we lay the groundwork for the entire unit by presenting the basic principles of exponential growth, how the environment affects growth, and some principles of microbial growth control. Then, after we consider the important topic of microbial regulation in Chapter 6, we will revisit microbial growth in Chapter 7 and reexamine the process from a molecular and regulatory perspective. We finish the unit by delving into the world of viruses and their replication. Although not cells, viruses are critically important microbes whose replication shows parallels with the growth of microbial cells.

5.1 Binary Fission, Budding, and Biofilms

Growth is the result of cell division and is the ultimate process in the life of a microbial cell. In microbiology, growth is defined as *an increase in the number of cells*. Microbial cells have a finite life span, and a species is maintained only as a result of continued growth of its population. As macromolecules accumulate in the cytoplasm of a cell, they assemble into major cell structures, such as the cell wall, cytoplasmic membrane, flagella, ribosomes, enzyme complexes, and so on, eventually leading to the events of cell division itself. In a growing culture of a rod-shaped bacterium such as *Escherichia coli*, cells elongate to approximately twice their original length and then form a partition that constricts the cell into two daughter cells (Figure 5.1). This process is called **binary fission** ("binary" to indicate that two cells have arisen from one).

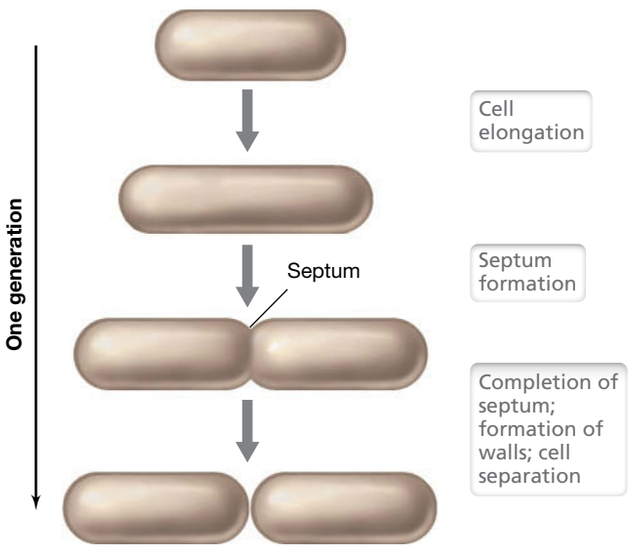


Figure 5.1 Binary fission in a rod-shaped bacterium. Cell numbers (and all components of the cells) double every generation.

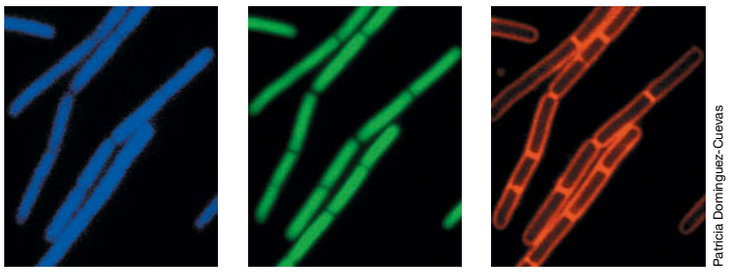


Figure 5.2 Septa. The septum that separates dividing cells of the bacterium *Bacillus subtilis* is clearly visible in this series of fluorescent micrographs. *(a)* DAPI stains the entire cell. *(b)* The green fluorescent protein lights up entire cells. *(c)* A dye that stains only the cytoplasmic membrane shows that septa contain membrane (and cell wall) material.

The partition that forms between dividing cells is called a *septum* and results from the inward growth of the cytoplasmic membrane and cell wall from opposing directions; septum formation continues until the two daughter cells are pinched off. There are some variations in this general pattern of binary fission. In some bacteria, such as *Bacillus subtilis*, a septum forms without cell wall constriction (Figure 5.2), while in the budding bacterium *Caulobacter* (see Figure 5.3) constriction occurs but no septum is formed.

Cell Generations and Generation Time

When one cell eventually separates to form two cells, we say that one *generation* has occurred, and the time required for this process is called the **generation time** (Figure 5.1 and see Figure 5.6). During one generation, all cellular constituents increase proportionally. Each daughter cell receives a copy of the chromosome(s) and sufficient copies of ribosomes and all other macromolecular complexes, monomers, and inorganic ions to begin life as an independent entity. Partitioning of the replicated DNA molecule between the two daughter cells depends on the DNA remaining attached to the cytoplasmic membrane during division, with constriction leading to separation of the chromosomes, one to each daughter cell (↪ Figure 7.1).

The generation time of a given bacterial species is variable and depends on nutritional and genetic factors, and on temperature. Under the best nutritional conditions, the generation time of a laboratory culture of *E. coli* is about 20 min. A few bacteria can grow even faster than this, but many bacteria grow much slower, with generation times of hours or days being more common. In nature, microbial cells probably grow much slower than their maximum rates observed in the laboratory. This is because the conditions and resources necessary for optimal growth in the laboratory are often not present in a natural habitat, and unlike growth in laboratory pure cultures, microbes in nature coexist with other microbes in microbial communities (↪ Figure 1.1) and must compete with their neighbors for resources and space.

Budding Cell Division

Although cell division in most bacteria occurs by binary fission, in a few bacteria other forms of growth and cell division occur. Budding bacteria are the primary examples here, and these are cells

that divide as a result of unequal cell growth. In contrast to binary fission that yields two equivalent cells (Figure 5.1), **budding division** forms a totally new daughter cell, with the mother cell retaining its original identity (Figure 5.3 and ↔ Section 7.4).

A fundamental difference between budding bacteria and bacteria that divide by binary fission is the formation of new cell wall material from a single point (polar growth) rather than throughout the whole cell (intercalary growth) as in binary fission. An important consequence of polar growth is that large cytoplasmic structures, such as internal membrane complexes, are not partitioned during the cell division process and must be formed de novo in the developing bud. However, this has an advantage in that more complex internal structures can be formed in budding cells than in cells that divide by binary fission, since the latter cells would have to partition these structures between the two daughter cells. Not coincidentally, many budding bacteria, particularly phototrophic and chemolithotrophic species, contain extensive internal membrane systems that house specific enzymes required to perform their particular metabolic specialties.

Some budding bacteria form cytoplasmic extensions such as stalks or hyphae, and classic examples are the genera *Caulobacter* and *Hyphomicrobium* (Figure 5.3). These organisms form cellular extrusions from which new cells bud off. Other budding bacteria such as the aquatic bacterium *Ancalomicrobium* produce multiple appendages that resemble arms extending away from the cell (↔ Figure 15.57*b*). The appendages increase the surface-to-volume ratio of the cell (↔ Section 2.2), which increases its ability to extract nutrients from oligotrophic (very dilute) habitats. Many budding bacteria also have distinctive life cycles, and we consider these and the group as a whole in Section 15.20.

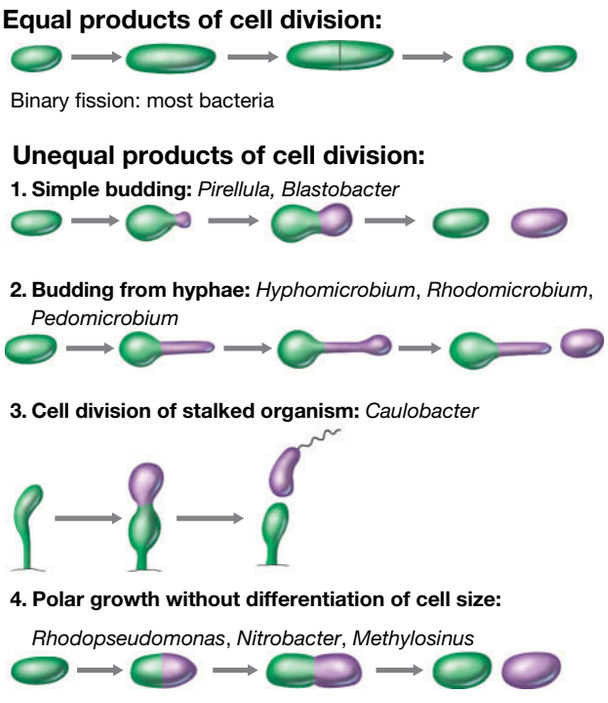
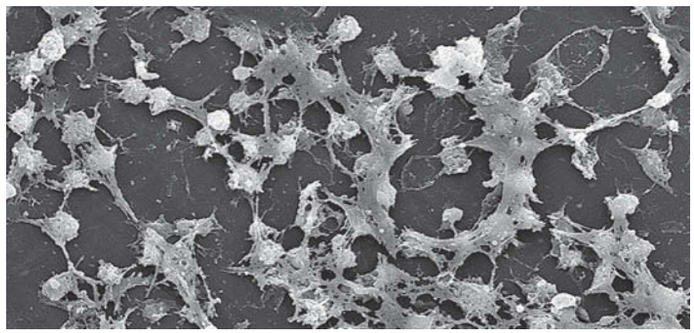
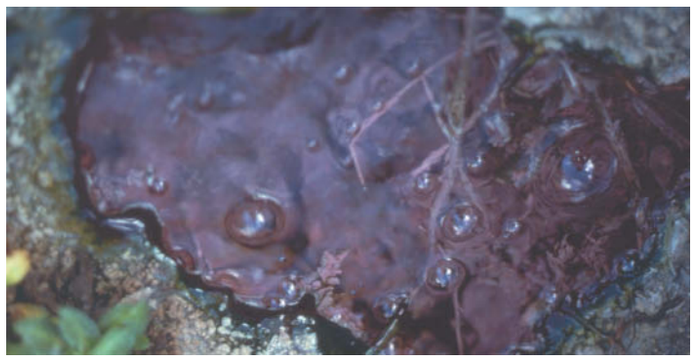


Figure 5.3 Cell division in different morphological forms of bacteria. The contrast is shown between cell division in conventional bacteria (cells that divide by binary fission) and in various budding and stalked bacteria.



(a)



(b)

Figure 5.4 Biofilms. *(a)* Scanning electron micrograph of a biofilm of cells of *Staphylococcus aureus* that formed on an indwelling catheter. *(b)* A microbial mat of the purple phototrophic bacterium *Thermochromatium tepidum* that developed in a small sulfidic hot spring in Yellowstone National Park.

Biofilms

Whether dividing by binary fission (Figure 5.1) or some form of budding (Figure 5.3), microbial cells can grow either in suspension or attached to surfaces. The suspended lifestyle, called *planktonic growth*, is the way many bacteria live in nature, for example, organisms that inhabit the water column of a lake. However, many other microorganisms show *sessile growth*, meaning that they grow attached to a surface. These attached cells can then develop into **biofilms** (Figure 5.4).

A biofilm is an attached polysaccharide matrix containing embedded bacterial cells (Figure 5.4*a*). Biofilms form in stages, beginning with the attachment of planktonic cells. This is followed by the production of a sticky matrix and further growth and development to form the tenacious and nearly impenetrable mature biofilm. Some biofilms form multilayered sheets with different organisms present in the individual layers. These biofilms are called *microbial mats*; mats composed of various phototrophic and chemotrophic bacteria are common in the outflows of hot springs (Figure 5.4*b*) and in marine intertidal regions. Biofilms are a common growth form for bacteria in nature because the intensely interwoven nature of the structure prevents harmful chemicals (for example, antibiotics or other toxic substances) from penetrating. Biofilms are also a barrier to bacterial grazing by protists and prevent cells from being washed away into a potentially less favorable habitat.

Bacterial biofilms affect many aspects of our lives, including human health. For example, biofilms have been implicated in difficult-to-treat infections of implanted medical devices, such as artificial heart valves and joints, and indwelling devices, such as catheters (Figure 5.4*a*). Moreover, symptoms of the disease cystic fibrosis are caused by a tenacious bacterial biofilm that fills the lungs and prevents gas exchange. Biofilms also cause fouling and plugging of water distribution systems and can form in fuel storage tanks, where they contaminate the fuel by producing souring agents such as H_2S.

We examine biofilms in more detail elsewhere in this book, including Sections 7.9, 20.4, and 20.5.

MINIQUIZ

- Define the term generation. What is meant by the term generation time?
- How do binary fission and budding cell division differ?
- How does the biofilm growth mode differ from that of planktonic cells? Which growth mode better protects the bacterial cells from harm?

5.2 Quantitative Aspects of Microbial Growth

During cell division, one cell becomes two. During the time that it takes for this to occur (the generation time), both total cell *number* and *mass* double (Figure 5.1). As we will see, cell numbers in a bacterial culture can quickly become very large, and so it is necessary to deal with the topic of microbial growth using quantitative methods.

Plotting Growth Data

A growth experiment beginning with a single cell having a generation time of 30 min is presented in Figure 5.5. This repetitive pattern, where the number of cells doubles in a constant time interval, is called **exponential growth**. When the cell number from such an experiment is graphed on arithmetic (linear) coordinates as a function of time, one obtains a curve with a continuously increasing slope (Figure 5.5*b*). By contrast, when the cell number is plotted on a logarithmic ($\log_{10}$) scale as a function of time (a *semilogarithmic* graph), as shown in Figure 5.5*b*, the points fall on a straight line. This straight-line function reflects the fact that the cells are growing exponentially and that the population is doubling in a constant time interval.

Semilogarithmic graphs are also convenient for estimating the generation time of a culture from actual growth data, since generation times may be inferred directly from the graph as shown in Figure 5.6. For example, when two points on the curve that represent one cell doubling on the Y axis are selected and vertical lines drawn from them to intersect the X axis, the time interval measured on the X axis is the generation time (Figure 5.6*b*).

The Mathematics of Bacterial Growth

The increase in cell number in an exponentially growing bacterial culture can be expressed mathematically as a geometric progression of the number 2. As one cell divides to become two cells, we express this as $2^0 \rightarrow 2^1$. As two cells become four, we express this as $2^1 \rightarrow 2^2$ and so on (Figure 5.5*a*). A fixed relationship exists between the initial number of cells in a culture and the number present after a period of exponential growth, and this relationship is expressed as

$$N = N_0 2^n$$

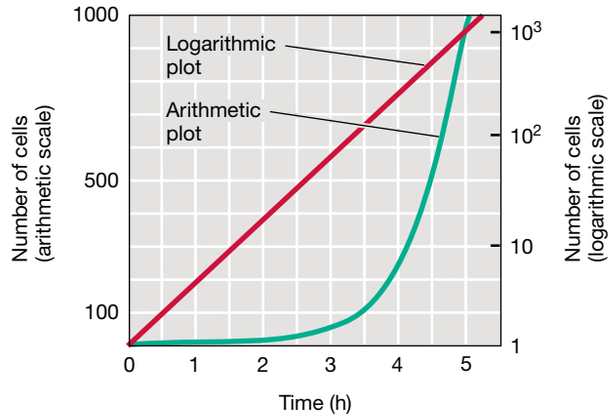
where N is the final cell number, N_0 is the initial cell number, and n is the number of generations (a single generation is shown in Figure 5.1) during the period of exponential growth. The generation time (g) of the exponentially growing population is t/n, where t is the duration of exponential growth expressed in days, hours, or minutes. From knowledge of the initial and final cell numbers in an exponentially growing cell population, it is possible to calculate n, and from n and knowledge of t, the generation time, g.

The equation $N = N_0 2^n$ can be expressed in terms of n by taking the logarithms of both sides and doing some simple algebra to yield the expression $n = [3.3(\log N - \log N_0)]$. Using this expression, it is possible to calculate generation times in terms of measurable quantities, N and N_0 (Sections 5.6–5.8 describe methods for quantifying cell numbers). As an example, consider actual growth data from the graph in Figure 5.6*b*, in which $N = 10^8$, $N_0 = 5 \times 10^7$, and $t = 2$:

$$n = 3.3[\log(10^8) - \log(5 \times 10^7)]$$
$$= 3.3\ (8 - 7.69) = 3.3(0.301) = 1$$

Time (h)	Total number of cells	Time (h)	Total number of cells
0	1	4	256 (2^8)
0.5	2	4.5	512 (2^9)
1	4	5	1,024 (2^{10})
1.5	8	5.5	2,048 (2^{11})
2	16	6	4,096 (2^{12})
2.5	32	.	.
3	64	.	.
3.5	128	10	1,048,576 (2^{20})

(a)



(b)

Figure 5.5 The rate of growth of a microbial culture. *(a)* Data for a population that doubles every 30 min. *(b)* Data plotted on arithmetic (left ordinate) and logarithmic (right ordinate) scales.

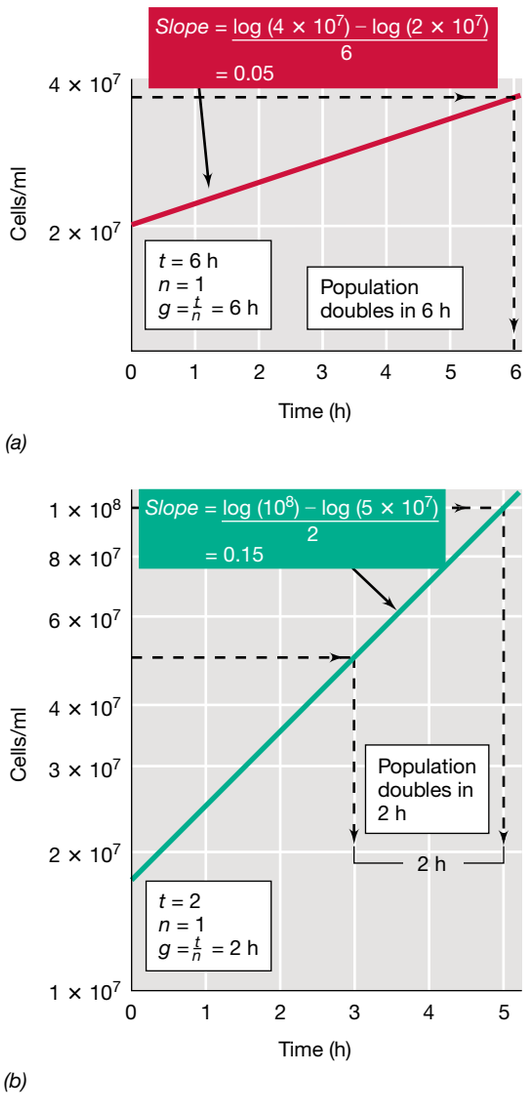


Figure 5.6 Calculating microbial growth parameters. Method of estimating the generation times (g) of exponentially growing populations with g of *(a)* 6 h and *(b)* 2 h from data plotted on semilogarithmic graphs. The slope of each line is equal to $0.301/g$, and n is the number of generations in the time t. All numbers are expressed in scientific notation; that is, 10,000,000 is 1×10^7, 60,000,000 is 6×10^7, and so on.

Thus, in this example, $g = t/n = 2/1 = 2$ h. If exponential growth continued for another 2 h, the cell number would be 2×10^8. Two hours later the cell number would be 4×10^8, and so on. Besides determining the generation time of an exponentially growing culture by inspection of graphical data (Figure 5.6*b*), g can be calculated directly from the slope of the straight-line function obtained in a semilogarithmic plot of exponential growth. The slope of this line is equal to $\log(\Delta N)/\Delta t$ and this equals $0.301\ n/t$ (or $0.301/g$). In the example of Figure 5.6*b*, the slope would thus be 0.301/2, or 0.15. Since g is equal to 0.301/slope, we arrive at the same value of 2 for g.

The Instantaneous Growth Rate Constant

Other expressions are often useful in describing exponential growth, and chief among these is the *instantaneous growth rate constant*, abbreviated k. The instantaneous growth rate constant expresses the rate at which the population is growing *at any instant* (by contrast, g is the mean time required for the cell population to double); k is expressed in units of reciprocal hours (h^{-1}).

The instantaneous rate of growth is a function of the number of cells at a given time (N) multiplied by k, or $dN/dt = kN$. By integration using natural logarithms ($\log_e$), this equation can be reexpressed as $N = N_0 e^{kt}$. Taking the $\log_{10}$ of both sides of this equation converts natural logs to $\log_{10}$ (so that N can be plotted against t on semilog paper, Figure 5.5*b* and Figure 5.6) and yields the expression $\log N = kt/2.303 + \log N_0$. The slope of this function ($k/2.303$) is also equal to $0.301/g$ (Figure 5.6), and thus k can be expressed in terms of g by the expression $k = 0.693/g$.

Armed with knowledge of n and t, one can calculate g and k for different microorganisms growing under various conditions. This is often useful for optimizing culture conditions for a newly isolated organism and also for testing the positive or negative effect of some treatment on a bacterial culture. For example, comparison with an unamended control allows factors that stimulate or inhibit growth to be identified by measuring their effect on the various growth parameters presented here.

Consequences of Exponential Growth

During exponential growth, the increase in cell number is initially rather slow but increases at an ever-faster rate. In the later stages of exponential growth, this results in an explosive increase in cell numbers. For example, in the experiment shown in Figure 5.5, the rate of cell production in the first 30 min of growth is 1 cell per 30 min. However, between 4 and 4.5 h of growth, the rate of cell production is 256 cells per 30 min, and between 5.5 and 6 h of growth it is 2048 cells per 30 min. Because of this, cell numbers in laboratory cultures of bacteria can quickly become very large, with final population sizes of $>10^9$ cells/ml not uncommon.

Besides being a theoretical consideration, exponential growth can have implications in everyday life. Consider something we have all experienced, the spoilage of milk. The lactic acid bacteria responsible for the soured flavor of spoiled milk contaminate the milk during its collection and exist in fresh, pasteurized milk in low numbers; these organisms grow slowly at refrigerator temperature (4°C) but much faster at room temperature. If a bottle of fresh milk is left to stand at room temperature overnight, some lactic acid is made, but not enough to affect milk quality. However, if week-old milk, which now contains a week's worth of slow bacterial growth (and thus much higher cell numbers), is left standing under the same conditions, a huge amount of lactic acid is made, and spoilage results.

MINIQUIZ

- What is a *semilogarithmic* plot and what information can we derive from it?
- For an exponentially growing culture that increases from 5×10^6 cells/ml to 5×10^8 cells/ml in 8 h, calculate g, n, and k for this culture.
- For testing a bacterium's response to a toxic substance, why would g be useful information?

5.3 The Microbial Growth Cycle

The data presented in Figures 5.5 and 5.6 are a reflection of exponentially growing cells. But exponential growth is only part of the *microbial growth cycle*. For several reasons, an organism growing in an enclosed vessel, such as a tube or a flask (a **batch culture**), cannot grow exponentially indefinitely. Instead, a typical *growth curve* for the population is obtained, as illustrated in Figure 5.7. The growth curve describes the entire growth cycle and is made up of four phases: lag, exponential, stationary, and death.

Lag and Exponential Phases

When a microbial culture is inoculated into fresh growth media (see Section 5.5), growth begins only after a period of time called the *lag phase*. This interval may be brief or extended depending on the history of the cells used as inocula and the composition of the growth medium and growth conditions (see Sections 5.9–5.14). If an exponentially growing culture is transferred into the same medium under the same conditions of growth, there will be essentially no lag and exponential growth will begin immediately. However, if the inoculum is taken from an old culture, there is usually a lag because the cells are depleted of various essential constituents and time is required for their biosynthesis.

A lag is also observed when a microbial culture is transferred from a nutrient-rich culture medium to one that is nutrient-poor. In order to grow, cells must have a complete complement of enzymes for synthesis of the essential metabolites not present in that medium. Hence, when transferred to a nutrient-poor medium, time is needed for the biosynthesis of new enzymes and for these to produce a sufficient pool of required metabolites before growth can actually begin. These events occur during the lag period.

When a growing cell population doubles at regular intervals (Section 5.2) the cells are said to be in the *exponential phase* of growth. Exponential phase cells are typically in their healthiest state and are thus most desirable for studies of their enzymes or other cell components. Rates of exponential growth vary greatly. Exponential growth rates are influenced by the growth conditions an organism is experiencing as well as genetic characteristics of the organism itself.

In general, prokaryotic cells grow faster than eukaryotes, and small eukaryotes tend to grow faster than large ones. But when all organisms are considered, doubling times for exponential growth vary enormously, from as little as a few minutes to days or weeks. Organisms living under very stressful conditions, such as those in Earth's nutrient-poor deep subsurface, may divide every few months (or even years) (Sections 20.7 and 20.13). Much plays into an organism's exponential growth rate and it is hard to predict how fast an organism can grow until it is brought into laboratory culture and its ideal growth conditions have been identified.

Stationary and Death Phases

In a batch culture, exponential growth cannot be maintained indefinitely. Consider the fact that a single cell of a bacterium weighing one-trillionth (10^{-12}) of a gram and growing exponentially with a 20-min generation time would produce, if allowed to grow exponentially in batch culture for 48 h, a population of cells that weighed 4000 times the weight of planet Earth! Obviously this is impossible, and growth becomes limited in such cultures because either an essential nutrient in the culture medium is depleted or the organism's waste products accumulate. When exponential growth ceases for one (or both) of these reasons, the population enters *stationary phase* (Figure 5.7).

In the stationary phase, there is no net increase or decrease in cell number and thus the growth rate of the population is zero. Despite growth arrest, energy metabolism and biosynthetic processes in stationary phase cells may continue, but typically at a greatly reduced rate. Some cells may even divide during stationary

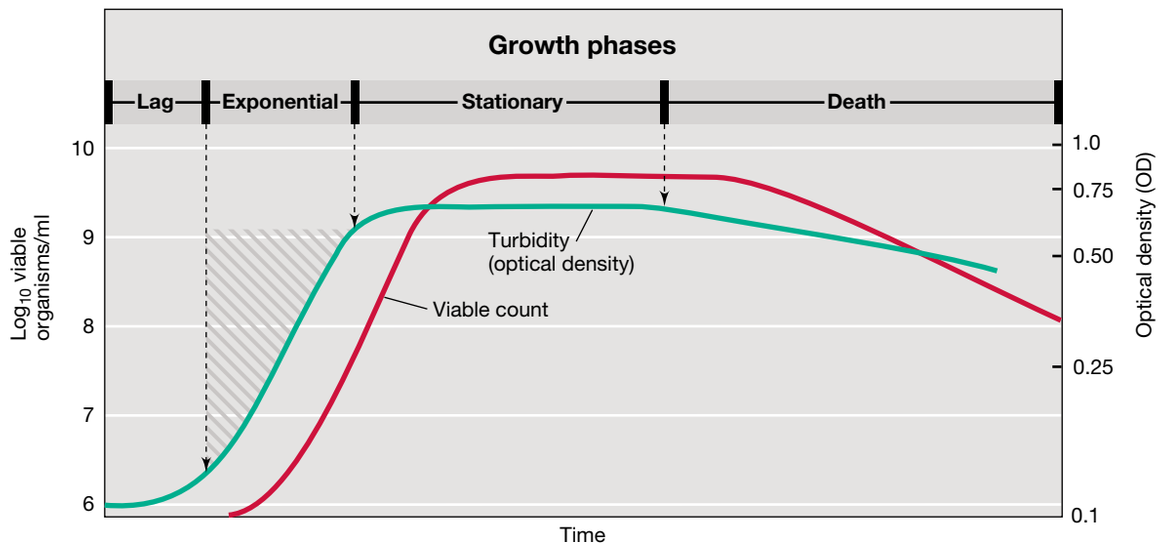


Figure 5.7 Typical growth curve for a bacterial population. A viable count measures the cells in the culture that are capable of reproducing. Optical density (turbidity), a quantitative measure of light scattering by a liquid culture (see Figure 5.16), increases with the increase in cell number.

phase, but no net increase in cell number occurs. This is because some cells in the population grow while others die, the two processes balancing each other out (cryptic growth). Eventually, the population will enter the *death phase* of the growth cycle, which, like the exponential phase, occurs as an exponential function (Figure 5.7). Typically, however, the rate of cell death is much slower than the rate of exponential growth and viable cells may remain in a culture for months or even years.

The phases of bacterial growth shown in Figure 5.7 are reflections of the events in a *population* of cells, not in individual cells. Thus, the terms lag phase, exponential phase, and so on have no meaning with respect to individual cells but only to cell populations. Growth of an individual cell (Section 5.1) is a prerequisite for population growth. But it is population growth that is most relevant to the ecology of microorganisms, because measurable microbial activities require microbial populations, not just an individual microbial cell.

MINIQUIZ

- In which phase of the growth curve do cells divide in a constant time period?
- Under what conditions would a lag phase not occur?
- Why do cells enter stationary phase?

5.4 Continuous Culture

Up to this point our consideration of microbial population growth has been confined to *batch cultures*. The environment in a batch culture is constantly changing because of nutrient consumption and waste production. These limitations can be circumvented in a *continuous culture device*. Unlike a batch culture, which is a *closed* system, a continuous culture is an *open* system. In the continuous culture growth vessel, a known volume of sterile medium is added at a constant rate while an equal volume of spent culture medium (which also contains cells) is removed at the same rate. Once in equilibrium, the culture volume, cell number, and nutrient/waste product status remain constant, and the culture attains *steady state*.

The Chemostat and the Concept of Steady State

The most common type of continuous culture is the **chemostat**, a device wherein both specific growth rate (how fast the cells grow) and cell density (how many cells per ml are obtained) can be controlled independently (Figure 5.8). In the chemostat, two factors govern the specific growth rate and cell density, respectively: (1) the *dilution rate* (D) which is expressed as F/V, where F is the flow rate (the rate at which fresh medium is pumped in and spent medium is removed), and V is the culture volume; and (2) the *concentration of a limiting nutrient*, such as a carbon or nitrogen source, present in the sterile medium entering the chemostat vessel.

When a chemostat filled with sterile medium is inoculated, the cells begin growing and increase in number more rapidly than they are removed in the overflow. As cell numbers increase, the level of the limiting nutrient in the culture decreases. This reduction in the growth-limiting nutrient serves as a feedback loop to reduce the specific growth rate, leading to a decrease in cell density as cells are removed in the overflow. However, once the limiting nutrient has decreased to a value just sufficient to support a specific growth rate that compensates for losses of cells through outflow, the chemostat reaches steady state, a condition where cell density and substrate concentration do not change over time.

In steady state, the specific growth rate of the culture is equal to D; that is, the rate of increase in cell numbers due to growth is equal to the rate of decrease in cell numbers due to dilution (outflow). The chemostat steady state is thus a dynamic condition in which cells are continuously growing and continuously being removed. Indeed, this dependency of specific growth rate on substrate concentration (Figure 5.9) drives the feedback loop that allows the chemostat to be self-regulating and the experimenter to choose the growth rate of the culture by simply changing the speed of the pump. In a batch culture, the nutrient concentration also affects both growth rate and cell yield, but when the nutrient level exceeds that which supports the maximal growth rate, only cell yield is increased by additional substrate (Figure 5.9).

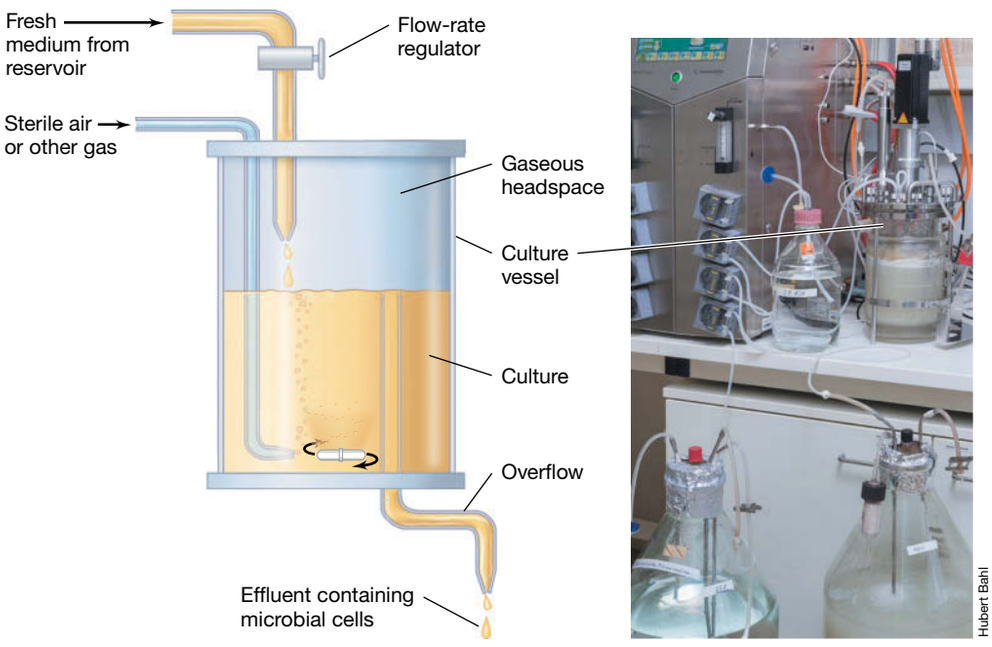


Figure 5.8 Continuous culture device (chemostat). The population density is controlled by the concentration of a limiting nutrient in the reservoir, and the growth rate is controlled by the dilution rate; both parameters are set by the experimenter. *(a)* Chemostat components. *(b)* Photo of a chemostat setup.

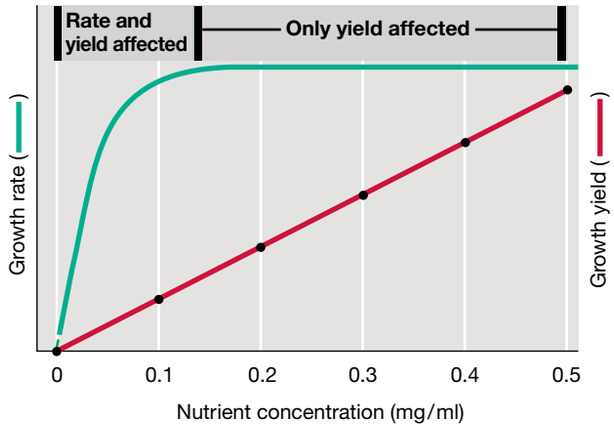


Figure 5.9 The effect of nutrients on growth. Relationship between nutrient concentration, growth rate (green curve), and growth yield (red curve). Only at low nutrient concentrations are both growth rate and growth yield affected.

The effects of varying *D* in a chemostat culture are shown schematically in **Figure 5.10**. As seen, there are rather wide limits over which *D* controls growth rate, although at both very low and very high *D*, the desired steady state with actively growing cells breaks down. In steady state, if the concentration of the limiting nutrient in the inflowing medium is increased at a constant *D*, cell density will increase but the growth rate will remain the same. Thus, by varying *D* or the concentration of the growth-limiting nutrient, one can establish dilute (for example, 10^5 cells/ml), moderate (for example, 10^7 cells/ml), or dense (for example, 10^9 cells/ml) cell populations growing at any specific growth rate.

Experimental Uses of the Chemostat

A practical advantage of the chemostat is that a cell population can be maintained in the exponential growth phase for long periods—weeks or even months. Exponential phase cells are usually most desirable for physiological experiments. Such cells are available at any time in the chemostat, and the vessel can be repeatedly sampled. Chemostat cultures have been used to study the growth and physiology of cells at submaximal growth rates, and from such studies, several tenets of microbial physiology have emerged; these include the fact that the ribosome content of cells increases in proportion to their specific growth rate and that nutrient concentration controls both specific growth rate and cell yield (Figure 5.9).

The chemostat has also been used in studies of microbial ecology and evolution. For example, because the chemostat can mimic the low substrate concentrations that are often found in nature, it is possible to ask which organisms in mixed cultures of known composition compete best at various specific growth rates or when particular nutrients are limiting. This can be done by monitoring changes in the diversity of the microbial community as a function of variations in *D* or the limiting nutrient. One can study the evolution of a pure culture in the chemostat by subjecting the culture to a growth or nutrient challenge and asking whether these conditions more rapidly select for particular spontaneous mutants displaying new physiological properties than in batch cultures where all nutrients are in excess.

Chemostats have also been used for the direct enrichment and isolation of bacteria from nature. From a natural sample, one can select a stable population under the chosen conditions of nutrient concentration and *D* and then slowly increase *D* until a single organism remains. In this way, microbiologists studying the growth rates of various soil bacteria isolated a bacterium with a 6-min doubling time—the fastest-growing bacterium known!

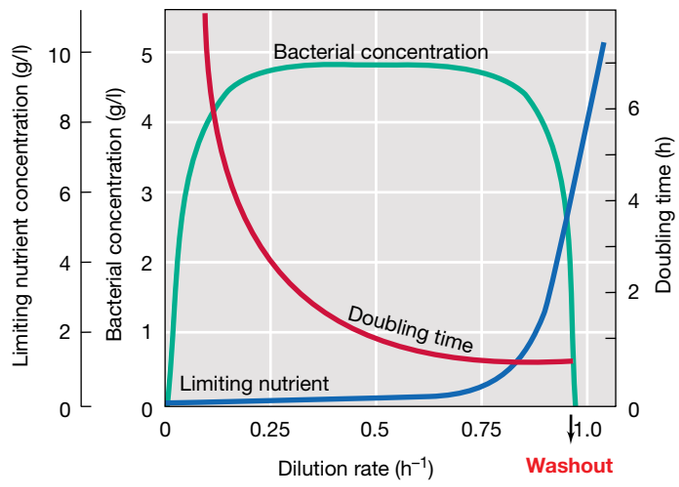


Figure 5.10 Steady-state relationships in the chemostat. The dilution rate (*D*) is determined from the flow rate and the volume of the culture vessel. Note that at high *D*, growth cannot balance dilution, and the population washes out. Note also that although both the population density remains constant and the concentration of the growth-limiting nutrient remains near zero during steady state, the specific growth rate (as reflected in the doubling time) can vary over a wide range.

MINIQUIZ

- How do microorganisms in a chemostat differ from microorganisms in a batch culture?
- What happens in a chemostat if the dilution rate exceeds the maximal specific growth rate of the organism?
- Do pure cultures have to be used in a chemostat?

II • Culturing Microbes and Measuring Their Growth

In the next few sections we consider how microbes are grown in laboratory culture and how microbial growth is measured. Culturing microbes and assessing their growth are common events in the daily routine of many microbiologists and microbiology laboratories.

5.5 Growth Media and Laboratory Culture

Laboratory cultures of microorganisms are grown in **culture media**, nutrient solutions tailored to the particular organism to be grown. Because laboratory culture is required for the detailed study of any microorganism, careful attention must be paid to the selection and preparation of media for laboratory culture to be

successful. Culture media must be sterilized before use, and sterilization is typically achieved by heating the medium under pressure in an *autoclave*. We discuss the operation and principles of the autoclave in Section 5.15, along with other methods for sterilizing culture media and laboratory devices.

Classes of Culture Media

Two broad classes of culture media are used in microbiology: *defined media* and *complex media*. **Defined media** are prepared by adding precise amounts of pure inorganic or organic chemicals to distilled water. Therefore, the *exact composition* of a defined medium (in both a qualitative and quantitative sense) is known. Of major importance in any culture medium is the carbon source because all cells need large amounts of carbon to make new cell material (Section 3.1). The particular carbon source and its concentration depend on the organism to be cultured. Table 5.1 lists recipes for four different culture media. Some defined media, such as the one listed for *Escherichia coli*, are considered "simple" because they contain only a single carbon source. In such a medium, *E. coli* must biosynthesize all organic molecules from glucose.

For culturing many microorganisms, knowledge of the exact composition of a medium is not essential. In these instances complex media may suffice and may even be advantageous. **Complex media** are made from digests of microbial, animal, or plant products, such as milk protein (casein), beef (beef extract), soybeans (tryptic soy broth), yeast cells (yeast extract), or any of a number of other highly nutritious substances (Table 5.1). Such digests are commercially available in dehydrated form and need only be hydrated with distilled water to form a culture medium. However, the disadvantage of a complex medium is that its exact nutritional composition is unknown. An *enriched medium*, used for the culture of nutritionally demanding (fastidious) microbes, many of which are pathogens, is a complex medium to which additional highly nutritious substances (such as serum or blood) are added.

Culture media are sometimes prepared that are selective or differential (or both), especially media used in diagnostic microbiology. A *selective medium* contains compounds that inhibit the growth of some microorganisms but not others. For example, selective media are available for the isolation of certain pathogens, such as *Salmonella* or those strains of *E. coli* that cause foodborne illnesses. A *differential medium* is one to which an indicator (typically a dye) is added, which reveals by a color change whether a particular metabolic reaction has occurred during growth. Differential media are useful for distinguishing bacteria and are widely used in clinical diagnostics and microbial taxonomy. Differential and selective media are further discussed in Chapter 28.

Nutritional Requirements and Biosynthetic Capacity

Of the four culture medium recipes in Table 5.1, three are defined and one is complex. The complex medium is easiest to prepare and

TABLE 5.1 Examples of culture media for microorganisms with simple and demanding nutritional requirements[a]

Defined culture medium for Escherichia coli	*Defined culture medium for* Leuconostoc mesenteroides	*Complex culture medium for either* E. coli *or* L. mesenteroides	*Defined culture medium for* Thiobacillus thioparus
K_2HPO_4 7 g	K_2HPO_4 0.6 g	Glucose 15 g	KH_2PO_4 0.5 g
KH_2PO_4 2 g	KH_2PO_4 0.6 g	Yeast extract 5 g	NH_4Cl 0.5 g
$(NH_4)_2SO_4$ 1 g	NH_4Cl 3 g	Peptone 5 g	$MgSO_4$ 0.1 g
$MgSO_4$ 0.1 g	$MgSO_4$ 0.1 g	KH_2PO_4 2 g	$CaCl_2$ 0.05 g
$CaCl_2$ 0.02 g	Glucose 25 g	Distilled water 1000 ml	KCl 0.5 g
Glucose 4–10 g	Sodium acetate 25 g	pH 7	$Na_2S_2O_3$ 2 g
Trace elements (Fe, Co, Mn, Zn, Cu, Ni, Mo) 2–10 µg each	Amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) 100–200 µg of each		Trace elements (as in first column) 2–10 µg each
Distilled water 1000 ml	Purines and pyrimidines (adenine, guanine, uracil, xanthine) 10 mg of each		Distilled water 1000 ml
pH 7	Vitamins (biotin, folate, nicotinic acid, pyridoxal, pyridoxamine, pyridoxine, riboflavin, thiamine, pantothenate, *p*-aminobenzoic acid) 0.01–1 mg of each		pH 7
	Trace elements (as in first column) 2–10 µg each		Carbon source: CO_2 from air
(a)	Distilled water 1000 ml	*(b)*	
	pH 7		

[a]The photos are tubes of *(a)* the defined medium described, and *(b)* the complex medium described. Note how the complex medium is colored from the various organic extracts and digests that it contains. Photo credits: Cheryl L. Broadie and John Vercillo, Southern Illinois University at Carbondale.

supports growth of both *Escherichia coli* and *Leuconostoc mesenteroides*, the examples used in Table 5.1. By contrast, the simple defined medium supports growth of *E. coli* but not of *L. mesenteroides*. Growth of the latter in a defined medium requires the addition of several nutrients not needed by *E. coli*. The nutritional needs of *L. mesenteroides* can be satisfied by preparing either a highly supplemented defined medium, a rather laborious undertaking because of all the individual nutrients that need to be added (Table 5.1), or by preparing a complex medium, a much less demanding operation.

The fourth medium listed in Table 5.1 supports growth of the bacterium *Thiobacillus thioparus* but would not support the growth of any of the other organisms. This is because *T. thioparus* is both a chemolithotroph and an autotroph (Section 3.3) and thus has no organic carbon requirements. *T. thioparus* derives all of its carbon from CO_2 and conserves energy from the oxidation of the sulfur compound thiosulfate ($Na_2S_2O_3$). Thus, *T. thioparus* has the greatest biosynthetic capacity of all the organisms listed in the table, surpassing even *E. coli* in this regard.

The take-home lesson from Table 5.1 is that different microorganisms may have vastly different nutritional requirements. For successful cultivation, it is necessary to understand an organism's physiology and nutritional requirements and then supply it with the nutrients it needs in both the proper form and amount.

Laboratory Culture

Once a sterile culture medium has been prepared, microbes can be inoculated and the culture can be incubated under conditions that will support growth. In a laboratory situation, inoculation will typically be with a pure culture and into a culture medium that is either liquid (Table 5.1) or solid (**Figure 5.11**). Liquid culture media are solidified with *agar*, an algal polysaccharide. Solid media immobilize cells, allowing them to grow and form visible, isolated masses called *colonies* (Figure 5.11). Microbial colonies are of various shapes and sizes depending on the organism, the culture conditions, the nutrient supply, and other physiological parameters. Some microorganisms produce pigments that cause the colony to be colored (Figure 5.11). Colonies permit the microbiologist to visualize the composition and presumptive purity of a culture. Plates inoculated from a mixed culture (such as a natural sample, Figure 5.11*e*) or from a contaminated pure culture will usually contain more than one colony type.

Once a sterile culture medium has been prepared, it is ready for inoculation. This requires **aseptic technique** (**Figure 5.12**), a series of steps to prevent contamination during manipulations of cultures and sterile culture media, both liquid and solid. With liquid medium, the goal is to transfer a culture while protecting the tube or bottle rim from air currents or contact with nonsterile surfaces (Figure 5.12*a*). With agar Petri plates, the plan is basically the same but with a greater emphasis on keeping the surface of

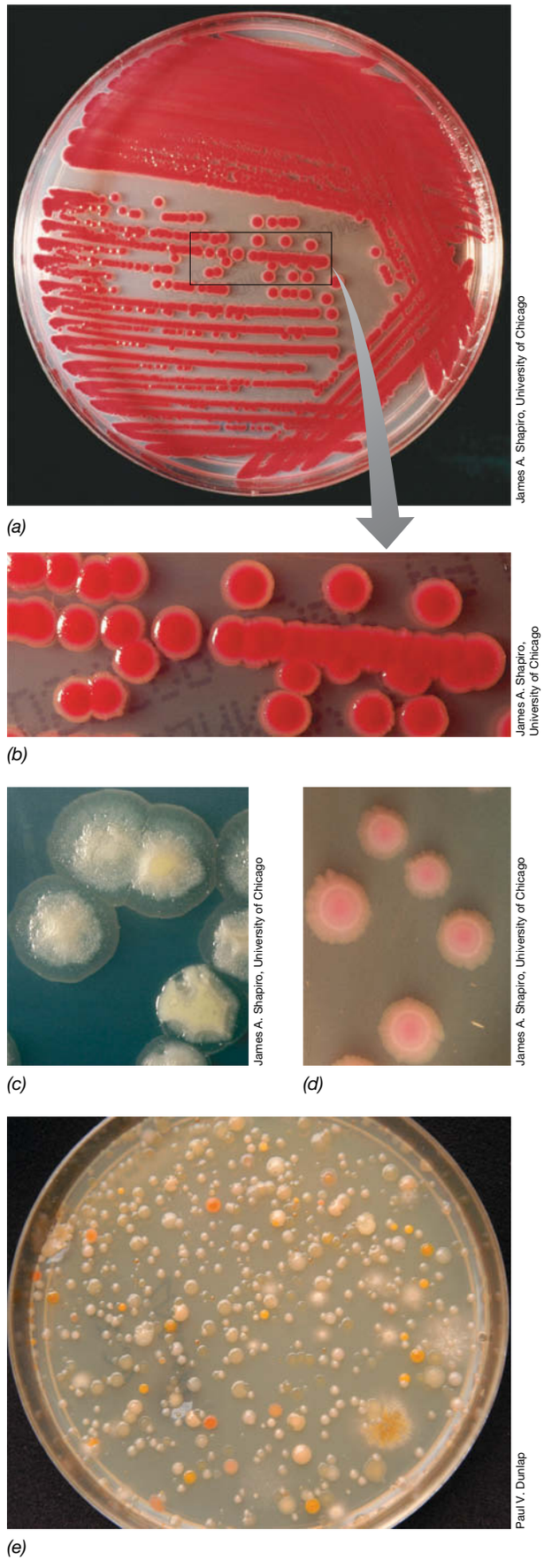
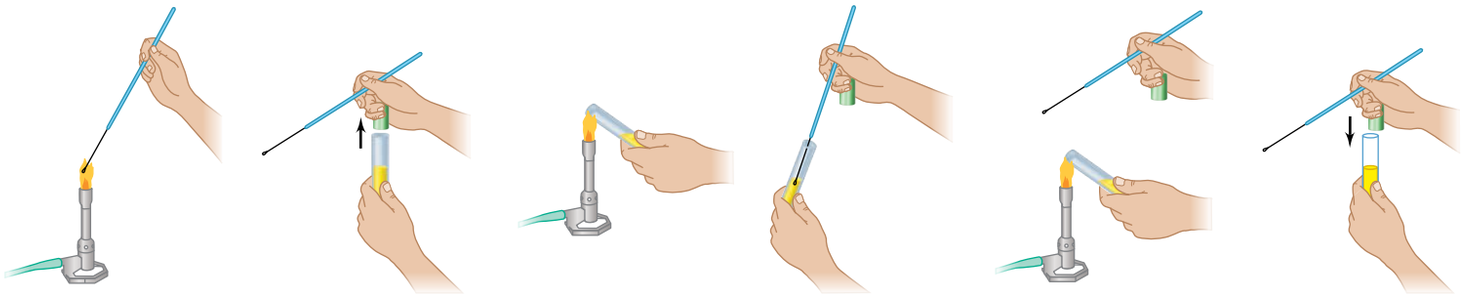
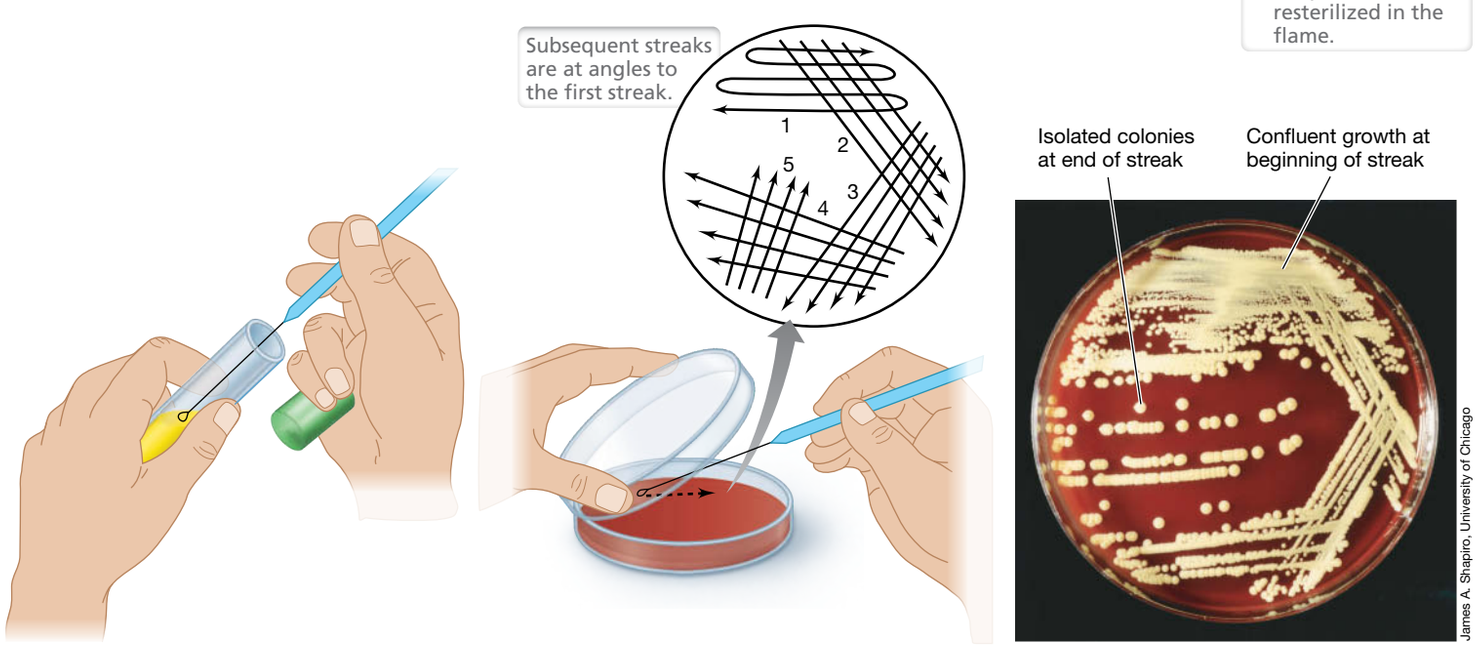


Figure 5.11 Bacterial colonies. Colonies are visible masses of cells formed from the division of one or a few cells and can contain over a billion (10^9) individual cells. *(a) Serratia marcescens*, grown on MacConkey agar. *(b)* Close-up of colonies outlined in part *a*. *(c) Pseudomonas aeruginosa*, grown on trypticase soy agar. *(d) Shigella flexneri*, grown on MacConkey agar. *(e)* An agar plate containing many different bacterial colonies that developed from plating a dilution of seawater.



1. Flaming the loop sterilizes it.
2. Tube cap is removed.
3. Flaming the tube tip sterilizes the surface.
4. Only sterilized portion of loop enters tube.
5. The tube is reflamed.
6. Tube is recapped and then steps 2–6 repeated with tube of fresh medium. Loop is then resterilized in the flame.

(a) **Transfering a liquid culture**



1. Loop is sterilized and a loopful of inoculum is removed from tube.
2. Initial streak is worked in well in one corner of the agar plate.
3. Appearance of well-streaked plate after incubation shows colonies of the bacterium *Micrococcus luteus* on a blood agar plate.

(b) **Streaking a Petri plate**

Figure 5.12 Aseptic transfer. *(a)* Liquid media: After the tube is recapped at the end, the loop is resterilized. *(b)* Solid media: making a streak plate to obtain pure cultures. The plate cover is opened just enough to permit streaking manipulations. In streaking a plate, the microbial cells are separated by the streaking process to yield widely separated single cells that then grow and divide to form colonies.

the agar protected from aerosols or particulate matter that could drop in (Figure 5.12*b*).

A mastery of aseptic technique is required for maintaining pure cultures, as airborne contaminants are virtually everywhere, even in what may appear to be a very clean microbiology laboratory. Picking an isolated colony and restreaking it is the main method for obtaining pure cultures from mixed liquid or plate (Figure 5.11*e*) cultures and is a common procedure in the microbiology laboratory. Other techniques for obtaining pure cultures have been developed that are especially suited for particular groups of bacteria with unusual growth requirements and these will be discussed in Sections 19.2 and 19.3.

MINIQUIZ

- Why would a complex culture medium for *Leuconostoc mesenteroides* be easier to prepare than a chemically defined medium?
- In which medium shown in Table 5.1, defined or complex, do you think *Escherichia coli* would grow the fastest? Why? *E. coli* will not grow in the medium described for *Thiobacillus thioparus*. Why?
- What is meant by the word sterile? Why is aseptic technique necessary for successful maintenance of pure cultures in the laboratory?
- How many cells could be present in a single bacterial colony?

5.6 Microscopic Counts of Microbial Cell Numbers

Assessing cell numbers gives quantitative information on the state of a microbial culture or community. Several methods for enumerating a microbial population have been developed, each having their own strengths and caveats. We begin with the classic "total count" carried out by microscopic examination of a culture or natural sample.

Total Cell Count

Total counts of microbial numbers in a culture or natural sample can be done by simply observing and enumerating the cells present by a *microscopic cell count*. Microscopic counts can be performed either on samples dried on slides or on liquid samples. Dried samples can be stained to increase contrast between cells and their background (↔ Sections 1.6 and 19.3). With liquid samples, counting chambers consisting of a grid with squares of known area etched on the surface of a glass slide are used (**Figure 5.13**). When the coverslip is placed on the chamber, each square on the grid has a precise volume. The number of cells per unit area of grid can be counted under the microscope, giving a measure of the number of cells per small chamber volume. The number of cells per milliliter of suspension is calculated by employing a conversion factor based on the volume of the chamber sample (Figure 5.13).

Microscopic counting is a quick and easy way of estimating microbial cell numbers. However, it has several limitations that restrict its usefulness. For example, without special staining techniques (↔ Section 19.3), dead cells cannot be distinguished from live cells, and precision is difficult to achieve, even when replicate counts are made. Moreover, small cells are often difficult to see under the microscope, which can lead to erroneous counts, and cell suspensions of low density (less than about 10^6 cells/milliliter) will have few if any cells in a microscope field unless the sample is first concentrated and resuspended in a small volume. Finally, motile cells must be killed (usually with formaldehyde) or otherwise immobilized before counting, and debris in the sample may easily be mistaken for microbial cells.

Microscopic Cell Counts in Microbial Ecology

Despite its many potential caveats, microbial ecologists often use microscopic cell counts on natural samples. But they do so using stains to visualize the cells, often stains that yield phylogenetic or other key information about the cells, such as their metabolic properties.

There are many stains that can be used in a general way. For example, the stain DAPI (↔ Section 1.6 and Figure 1.20*e*) stains all cells because it reacts with DNA. Other general fluorescent stains can differentiate live from dead cells by detecting whether the cytoplasmic membrane is intact or not. By contrast, fluorescent stains that are highly specific for certain organisms or groups of related organisms can be prepared by attaching fluorescent dyes to specific nucleic acid probes. For example, *phylogenetic stains* that stain only species of *Bacteria* or only species of *Archaea* can be used in combination with nonspecific stains to determine the proportion of each domain present in a sample (↔ Section 19.4). Other fluorescent probes have been developed that target genes encoding enzymes that catalyze specific metabolic processes; if a cell is stained by one of these probes, a metabolism can be inferred that may reveal the cell's ecological role in the microbial community. In all of these cases, if cells in the sample are present in only low numbers, for example in a sample of ocean water, this limitation can be overcome by first concentrating the cells on a filter and then counting them after staining.

Because they are easy to do and often yield useful baseline information, microscopic cell counts are common in ecological studies of natural microbial environments. We pursue this theme in more detail in Chapter 19.

MINIQUIZ

- What are some of the problems that can arise when unstained preparations are enumerated in microscopic counts?
- Using microscopic techniques, how could you tell whether *Archaea* were present in an alpine lake where total cell numbers were only 10^5/ml?

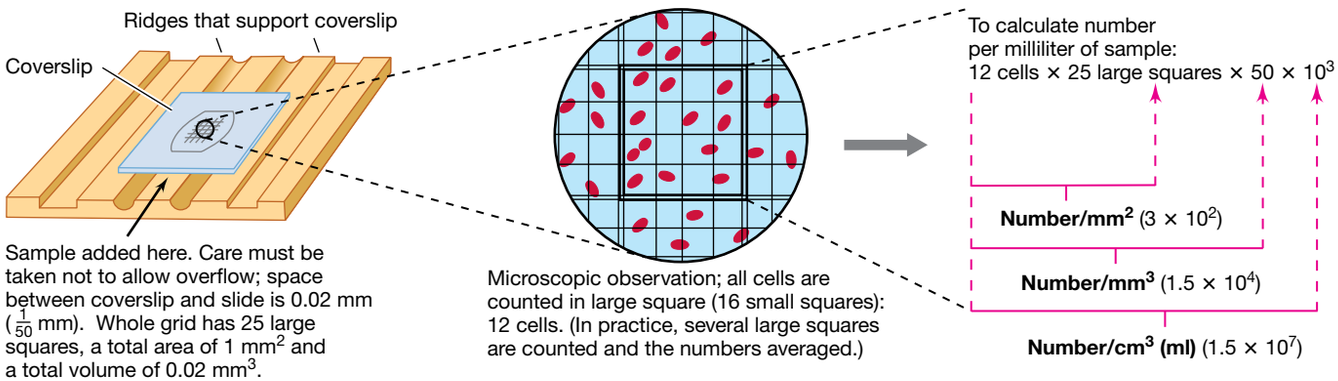


Figure 5.13 Direct microscopic counting procedure using the Petroff–Hausser counting chamber.
A phase-contrast microscope is typically used to count the cells to avoid the necessity for staining.

5.7 Viable Counting of Microbial Cell Numbers

A **viable** cell is one that is able to divide and form offspring, and in most cell-counting situations, these are the cells we are most interested in. For these purposes, one would use a **viable count**, also called a **plate count** because agar plates are required. The assumption made in a viable count is that each viable cell will grow and divide to yield one colony, and hence, colony numbers are a reflection of cell numbers (Section 5.5 and Figure 5.11).

Methods for Viable Counts

There are at least two ways of performing a plate count: the *spread-plate* method and the *pour-plate* method (Figure 5.14). In the spread-plate method, a volume (usually 0.1 ml or less) of an appropriately diluted culture is spread over the surface of an agar plate using a sterile glass spreader. In the pour-plate method, a known volume (usually 0.1–1.0 ml) of culture is pipetted into a sterile Petri plate. Molten agar medium, tempered to just above gelling temperature (~50°C), is then added and gently mixed before allowing the agar to solidify.

With both the spread-plate and pour-plate methods, it is important that the number of colonies developing on or in the medium not be too many or too few. On crowded plates some cells may not form colonies, and some colonies may fuse, leading to erroneous measurements. If the number of colonies is too small, the statistical significance of the calculated count will be low. The usual practice, which is most valid statistically, is to count colonies only on plates that contain between 30 and 300 colonies.

To obtain a countable colony number, the sample must almost always be diluted. Because one may not know the approximate viable count ahead of time, it is usually necessary to make more than one dilution. Several 10-fold dilutions of the sample are commonly used (Figure 5.15). To make a 10-fold (10^{-1}) dilution, one can mix 0.5 ml of sample with 4.5 ml of diluent, or 1.0 ml of sample with 9.0 ml of diluent. If a 100-fold (10^{-2}) dilution is needed, 0.05 ml can be mixed with 4.95 ml of diluent, or 0.1 ml with 9.9 ml of diluent. Alternatively, a 10^{-2} dilution can be achieved by making two successive 10-fold dilutions. With dense cultures, such *serial* dilutions are needed to reach a suitable dilution for plating to yield countable colonies. Thus, if a 10^{-6} ($1/10^6$) dilution is needed, it can be achieved by making three successive 10^{-2} ($1/10^2$) dilutions or six successive 10^{-1} dilutions (Figure 5.15).

Sources of Error in Plate Counting

The number of colonies obtained in a viable count depends not only on the inoculum size and the viability of the culture, but also on the culture medium and the incubation conditions. The colony number can also change with the length of incubation. For example, if a mixed culture is counted, the cells deposited on the plate will not all form colonies at the same rate; if a short incubation time is used, fewer than the maximum number of colonies will be obtained. Furthermore, the size of colonies may vary. If some tiny colonies develop, they may be missed during the counting. With pure cultures, colony development is a more synchronous process and uniform colony morphology is the norm.

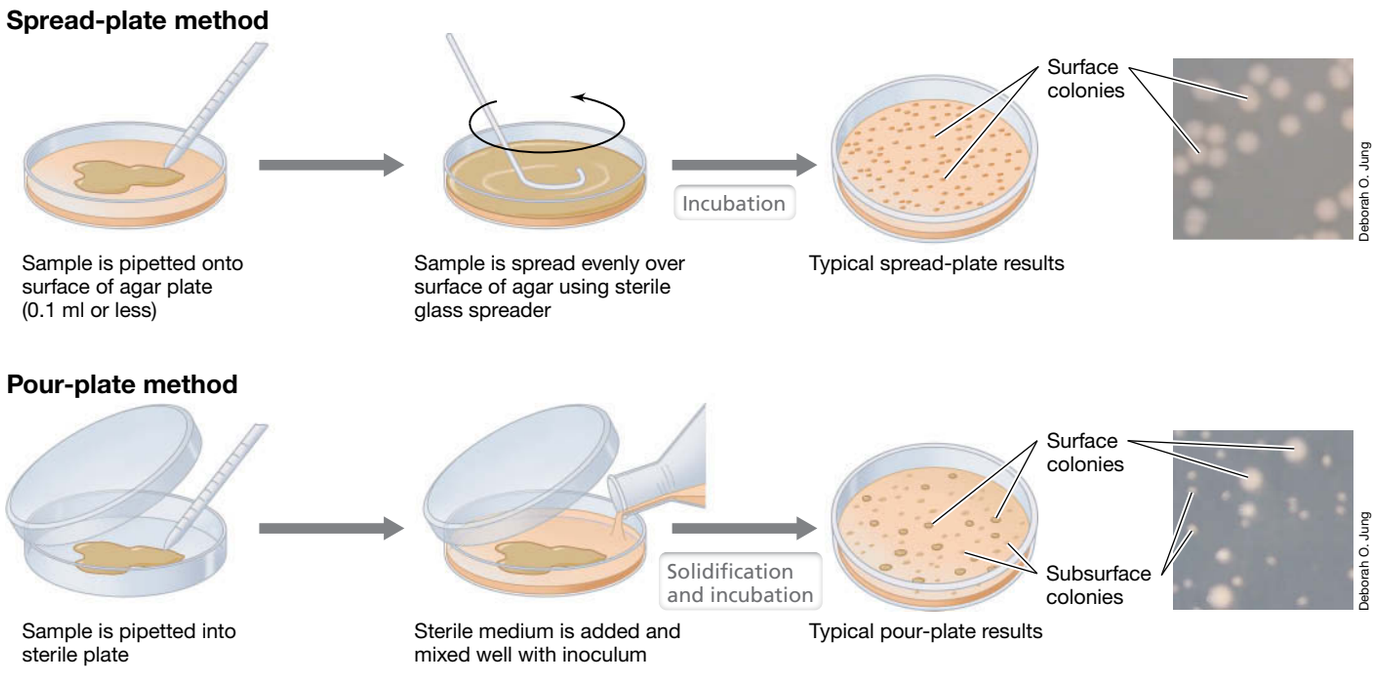


Figure 5.14 Two methods for the viable count. Only surface colonies form in the spread plate technique. By contrast, in the pour-plate method, colonies form within the agar as well as on the agar surface. On the far right are photos of colonies of *Escherichia coli* formed from cells plated by the spread-plate method (top) or the pour-plate method (bottom).

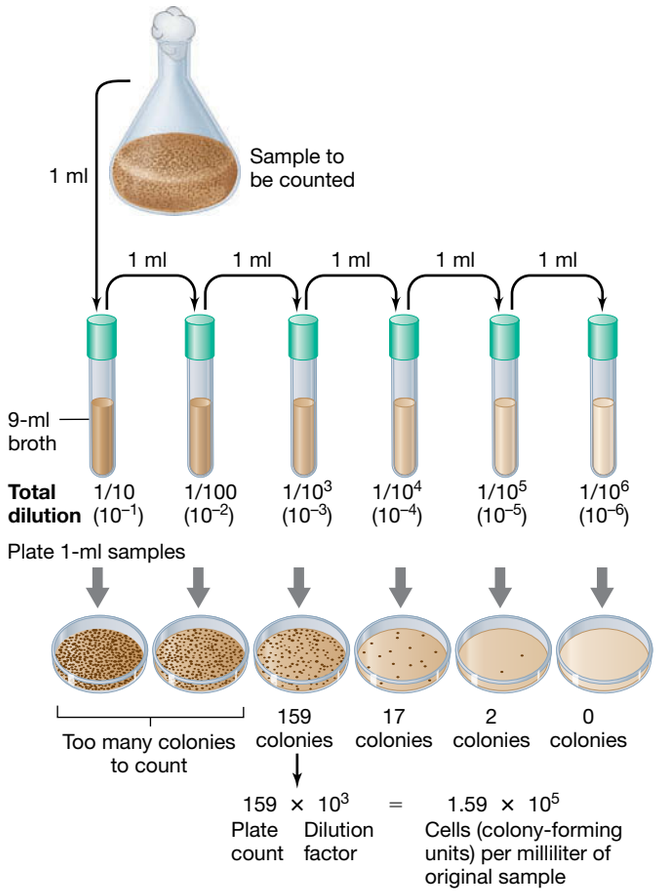


Figure 5.15 Procedure for viable counting using serial dilutions of the sample and the pour-plate method. The sterile liquid used for making dilutions can simply be water, but a solution of mineral salts or actual growth medium may yield a higher recovery. The dilution factor is the reciprocal of the dilution.

Viable counts can be subject to error for several reasons. These include plating inconsistencies, such as inaccurate pipetting of a liquid sample, a nonuniform sample (for example, a sample that has formed cell clumps of differing cell number), insufficient mixing, and heat intolerance if the pour plate method is used. Hence, if accurate counts are to be obtained, great care and consistency must be taken in sample preparation and plating, and replicate plates of key dilutions must be prepared. Because sources of error in plate counting are often considerable, data are typically expressed as the number of *colony-forming units* obtained rather than the actual number of viable cells, to account for clumps containing more than one viable cell.

Applications of the Plate Count

Despite the caveats associated with viable counting, the procedure is quick and easy to do and so is widely used in many subdisciplines of microbiology. For example, in food, dairy, medical, and aquatic microbiology, viable counts are employed routinely. The method has the virtue of high sensitivity, because as few as one viable cell per sample plated can be detected. This feature allows for the sensitive detection of microbial contamination of foods or other materials.

The use of highly selective culture media and growth conditions allows the plate count to be used to target particular species in a sample containing many organisms. For example, a complex medium containing 10% NaCl is very useful in isolating species of potentially pathogenic *Staphylococcus* from skin, because the salt inhibits growth of most other skin bacteria. In practical applications such as in the food industry, viable counting performed using both a complex medium and a selective medium (Section 5.5) on the same sample allows for simultaneous quantitative and qualitative assessments of food quality and safety. That is, with a single food sample, the complex medium yields a total cell count—a relative indicator of freshness and shelf life—while the selective medium indicates the presence or absence of a particular pathogen that may be transmitted in this particular food.

Targeted counting is also common in wastewater and other water analyses. For instance, enteric bacteria such as *Escherichia coli* originate from feces and are easy to recover from natural samples using selective media; if enteric bacteria are detected in a water sample from a swimming site, for example, their presence is a signal that the water contains fecal matter and is therefore unsafe for human contact.

The Great Plate Count Anomaly

Direct microscopic counts of natural samples typically reveal far more microbes than are recoverable on plates of any single culture medium. Thus, although a very sensitive technique, plate counts can be highly unreliable when used to assess total cell numbers of natural samples, such as soil and water. In microbiology, this is referred to as "the great plate count anomaly."

Why do plate counts reveal lower numbers of cells than direct microscopic counts? One obvious factor is that microscopic methods count dead cells, whereas by definition, viable methods do not. More important, however, is the fact that different organisms, even those present in a very small natural sample, may have vastly different requirements for nutrients and growth conditions in laboratory culture (Sections 3.1 and 5.5 and Table 5.1). Thus, one medium and set of growth conditions can only be expected to support the growth of one subset of the total microbial community. If this subset makes up, for example, 10^6 cells/g of a total viable community of 10^9 cells/g, the plate count will reveal only 0.1% of the viable cell population, a vast underestimation of the actual number of organisms present in the sample.

Plate count results thus carry a large caveat. Plate counts targeted to specific organisms using highly selective media (Section 5.5), as in, for example, the microbial analysis of sewage or food, can often yield quite reliable data, since the physiology of the targeted organisms is known and so the recovery of viable cells is near 100%. By contrast, "total" cell counts of the same samples using a single medium and set of growth conditions may be, and usually are, underestimates of actual cell numbers by one to several orders of magnitude. These days, however, counting cells using a cell proxy has become common, as a wide variety of molecular methods have been developed to both detect and quantify specific organisms in natural samples. We explore these methods and what they can tell us in Chapter 19.

MINIQUIZ

- Why is a viable count more sensitive than a microscopic count? What major assumption is made in relating plate count results to cell number?
- Describe how you would dilute a bacterial culture by 10^{-7}.
- Explain the "great plate count anomaly."

5.8 Turbidimetric Measures of Microbial Cell Numbers

During exponential growth, all cellular components increase in proportion to the increase in cell numbers. One such cellular component is cell mass itself. Cells scatter light, and a rapid and widely used technique in microbiology for estimating cell mass is *turbidity*. A cell suspension looks cloudy (turbid) to the eye because cells scatter light that passes through the suspension. The more cells that are present, the more light is scattered, and hence the more turbid the suspension. Because cell mass is proportional to cell number, turbidity measurements can quickly estimate cell numbers in laboratory cultures.

Optical Density and Its Relationship to Cell Numbers

Turbidity is measured with a spectrophotometer, an instrument that passes light through a cell suspension and measures the unscattered light that emerges (**Figure 5.16**). A spectrophotometer employs a prism or diffraction grating to generate incident light of a specific wavelength (Figure 5.16*a*). Commonly used wavelengths

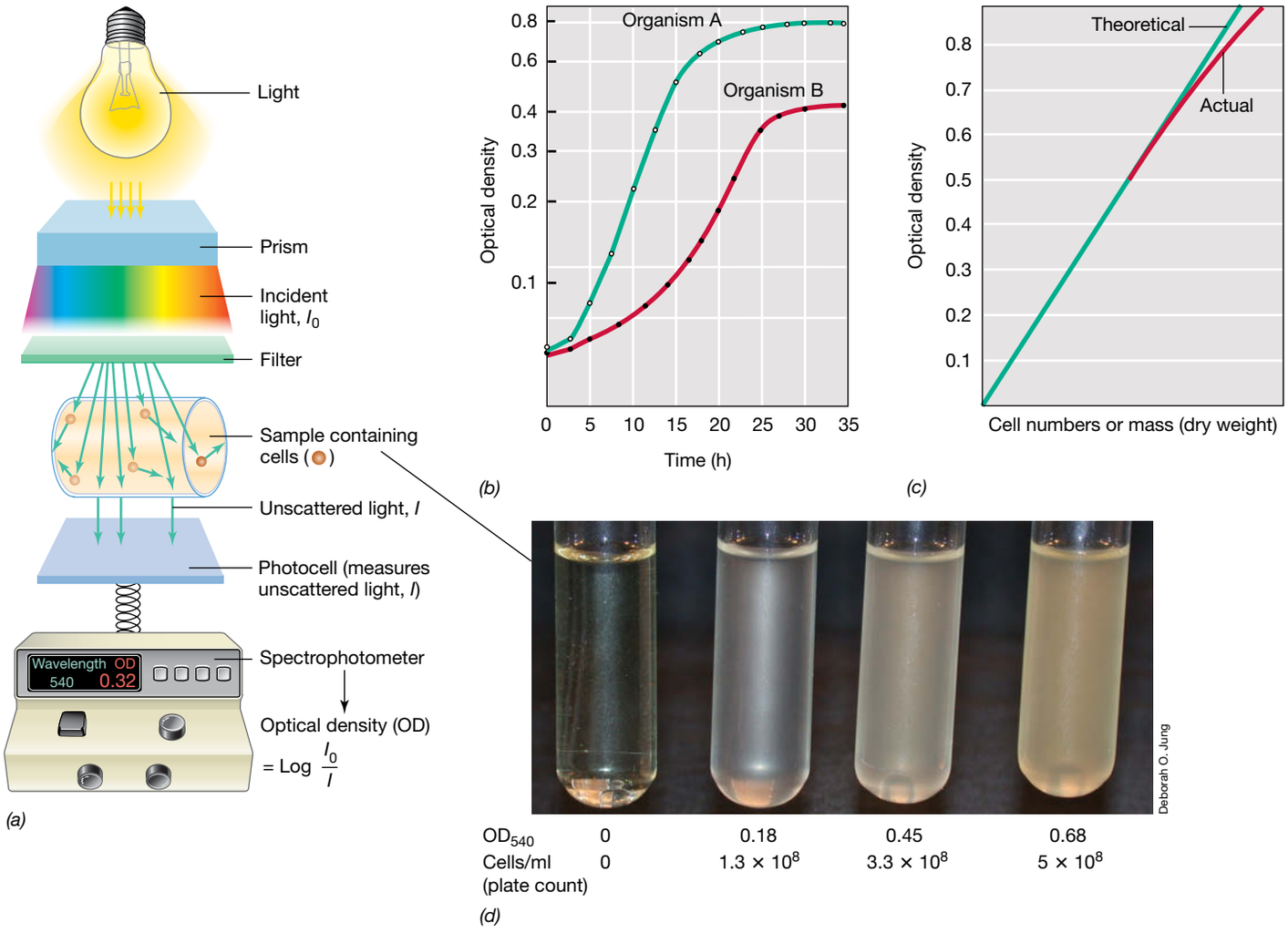


Figure 5.16 Turbidity measurements of microbial growth. *(a)* Measurements of turbidity are made in a spectrophotometer. The photocell measures incident light unscattered by cells in suspension and gives readings in optical density units. *(b)* Typical growth curve data for two organisms growing at different growth rates. For practice, calculate the generation time of the two cultures using the formula $n = 3.3 (\log N - \log N_0)$ where N and N_0 are two different OD readings with a time interval t between the two. Which organism is growing faster, A or B? *(c)* Relationship between cell number or dry weight and turbidity readings. Note that the one-to-one correspondence between these relationships breaks down at high turbidities. *(d)* Liquid cultures of *Escherichia coli*. The increasing (left to right) optical density (OD_{540}) of each culture is shown below the tube, as is the actual cell number measured in a viable count.

for microbial turbidity measurements include 480 nm (blue), 540 nm (green), and 660 nm (red). Sensitivity is best at shorter wavelengths, but measurements of dense cell suspensions are more accurate at longer wavelengths.

The unit of turbidity is *optical density* (*OD*) at the wavelength specified, for example, OD_{540} for measurements at 540 nm (Figure 5.16). For unicellular organisms, optical density is proportional, within certain limits, to cell number. Turbidity readings can therefore be used as a substitute for total or viable counting methods. However, before this can be done, a standard curve must be prepared that relates cell number (microscopic or viable count) to turbidity. As can be seen in such a plot, proportionality only holds within limits (Figure 5.16*c*). At high cell densities, light scattered away from the spectrophotometer's photocell by one cell can be scattered back toward the photocell by another, and as a result, the one-to-one correspondence between cell number and turbidity deviates from linearity. Nevertheless, when a standard has been prepared, turbidimetric estimates of bacterial abundance are very useful.

Other Issues with Turbidimetric Growth Estimates

On the one hand, turbidity measurements are quick and easy to perform and can be made without destroying or significantly disturbing the sample. For these reasons, turbidity measurements are widely employed to monitor growth of pure cultures of *Bacteria*, *Archaea*, and many microbial eukaryotes. With turbidimetric assays, the same sample can be checked repeatedly (Figure 5.16*d*) and the measurements plotted on a semilogarithmic plot versus time (Figure 5.16*b*) to calculate the generation time and other growth parameters (Section 5.2).

On the other hand, turbidity measurements can occasionally be problematic. Although many microorganisms grow evenly distributed in suspensions in liquid medium, many do not. Some bacteria routinely form small to large clumps, and in such instances, OD measurements may be quite inaccurate as a measure of total microbial mass. In addition, many bacteria form biofilms on the sides of tubes or other growth vessels (Section 5.1). Hence for OD measurements to be an accurate reflection of cell mass (and thus cell numbers) in a liquid culture, clumping and biofilms have to be minimized. This can be accomplished by stirring, shaking, or in some way keeping the cells well mixed during the growth process to prevent the formation of cell aggregates and the sticking of swimming cells to surfaces. Some bacteria are just naturally planktonic and stay well suspended in liquid medium for long periods. But if a solid surface is available, most bacteria will eventually develop a static biofilm, and accurately quantifying cell numbers by turbidity in such a case can be difficult or even impossible.

MINIQUIZ

- List two advantages of using turbidity as a measure of cell growth.
- Describe how you could use a turbidity measurement to tell how many colonies you would expect from plating a culture of a given OD.

III • Environmental Effects on Growth: Temperature

Even when a microbe is provided with an optimal array of its required nutrients, growth is not a sure thing unless the chemical and physical state of its environment is also suitable. Four environmental factors control microbial growth in a major way: temperature, pH, water availability, and oxygen. If any one of these factors is beyond the limits that an organism can tolerate, growth will not occur, even in an ideal culture medium. In Parts III and IV of this chapter we examine these important environmental factors, beginning with temperature, the key factor affecting the growth and survival of microorganisms.

5.9 Temperature Classes of Microorganisms

At either too cold or too hot a temperature, microorganisms will not be able to grow and may even die. The minimum and maximum temperatures supporting growth vary greatly among different organisms and usually reflect the temperature range and average temperature of the environments the organisms inhabit.

Cardinal Temperatures

Temperature affects microorganisms in two opposing ways. As temperatures rise, the rate of enzymatic reactions increases and growth becomes faster. However, above a certain temperature, proteins or other cell components may be denatured or otherwise irreversibly damaged. For every microorganism there is a *minimum* temperature below which growth is not possible, an *optimum* temperature at which growth is most rapid, and a *maximum* temperature above which growth is not possible. These three temperatures, called the **cardinal temperatures** (Figure 5.17), are characteristic for any given microorganism and can differ dramat-

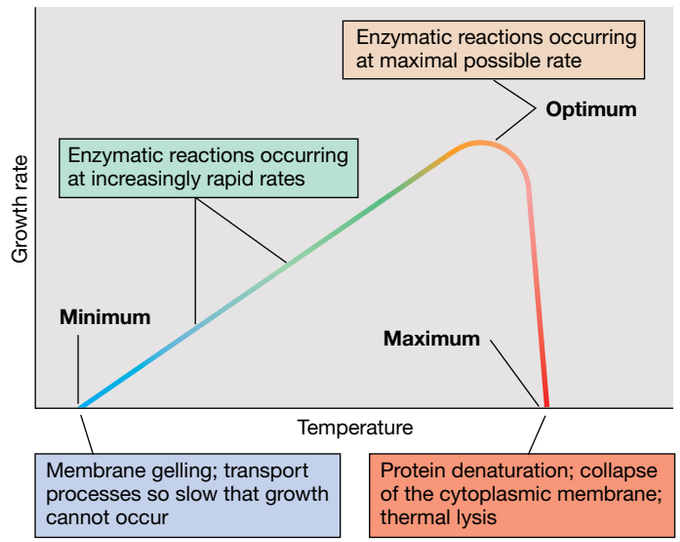


Figure 5.17 The cardinal temperatures: minimum, optimum, and maximum. The actual values may vary greatly for different organisms (see Figure 5.18).

ically between species. For example, some organisms have growth temperature optima near 0°C, whereas the optima for others can be higher than 100°C. The temperature range throughout which microbial growth is possible is even wider than this, from as low as −15°C to at least 122°C. However, no single organism can grow over this whole temperature range, as the range for any given organism is typically less than 40°C.

The maximum growth temperature of an organism reflects the temperature above which denaturation of one or more essential cell components, such as a key enzyme, occurs. The factors controlling an organism's minimum growth temperature are not as clear. However, the cytoplasmic membrane must remain in a semifluid state for nutrient transport and bioenergetic functions to take place. That is, if an organism's cytoplasmic membrane stiffens to the point that it no longer functions properly in transport or can no longer develop or consume a proton motive force, the organism cannot grow. In contrast to the minimum and maximum, the growth temperature *optimum* reflects a state in which all or most cellular components are functioning at their maximum rate and typically lies closer to the maximum than to the minimum (see Figure 5.18).

Temperature Classes of Organisms

Although there is a continuum of organisms, from those with very low temperature optima to those with high temperature optima, it is possible to distinguish four broad classes of microorganisms in relation to their growth temperature optima: **psychrophiles**, with low temperature optima; **mesophiles**, with midrange temperature optima; **thermophiles**, with high temperature optima; and **hyperthermophiles**, with very high temperature optima (Figure 5.18).

Mesophiles are widespread in nature and are the most commonly studied microorganisms. Mesophiles are found in the intestines of endothermic (warm-blooded) animals and in terrestrial and aquatic environments in temperate and tropical latitudes. *Escherichia coli* is a typical mesophile, and its cardinal temperatures have been precisely defined. The optimum temperature for most strains of *E. coli* is near 39°C, the maximum is 48°C, and the minimum is 8°C. Thus, the temperature *range* for *E. coli* is about 40 degrees (Figure 5.18).

Psychrophiles and thermophiles are found in unusually cold and unusually hot environments, respectively. Hyperthermophiles are found in extremely hot habitats such as hot springs, where temperatures can be as hot as 100°C, and deep-sea hydrothermal vents, where temperatures can exceed 100°C. We now consider these fascinating microbes and examine some of the physiological problems they face and some of the biochemical solutions they have evolved to thrive under these extreme conditions.

MINIQUIZ

- How does a hyperthermophile differ from a psychrophile?
- What are the cardinal temperatures for *Escherichia coli*? To what temperature class does it belong?
- *E. coli* can grow at a higher temperature in a complex medium than in a defined medium. Why?

5.10 Microbial Life in the Cold

Because humans live and work in places where temperatures are moderate, it is natural to consider very hot and very cold environments as "extreme." However, many microbial habitats are indeed very hot or very cold, and organisms that inhabit these environments—called *extremophiles* (Section 1.3 and Table 1.1)—actually thrive in these punishing environments. We consider the biology of these fascinating organisms here and in the next section.

Cold Environments

Much of Earth's surface is cold. The oceans, which make up over half of Earth's surface, have an average temperature of 5°C, and

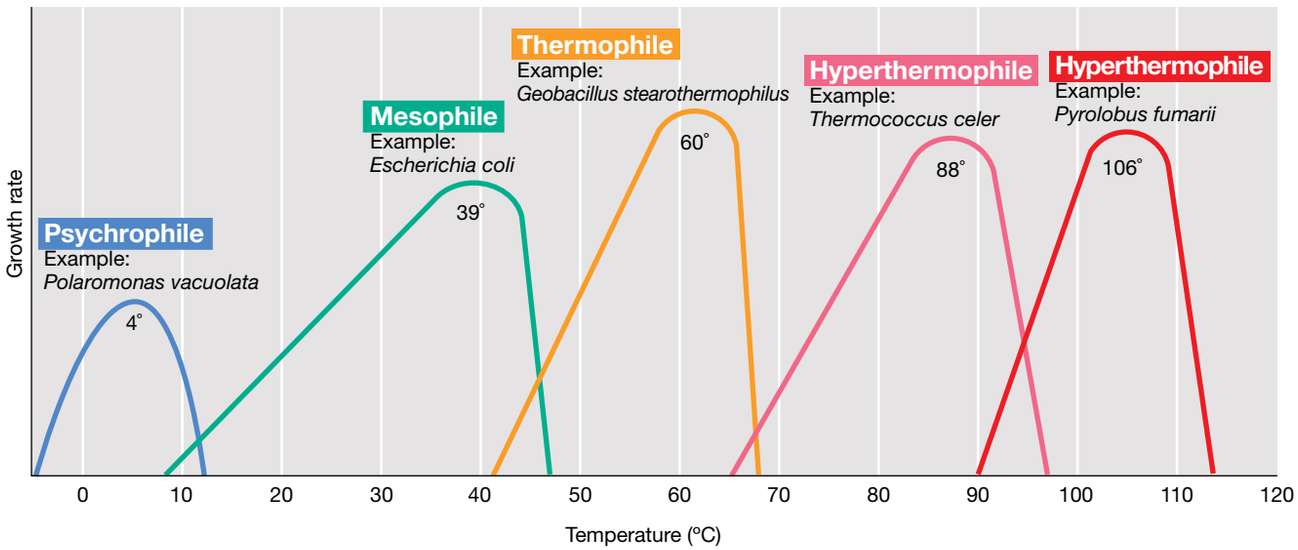


Figure 5.18 Temperature and growth response in different temperature classes of microorganisms.
The temperature optimum of each example organism is shown on the graph.

the depths of the open oceans have constant temperatures of 1–3°C. Vast areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer (**Figure 5.19**). These cold environments support diverse microbial life, as do glaciers (Figure 5.19*e*), where the networks of liquid water channels that run through and under the glacier are teeming with microorganisms. Even in solidly frozen materials there remain small pockets of liquid water where solutes have concentrated and microorganisms can metabolize and grow, albeit very slowly.

In considering cold environments as microbial habitats, it is important to distinguish between environments that are *constantly* cold and those that are only *seasonally* cold. The latter, characteristic of temperate climates, may have summer temperatures as high as 40°C. A temperate lake, for example, may have ice cover in the winter, but the water may remain at 0°C for only a relatively brief time. By contrast, Antarctic lakes contain a permanent ice cover several meters thick (Figure 5.19*d*), and the water column below the ice in these lakes remains at 0°C or colder year round. Marine sediments and glaciers are also constantly cold, as are subglacial lakes—lakes deep beneath the glacier surface—and all of these are teeming with microbial life. It is thus not surprising that the best examples of microbes well adapted to cold temperatures have emerged from these environments.

Psychrophilic and Psychrotolerant Microorganisms

A psychrophile is a microbe with an optimal growth temperature of 15°C or lower, a maximum growth temperature below 20°C, and a minimum growth temperature of 0°C or lower. By contrast, microbes that grow at 0°C but have optima of 20–40°C are called **psychrotolerant**. Psychrophiles are found in environments that

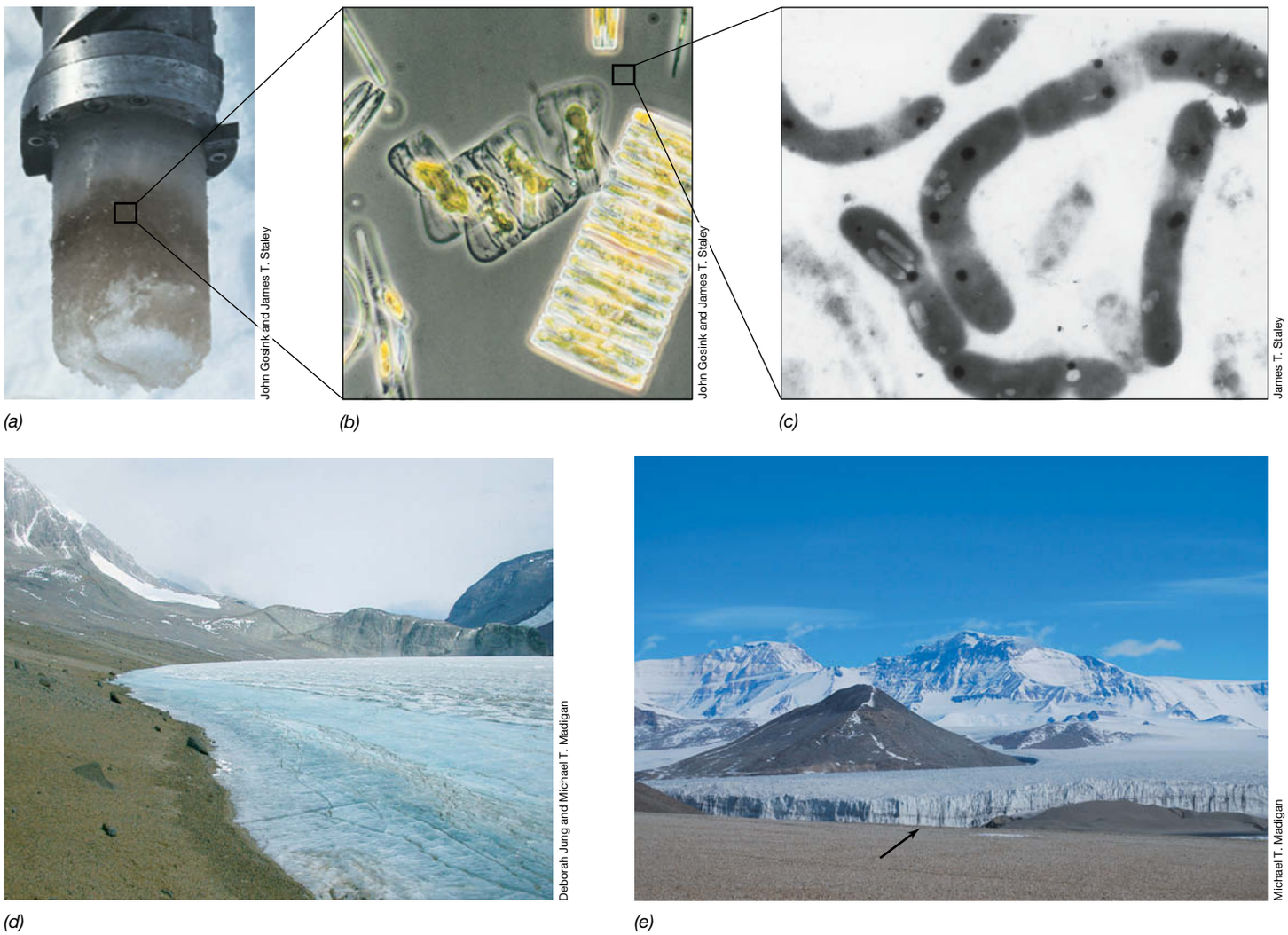


Figure 5.19 Antarctic microbial habitats and microorganisms. *(a)* A core of frozen seawater from McMurdo Sound, Antarctica. The core is about 8 cm wide. Note the dense coloration due to pigmented microorganisms. *(b)* Phase-contrast micrograph of phototrophic microorganisms from the core shown in part *a*. Most organisms are either diatoms or green algae (both eukaryotic phototrophs). *(c)* Transmission electron micrograph of *Polaromonas*, a gas vesiculate bacterium that lives in sea ice and grows optimally at 4°C. Cells are about 0.8 μm in diameter. *(d)* Lake Bonney, McMurdo Dry Valleys, Antarctica. Although the lake is permanently ice-covered, the water column under the ice contains a diverse array of *Bacteria* and *Archaea* and microbial eukaryotes. *(e)* Garwood Glacier, McMurdo Dry Valleys, Antarctica. The edge of the glacier (arrow) is about 20 m high. Glaciers and subglacial lakes are teeming with microbial life.

are constantly cold and may even be killed by warming to moderate temperatures. For this reason, the laboratory study of psychrophiles requires that great care be taken to ensure that they never warm up during sampling, transport to the laboratory, isolation, or other manipulations.

Psychrophilic algae and bacteria often grow in dense masses within and under sea ice (frozen seawater that forms seasonally) in polar regions (Figure 5.19*a–c*). They can also be found on the surfaces of permanent snowfields and glaciers where they impart a distinctive coloration to the surface (**Figure 5.20**). The snow alga *Chlamydomonas nivalis* is an example of this, the carotenoid pigment astaxanthin in its spores being responsible for the brilliant red color of the snow surface (Figure 5.20 inset). This alga grows within the snow as a green-pigmented vegetative cell and then sporulates. As the snow dissipates by melting, erosion, and ablation (evaporation and sublimation), the spores become concentrated on the surface. Related species of snow algae contain different carotenoid pigments, and thus fields of snow algae can also be green, orange, brown, or purple.

Several psychrophilic *Bacteria* and a few psychrophilic *Archaea* have been isolated, and some of these show very low growth temperature optima. The permafrost bacterium *Planococcus halocryophilus* grows slowly at −15°C, the lowest growth temperature documented for any bacterium. However, theoretical considerations of bacterial metabolism suggest that the lower temperature limit for bacterial metabolism is considerably colder than this. For example, microbial respiration (as measured by CO_2 production) has been measured in tundra soils at nearly −40°C. At a temperature of −20°C, pockets of liquid water can exist in "frozen" materials, and studies have shown that enzymes from cold-active bacteria still function under such conditions. Growth at such temperatures, if possible, would be extremely slow. However, if an organism can grow, even if only at a very low rate, it can remain competitive and maintain a population in its habitat.

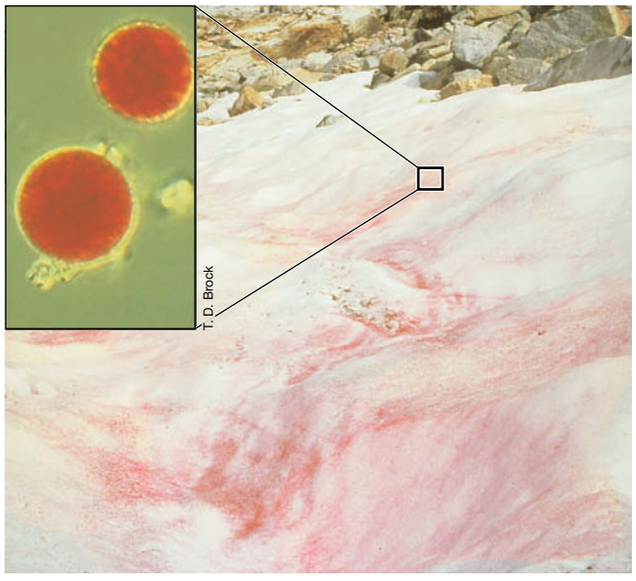


Figure 5.20 Snow algae. Snow bank in the Sierra Nevada, California, with red coloration caused by the presence of snow algae. Inset: photomicrograph of red-pigmented spores of the snow alga *Chlamydomonas nivalis*. Spores are about 18 µm in diameter. The spores germinate to yield motile green algal cells.

Psychrotolerant microorganisms are more widely distributed in nature than are psychrophiles and can be isolated from soils and water in temperate climates as well as from meat, dairy products, cider, vegetables, and fruit stored at standard refrigeration temperatures (4°C). Although psychrotolerant microorganisms grow at 0°C, most do not grow well, and one must often wait weeks before visible growth is seen in laboratory cultures. By contrast, the same organism cultured at 30°C may grow at rates similar to that of many mesophiles. Various *Bacteria*, *Archaea*, and microbial eukaryotes are psychrotolerant.

Molecular Adaptations to Life in the Cold

Psychrophiles produce enzymes that function—often optimally—in the cold and that may be denatured or otherwise inactivated at even very moderate temperatures. The molecular basis for this is not entirely understood, but clearly it is linked to protein structure. Several cold-active enzymes whose structure is known show a greater content of α-helix and lesser content of β-sheet secondary structure (↔ Section 4.7) than do enzymes that show little or no activity in the cold. Because β-sheet secondary structures tend to be more rigid than α-helices, the greater α-helix content of cold-active enzymes allows these proteins greater flexibility for catalyzing their reactions at cold temperatures. Cold-active enzymes also tend to have greater polar and lesser hydrophobic amino acid content (↔ Figure 4.28 for structures of amino acids) and lower numbers of weak bonds, such as hydrogen and ionic bonds, compared with the corresponding enzyme from mesophiles. Collectively, these molecular features are likely to keep cold-active enzymes flexible and functional under cold conditions.

Another characteristic feature of psychrophiles is that their cytoplasmic membranes remain functional at low temperatures. Cytoplasmic membranes from psychrophiles tend to have a higher content of unsaturated and shorter-chain fatty acids, and this helps the membrane remain in a semifluid state at low temperatures to carry out important transport and bioenergetic functions. Some psychrophilic bacteria even contain *polyunsaturated* fatty acids; unlike monounsaturated or fully saturated fatty acids that tend to stiffen at low temperatures, polyunsaturated fatty acids remain flexible even at very cold temperatures.

Other molecular adaptations to cold temperatures include "cold shock" proteins and cryoprotectants, and these are not limited to psychrophiles. Cold shock proteins are a type of molecular chaperone (↔ Section 4.11) and have several functions that include maintaining cold-sensitive proteins in an active form or binding specific mRNAs and facilitating their translation under cold conditions. Cryoprotectants include dedicated antifreeze proteins or specific solutes—such as glycerol or certain sugars—that are produced in large amounts at cold temperatures; these agents help prevent the formation of ice crystals that can puncture the cytoplasmic membrane. Highly psychrophilic bacteria often produce abundant levels of exopolysaccharide cell surface slime, and these slime layers confer cryoprotection as well.

Although freezing temperatures may prevent microbial growth, they do not necessarily cause death. Indeed, just the opposite may occur, and this has been exploited for the preservation of bacterial

cells in microbial culture collections. Cells suspended in growth medium containing 10% dimethyl sulfoxide (DMSO) or glycerol as a cryoprotectant and frozen at −80°C (ultracold freezer) or −196°C (liquid nitrogen) remain viable in the frozen state for years.

MINIQUIZ

- How do psychrotolerant organisms differ from psychrophilic organisms?
- What molecular adaptations to cold temperatures are seen in the cytoplasmic membrane of psychrophiles? Why are they necessary?

5.11 Microbial Life at High Temperatures

Microbial life flourishes in high-temperature environments, from sun-heated soils and pools of water to boiling hot springs, and the organisms that live in these environments are typically highly adapted to their environmental temperature. We examine these organisms now and pick up on them again in several later chapters.

Thermal Environments

Organisms whose growth temperature optimum exceeds 45°C are called *thermophiles* and those whose optimum exceeds 80°C are called *hyperthermophiles* (Figure 5.18). The surface of soils subject to full sunlight can be heated to above 50°C at midday, and some surface soils may warm to as high as 70°C. Fermenting materials such as compost piles and silage can also reach temperatures of 70°C. Thermophiles abound in such environments. The most extreme high-temperature environments in nature, however, are hot springs, and these are home to a huge diversity of thermophiles and hyperthermophiles.

Many terrestrial hot springs have temperatures at or near boiling, while those at the bottom of the ocean, called *hydrothermal vents*, can have temperatures of 350°C or greater. Hot springs are found throughout the world, but they are especially abundant in the western United States, New Zealand, Iceland, Japan, Italy, Indonesia, Central America, and central Africa. The largest concentration of hot springs in the world is in Yellowstone National Park, Wyoming (USA). Although some hot springs vary widely in temperature, many are nearly constant, varying less than a degree or two over many years. In addition, different springs have different chemical compositions and pH values. In habitats hotter than 65°C, only prokaryotic cells can thrive (Table 5.2), but the diversity of *Bacteria* and *Archaea* in such environments is often extensive.

Hyperthermophiles and Thermophiles

A variety of hyperthermophiles inhabit boiling hot springs (Figure 5.21), including both chemoorganotrophic and chemolithotrophic species. Growth rates of hyperthermophiles can be studied in the field by immersing a microscope slide into a spring and then examining it microscopically over time. The slide is an excellent surface for microbial attachment and subsequent growth, and so small microbial colonies form (Figure 5.21*b*) and growth rates can be calculated from cell number data. Ecological studies such as this have shown that growth rates in boiling springs are often quite high, with generation times (*g*) as short as 1 h not uncommon.

TABLE 5.2 Presently known upper temperature limits for growth of living organisms

Group	Upper temperature limits (°C)
Macroorganisms	
Animals	
Fish and other aquatic vertebrates	38
Insects	45–50
Ostracods (crustaceans)	49–50
Plants	
Vascular plants	45 (60 for one species)
Mosses	50
Microorganisms	
Eukaryotic microorganisms	
Protozoa	56
Algae	55–60
Fungi	60–62
Bacteria and Archaea	
Bacteria	
Cyanobacteria	73
Anoxygenic phototrophs	70–73
Chemoorganotrophs/chemolithotrophs	95
Archaea	
Chemoorganotrophs/chemolithotrophs	122

Cultures of diverse hyperthermophiles have been obtained, and a variety of morphological and physiological types of both *Bacteria* and *Archaea* are known. Some hyperthermophilic *Archaea* have growth-temperature optima above 100°C, while no species of *Bacteria* have yet been discovered that grow above 95°C. Growing laboratory cultures of organisms with optima above the boiling point requires pressurized vessels that permit temperatures in the growth medium to rise above 100°C without boiling. The most heat-tolerant organisms known inhabit hydrothermal vents, with the most thermophilic example thus far being *Methanopyrus*, a methane-producing genus of *Archaea* capable of growth at up to 122°C (Section 17.2).

In contrast to hyperthermophiles, thermophiles (optima 45–80°C) inhabit moderately hot or intermittently hot environments. As boiling water leaves a hot spring, it gradually cools, setting up a thermal gradient. Along this gradient, microorganisms become established, with different species growing in the different temperature ranges (Figure 5.22*a*). By studying the species distribution along such natural thermal gradients, it has been possible to determine the upper temperature limits for various classes of microbes (Table 5.2). Thermophilic *Bacteria* and *Archaea* have also been found in artificial thermal environments, such as hot water heaters. Hot water discharges from power plants and other artificial thermal sources also provide sites where thermophiles can flourish.

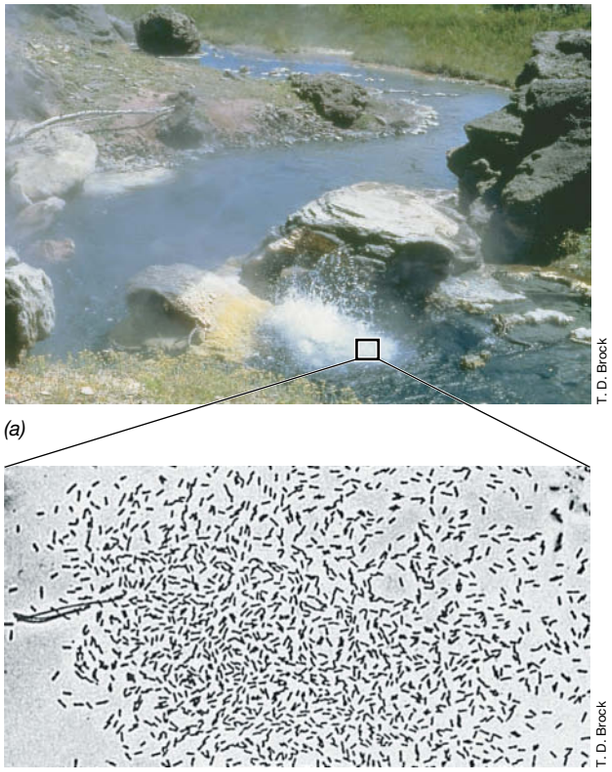


Figure 5.21 Growth of hyperthermophiles in boiling water. *(a)* Boulder Spring, a small boiling spring in Yellowstone National Park. This spring is superheated, having a temperature 1–2°C above the boiling point. The mineral deposits around the spring consist mainly of silica and sulfur. *(b)* Photomicrograph of a microcolony of *Archaea* that developed on a microscope slide immersed in such a boiling spring.

Protein and Membrane Stability at High Temperatures

How do thermophiles and hyperthermophiles survive high temperatures? First, their enzymes and other proteins are much more heat-stable than are those of mesophiles and actually function *optimally* at high temperatures. The heat stability of an enzyme from a hyperthermophile is often due to subtle changes in amino acid sequence from the corresponding enzyme from a mesophile, and these changes affect protein structure and function to resist heat denaturation. Heat-stable proteins also typically show increased ionic bonding between basic and acidic amino acids and have highly hydrophobic interiors, factors that also prevent unfolding. Finally, solutes such as di-inositol phosphate, diglycerol phosphate, and mannosylglycerate are produced at high levels in certain hyperthermophiles, and these are thought to help stabilize their proteins against thermal denaturation.

Enzymes from thermophiles and hyperthermophiles have significant commercial uses. Heat-stable enzymes catalyze biochemical reactions at high temperatures and are in general more stable than enzymes from mesophiles, thus prolonging the shelf life of commercial enzyme preparations (Figure 5.22*b*). A classic example of this is the DNA polymerase isolated from *Thermus aquaticus*—*Taq polymerase*—used to automate the repetitive steps in the polymerase chain reaction (PCR), a technique for amplifying

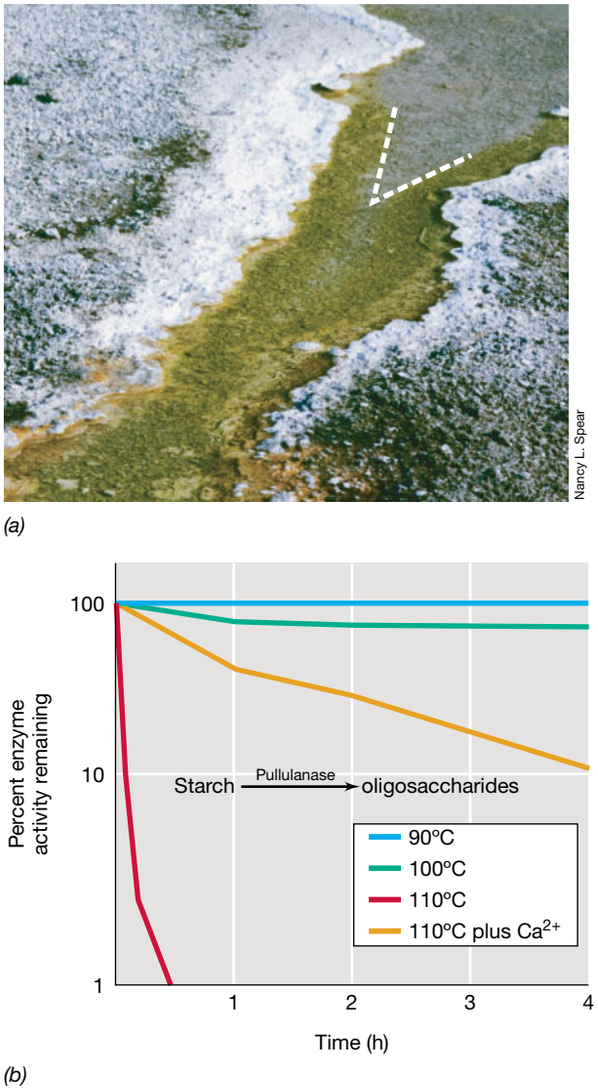


Figure 5.22 Hot spring microbes and their heat-stable enzymes. *(a)* Characteristic V-shaped pattern (shown by the dashed white lines) formed by cyanobacteria at the upper temperature for phototrophic life, 70–73°C, in the thermal gradient formed from a boiling hot spring in Yellowstone National Park (USA). The pattern develops because the water cools more rapidly at the edges than in the center of the channel. *(b)* In the spring source, hyperthermophiles thrive, and some have been used as sources of heat-stable enzymes, such as pullulanase from *Pyrococcus* (*Archaea*). Ca^{2+} stabilizes the enzyme above the boiling point.

DNA and a major tool of modern biology (Section 12.1). Several other heat-stable enzymes are commercially available for specific industrial applications (Figure 5.22*b*).

Besides enzymes and other macromolecules in the cell, the cytoplasmic membranes of thermophiles and hyperthermophiles must be heat-stable. Heat naturally works to peel apart the lipid bilayer that makes up the cytoplasmic membrane (Section 2.3). In thermophiles and most hyperthermophilic *Bacteria*, the cytoplasmic membrane has a higher content of long-chain and saturated fatty acids and a lower content of unsaturated fatty acids than are found in the cytoplasmic membranes of mesophiles. Saturated fatty acids form a stronger hydrophobic environment

than do unsaturated fatty acids, and longer-chain fatty acids have a higher melting point than shorter-chain fatty acids; collectively, these properties increase membrane stability.

Hyperthermophiles, most of which are *Archaea*, do not contain fatty acids in their membranes but instead have C_{40} hydrocarbons composed of repeating units of isoprene bonded by ether linkage to glycerol phosphate (Figure 2.6). In addition, however, the architecture of the cytoplasmic membranes of many hyperthermophiles takes a unique twist: The membrane forms a lipid *monolayer* rather than a lipid *bilayer* (Figure 2.6*e*). The monolayer structure covalently links both halves of the membrane and prevents it from melting at the high growth temperatures of hyperthermophiles. We consider other aspects of heat stability in hyperthermophiles, including that of DNA stability, in Chapter 17.

MINIQUIZ

- Which phylogenetic domain includes species with optima of >100°C? What special techniques are required to culture them?
- How does the membrane structure of hyperthermophilic *Archaea* differ from that of *Escherichia coli* and why is this structure helpful for growth at high temperature?
- What is *Taq* polymerase and why is it important?

IV • Environmental Effects on Growth: pH, Osmolarity, and Oxygen

As we have seen, temperature has a major effect on the growth of microorganisms. But many other environmental factors can affect microbial growth as well, including pH, osmolarity, and oxygen.

5.12 Effects of pH on Microbial Growth

Acidity or alkalinity of a solution is expressed by its **pH** on a logarithmic scale in which neutrality is pH 7 (**Figure 5.23**). pH values less than 7 are *acidic* and those greater than 7 are *alkaline*. In analogy to a temperature range (Figure 5.17), every microorganism has a pH range, typically about 2–3 pH units, within which growth is possible. Also, each organism shows a well-defined pH optimum, where growth occurs best. Most natural environments have a pH between 3 and 9, and organisms with pH growth optima in this range are most common. Terms used to describe organisms that grow best in particular pH ranges are shown in **Table 5.3**.

Acidophiles

Organisms that grow optimally at a pH value in the range termed *circumneutral* (pH 5.5 to 7.9) are called **neutrophiles**. For example, the bacterium *Escherichia coli* is a neutrophile (Table 5.3). By contrast, organisms that grow best below pH 5.5 are called **acidophiles**. There are different classes of acidophiles, some growing best at moderately acidic pH and others at very low pH. Many fungi and bacteria grow best at pH 5 or even below, while a more restricted number grow best below pH 3. An even more restricted group grow

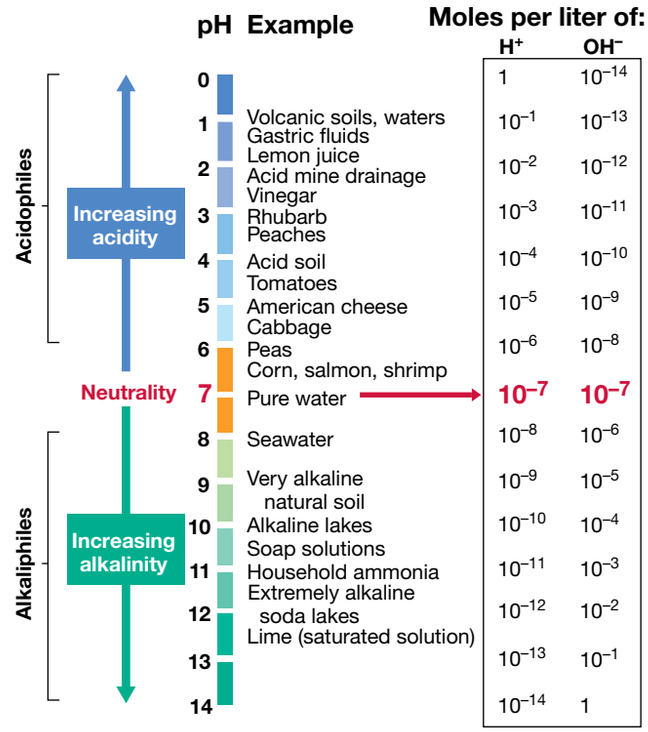


Figure 5.23 The pH scale. Although some microorganisms can live at very low or very high pH, the cell's internal pH remains near neutrality.

best below pH 2 and those with pH optima below 1 are extremely rare. Most acidophiles cannot grow at pH 7 and many cannot grow at pH values more than two units above their optimum.

A critical factor governing acidophily is the stability of the cytoplasmic membrane. When the pH is raised to neutrality, the cytoplasmic membranes of strongly acidophilic bacteria are destroyed and the cells lyse. This indicates that these organisms are not just acid-*tolerant* but that high concentrations of protons are actually *required* for cytoplasmic membrane stability. For example, the most acidophilic microbe known is *Picrophilus oshimae*, a species of *Archaea* that grows optimally at pH 0.7 and 60°C. Above pH 4, cells of *P. oshimae* spontaneously lyse. As one would predict, *P. oshimae* inhabits extremely acidic thermal soils associated with volcanic activity.

TABLE 5.3 Relationships of microorganisms to pH

Physiological class (optima range)	*Approximate pH optimum for growth*	*Example organism*[a]
Neutrophile (pH > 5.5 and < 8)	7	*Escherichia coli*
Acidophile (pH < 5.5)	5	*Rhodopila globiformis*
	3	*Acidithiobacillus ferrooxidans*
	1	*Picrophilus oshimae*
Alkaliphile (pH ≥ 8)	8	*Chloroflexus aurantiacus*
	9	*Bacillus firmus*
	10	*Natronobacterium gregoryi*

[a] *Picrophilus* and *Natronobacterium* are *Archaea*; all others are *Bacteria*.

Alkaliphiles

A few extremophiles have very high pH optima for growth, sometimes as high as pH 10, and some of these can still grow, albeit poorly, at even higher pH. Microorganisms showing pH optima of 8 or higher are called **alkaliphiles**. Alkaliphilic microorganisms are typically found in highly alkaline habitats, such as soda lakes and high-carbonate soils. The most well-studied alkaliphilic bacteria are certain *Bacillus* species, such as *Bacillus firmus*. This organism is alkaliphilic but has an unusually broad range for growth, from pH 7.5 to 11. Some extremely alkaliphilic microbes are also halophilic (salt-loving), and most of these are *Archaea* (Section 17.1). Some phototrophic purple bacteria (Sections 15.4 and 15.5) are also strongly alkaliphilic. Certain alkaliphiles have commercial uses because they excrete hydrolytic enzymes such as proteases and lipases that maintain their activities at alkaline pH. These enzymes are added to laundry detergents to remove protein and fat stains, respectively, from clothing.

Managing membrane bioenergetics is an obvious problem for alkaliphiles. *B. firmus* uses sodium (Na^+) rather than H^+ to drive transport reactions and rotate its flagellum; that is, it forms a *sodium* motive force instead of a *proton* motive force. Remarkably, however, *B. firmus* uses a proton motive force to drive ATP synthesis even though the external membrane surface is highly alkaline. Exactly how this happens is unclear, although it is thought that hydrogen ions are in some way kept very near the outer surface of the cytoplasmic membrane such that they cannot spontaneously combine with the abundant hydroxyl ions to form water.

Cytoplasmic pH and Buffers

The optimal pH for growth of an organism refers to the *extracellular* environment only; the *intracellular* pH must be maintained at a value consistent with the stability of macromolecules, a range of about 4 pH units from pH 5 to 9. Thus, despite conditions in their habitats, extreme acidophiles and alkaliphiles maintain cytoplasmic pH values nearer to neutrality.

To prevent major shifts in pH during microbial growth in batch cultures, *buffers* are commonly added to culture media along with the nutrients required for growth. However, any given buffer works over only a relatively narrow pH range. For neutrophilic species, potassium phosphate (KH_2PO_4) or sodium bicarbonate ($NaHCO_3$) is often employed. Various organic buffers are available for the growth of acidophiles and alkaliphiles and are widely used for assaying enzymes extracted from cells. The buffer keeps the enzyme solution at optimal pH during the assay, thus ensuring that the enzyme remains catalytically active and unaffected by any protons or hydroxyl ions generated in the enzymatic reaction.

MINIQUIZ

- How does the concentration of H^+ change when a culture medium at pH 5 is adjusted to pH 9?
- What terms are used to describe organisms whose growth pH optimum is very high? Very low?
- In terms of pH, what class of organism is the bacterium *Escherichia coli*?

5.13 Osmolarity and Microbial Growth

Water is the solvent of life, and water availability is an important factor affecting the growth of microorganisms. Water availability not only depends on how moist or dry an environment is but is also a function of the concentration of solutes (salts, sugars, or other substances) dissolved in the water that is present. Solutes bind water, making it less available to organisms. Hence, for organisms to thrive in high-solute environments, physiological adjustments are necessary. Water availability is expressed in terms of **water activity** (a_w), the ratio of the vapor pressure of air in equilibrium with a substance or solution to the vapor pressure of pure water. Values of a_w vary between 0 (no free water) and 1 (pure water); some a_w values are listed in Table 5.4.

Water diffuses from regions of high water concentration (low solute concentration) to regions of lower water concentration (higher solute concentration) in the process of osmosis. The cytoplasm of a cell typically has a higher solute concentration than the environment, so the tendency for water is to diffuse into the cell. Under such conditions, the cell is said to be in *positive water balance*, which is the normal state of the cell. However, when a cell is placed in an environment where the solute concentration exceeds that of the cytoplasm, water will flow out of the cell. If a cell has no strategy to counteract this, it will become dehydrated and unable to grow.

Halophiles and Related Organisms

In nature, osmotic effects are of interest mainly in habitats with high concentrations of salts. Seawater contains about 3% sodium chloride (NaCl) plus small amounts of many other minerals and elements. Microorganisms that inhabit marine environments almost always show an NaCl requirement and grow optimally at the a_w of seawater, 0.98 (Figure 5.24). Such organisms are called **halophiles**. The requirement for NaCl by halophiles is absolute and cannot be replaced by other salts, such as potassium chloride (KCl), calcium chloride ($CaCl_2$), or magnesium chloride ($MgCl_2$).

Although halophiles require at least some NaCl for growth, the NaCl optimum varies with the organism and is habitat dependent. For example, marine microorganisms typically grow best

TABLE 5.4 Water activity of several substances

Water activity (a_w)	*Material*	*Example organisms*[a]
1.000	Pure water	*Caulobacter, Spirillum*
0.995	Human blood	*Streptococcus, Escherichia*
0.980	Seawater	*Pseudomonas, Vibrio*
0.950	Bread	Most gram-positive rods
0.900	Maple syrup, ham	Gram-positive cocci such as *Staphylococcus*
0.850	Salami	*Saccharomyces rouxii* (yeast)
0.800	Fruit cake, jams	*Zygosaccharomyces bailii* (yeast), *Penicillium* (fungus)
0.750	Salt lakes, salted fish	*Halobacterium, Halococcus*
0.700	Cereals, candy, dried fruit	*Xeromyces bisporus* and other xerophilic fungi

[a]Selected examples of *Bacteria* and *Archaea* or fungi capable of growth in culture media adjusted to the stated water activity.

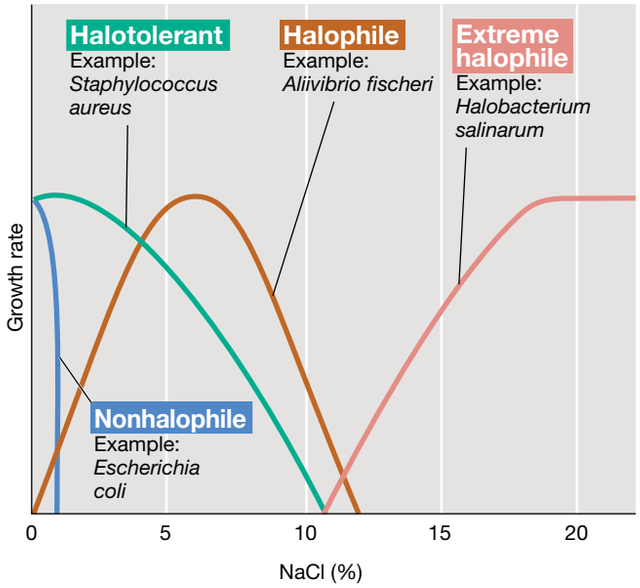


Figure 5.24 Effect of NaCl concentration on growth of microorganisms of different salt tolerances or requirements. The optimum NaCl concentration for marine microorganisms such as *Aliivibrio fischeri* is about 3%; for extreme halophiles, it is between 15 and 30%, depending on the organism.

with 1–4% NaCl, organisms from hypersaline environments (environments that are more salty than seawater) grow best at 3–12% NaCl, and organisms from extremely hypersaline environments require even higher levels of NaCl. Organisms isolated from brackish waters (a mixture of freshwater and seawater) may or may not be halophilic.

In contrast to halophiles, **halotolerant** organisms can tolerate some level of dissolved solutes but grow best in the absence of the added solute (Figure 5.24). Halophiles capable of growth in very salty environments are called **extreme halophiles** (Figure 5.24). These organisms require very high levels of NaCl, typically 15–30%, for optimum growth and are often unable to grow at all at NaCl concentrations below this. Organisms able to live in environments high in sugar are called **osmophiles**, and those able to grow in very dry environments (made dry by lack of water rather than by dissolved solutes) are called **xerophiles**. Examples of these various classes of organisms are given in Table 5.5.

From growth data obtained from extremely halophilic representatives of all three domains of life, there appears to be a common lower water activity limit for living organisms, and this limit is 0.61. This lower limit is likely set by the physiochemical constraints on obtaining water in osmotic environments of a_w less than 0.6 that cannot be overcome through biochemical adaptations by the cell. *Matric water activity*, a measure of water bound to a surface, is measured in the same way as osmotic water activity but can drop to significantly lower than 0.6 and still contain viable microbial communities. For example, hyper-arid hot desert soils can have matric a_w values as low as 0.1 during daylight hours. But these environments absorb moisture at night and during rain events, and these increase the water activity to above 0.6, making conditions suitable for microbial metabolism and growth.

Compatible Solutes

When an organism is transferred from a medium of high a_w to one of low a_w, it maintains positive water balance by increasing its internal solute concentration. This is possible either by pumping solutes into the cell from the environment or by synthesizing a cytoplasmic solute (Table 5.5). In either case, the solute must not inhibit biochemical processes in the cell and is thus called a **compatible solute**.

Compatible solutes are highly water-soluble organic molecules and include sugars, alcohols, and amino acid derivatives (Table 5.5). Glycine betaine, an analog of the amino acid glycine, is widely distributed among halophilic bacteria. Other common compatible solutes include sugars such as sucrose and trehalose, dimethylsulfoniopropionate (produced by marine algae), and glycerol, a common solute in xerophilic fungi, organisms that grow at the lowest water activities known (Table 5.5). In contrast to these organic solutes, KCl is the compatible solute of extremely halophilic *Archaea*, such as *Halobacterium* (Section 17.1), and of a few extremely halophilic *Bacteria*.

The concentration of compatible solute in a cell is a function of the levels of solute in its environment, and adjustments are made in response to the challenge from external solutes. However, in any given organism, the maximal level of compatible solute tolerated is a genetically encoded characteristic. As a result, different organisms have evolved to thrive in habitats of different salinities (Tables 5.4 and 5.5). In fact, organisms designated as *nonhalotolerant, halotolerant, halophilic*, or *extremely halophilic* (Figure 5.24) are to some extent a reflection of their genetic capacity to produce or accumulate compatible solutes.

MINIQUIZ

- What is the a_w of pure water? What is the lower limit of a_w for life?
- What are compatible solutes, and when and why are they needed by the cell? What is the compatible solute of *Halobacterium*?

5.14 Oxygen and Microbial Growth

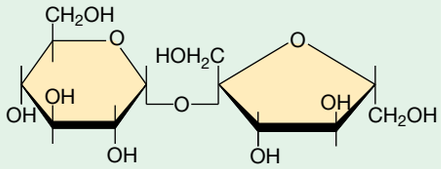
Oxygen (O_2) is an essential nutrient for many microbes; they are unable to metabolize or grow without it. Other microbes, by contrast, cannot grow in the presence of O_2 and may even be killed by it. We therefore see, just as we did for other environmental factors considered in this chapter, *classes* of microorganisms based on their needs or tolerance of O_2.

Oxygen Classes of Microorganisms

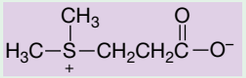
Microorganisms can be grouped according to their relationship with O_2 as outlined in Table 5.6. **Aerobes** can grow at full oxygen tensions (air is 21% O_2) and respire O_2 in their metabolism. **Microaerophiles**, by contrast, are aerobes that can use O_2 only when it is present at levels reduced from that in air (microoxic conditions). This is because of the limited capacity of these organisms to respire or because they contain some O_2-sensitive molecule such as an O_2-labile enzyme. Many aerobes are **facultative**, meaning that

TABLE 5.5 Compatible solutes of microorganisms

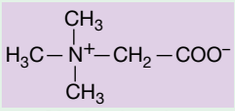
Organism group and example	Major cytoplasmic compatible solute(s)	Minimum a_w for growth[c]
Most nonphototrophic *Bacteria* (*Escherichia*) and freshwater cyanobacteria (*Anabaena*)	Amino acids (mainly glutamate or proline[a])/ sucrose, trehalose[b]	0.98
Marine cyanobacteria (*Synechococcus*)	α-Glucosylglycerol[b]	0.92
Marine algae (*Phaeocystis*)	Mannitol[b], various glycosides, dimethylsulfoniopropionate	0.92
Halotolerant *Bacteria* (*Staphylococcus*)	Amino acids	0.90
Salt lake cyanobacteria (*Aphanothece*)	Glycine betaine	0.75
Halophilic phototrophic purple *Bacteria* (*Halorhodospira*)	Glycine betaine, ectoine, trehalose[b]	0.75
Extremely halophilic *Archaea* (*Halobacterium*) and some *Bacteria* (*Salinibacter*)	KCl	0.75
Halophilic green algae (*Dunaliella*)	Glycerol	0.75
Haloalkaliphilic *Archaea* (*Natrinema*)	KCl	0.68
Xerophilic and osmophilic yeasts (*Zygosaccharomyces*)	Glycerol	0.62[d]
Xerophilic filamentous fungi (*Xeromyces*)	Glycerol	0.605[d]



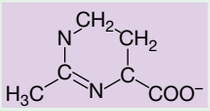
Sucrose



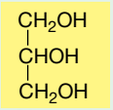
Dimethylsulfoniopropionate



Glycine betaine



Ectoine



Glycerol

[a] See Figure 4.28 for the structures of amino acids.
[b] Structures not shown. Like sucrose, trehalose is a C_{12} disaccharide; glucosylglycerol is a C_9 alcohol; mannitol is a C_6 alcohol.
[c] To achieve an osmotic a_w lower than about 0.77, solutes other than just NaCl are necessary; for example, other salts ($MgCl_2$, $MgSO_4$, or $CaCl_2$) or non-salts, such as glycerol or sucrose. For most organisms listed (other than for the xerophiles), the lower a_w for growth can be extended downward somewhat by additional solutes.
[d] Growth of *Zygosaccharomyces* tested in high-sucrose medium. Germination of *Xeromyces* spores tested using matric water potential.

TABLE 5.6 Oxygen relationships of microorganisms

Group	Relationship to O_2	Type of metabolism	Example[a]	Habitat[b]
Aerobes				
Obligate	Required	Aerobic respiration	*Micrococcus luteus* (B)	Skin, dust
Facultative	Not required, but growth better with O_2	Aerobic respiration, anaerobic respiration, fermentation	*Escherichia coli* (B)	Mammalian large intestine
Microaerophilic	Required but at levels lower than atmospheric	Aerobic respiration	*Spirillum volutans* (B)	Lake water
Anaerobes				
Aerotolerant	Not required, and growth no better when O_2 present	Fermentation	*Streptococcus pyogenes* (B)	Upper respiratory tract
Obligate	Harmful or lethal	Fermentation or anaerobic respiration	*Methanobacterium formicicum* (A)	Sewage sludge, anoxic lake sediments

[a] Letters in parentheses indicate phylogenetic status (B, *Bacteria*; A, *Archaea*). Representatives of either domain of prokaryotic cells are known in each category. Most eukaryotes are obligate aerobes, but facultative aerobes (for example, yeast) and obligate anaerobes (for example, certain protozoa and fungi) are known.
[b] Listed are typical habitats of the example organism; many others could be listed.

under the appropriate nutrient and culture conditions they can grow in the absence of O_2.

Some organisms cannot respire oxygen and are called **anaerobes**. There are two kinds of anaerobes: **aerotolerant anaerobes**, which can tolerate O_2 and grow in its presence even though they cannot respire, and **obligate anaerobes**, which are inhibited or even killed by O_2 (Table 5.6). *Anoxic* (O_2-free) microbial habitats are common in nature and include muds and other sediments, bogs, marshes, water-logged soils, intestinal tracts of animals, sewage sludge, the deep subsurface of Earth, and many other environments. Because there are many habitats for anaerobes, they are very common in nature and highly diverse. As far as is known, obligate anaerobiosis is characteristic of only three groups of microorganisms: a wide variety of *Bacteria* and *Archaea*, a few fungi, and a few protozoa.

Some of the best-known prokaryotic anaerobes are *Clostridium*, a genus of gram-positive endospore-forming *Bacteria*, and the methanogens, a group of methane-producing *Archaea*. Among obligate anaerobes, the sensitivity to O_2 varies greatly. Many clostridia, for example, although requiring anoxic conditions for growth, can tolerate traces of O_2 or even full exposure to air. Others, such as the methanogens, are killed rapidly by O_2 exposure.

Culture Techniques for Aerobes and Anaerobes

For the growth of aerobes, it is necessary to provide extensive aeration. This is because the O_2 that is consumed by the organisms during growth is not replaced fast enough by diffusion from the air. Therefore, forced aeration of liquid cultures is needed and can be achieved by either vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium through a fine glass tube or porous glass disc.

For the culture of anaerobes, the problem is not to *provide* O_2 but to *exclude* it. Bottles or tubes filled completely to the top with culture medium and fitted with leakproof closures provide suitably

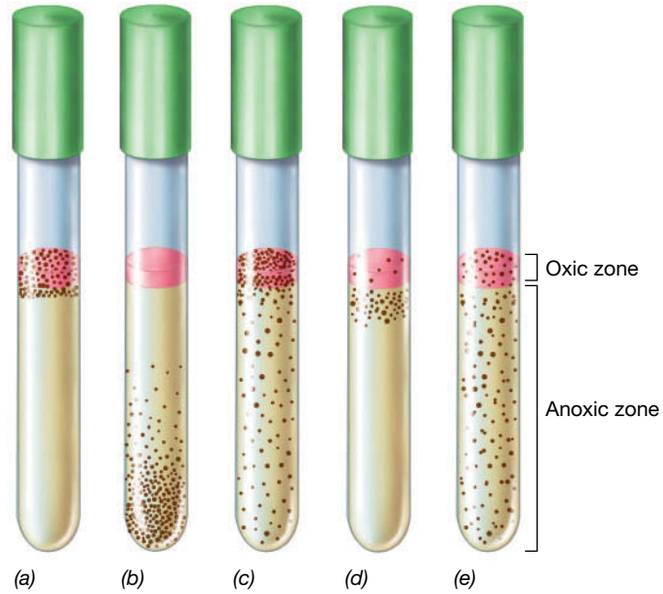


Figure 5.25 Growth versus O_2 concentration. From left to right, aerobic, anaerobic, facultative, microaerophilic, and aerotolerant anaerobe growth, as revealed by the position of microbial colonies (depicted here as black dots) within tubes of thioglycolate broth culture medium. A small amount of agar has been added to keep the liquid from becoming disturbed. The redox dye resazurin, which is pink when oxidized and colorless when reduced, has been added as a redox indicator. *(a)* O_2 penetrates only a short distance into the tube, so obligate aerobes grow only close to the surface. *(b)* Anaerobes, being sensitive to O_2, grow only away from the surface. *(c)* Facultative aerobes are able to grow in either the presence or the absence of O_2 and thus grow throughout the tube. However, growth is better near the surface because these organisms can respire. *(d)* Microaerophiles grow away from the most oxic zone. *(e)* Aerotolerant anaerobes grow throughout the tube. Growth is not better near the surface because these organisms can only ferment. In nature, many different habitats exist for each of these oxygen classes. In addition, a single habitat, such as a soil particle, may support growth of both aerobes and anaerobes (Section 20.1 and Figure 20.3).

Deborah O. Jung and M. T. Madigan

(a)

Coy Laboratory Products

(b)

Figure 5.26 Incubation under anoxic conditions. *(a)* Anoxic jar. A chemical reaction in the envelope in the jar generates $H_2 + CO_2$. The H_2 reacts with O_2 in the jar on the surface of a palladium catalyst to yield H_2O; the final atmosphere contains N_2, H_2, and CO_2. *(b)* Anoxic glove bag for manipulating and incubating cultures under anoxic conditions. The airlock on the right, which can be evacuated and filled with O_2-free gas, serves as a port for adding and removing materials to and from the glove bag.

anoxic conditions for organisms that are not overly sensitive to small amounts of O_2. A chemical called a *reducing agent* may be added to such vessels to remove traces of O_2 by reducing it to water (H_2O). An example is thioglycolate, which is present in thioglycolate broth, a medium commonly used to test an organism's requirements for O_2 (Figure 5.25).

Thioglycolate broth is a complex medium containing a small amount of agar, making the medium viscous but still fluid. After thioglycolate reacts with O_2 throughout the tube, O_2 can penetrate only near the top of the tube where the medium contacts air. Obligate aerobes grow only at the top of such tubes. Facultative organisms grow throughout the tube but grow best near the top. Microaerophiles grow near the top but not right at the top. Anaerobes grow only near the bottom of the tube, where O_2 cannot penetrate. The redox indicator dye *resazurin* is present in thioglycolate broth to signal oxic regions; the dye is pink when oxidized and colorless when reduced and so gives a visual assessment of the degree of penetration of O_2 into the medium (Figure 5.25).

To remove all traces of O_2 for the culture of strict anaerobes, one can incubate tubes or plates in a glass jar flushed with an O_2-free gas or fitted with an O_2 consumption system (Figure 5.26*a*). For manipulating cultures in an anoxic atmosphere, special enclosures called *anoxic glove bags* permit work with open cultures in completely anoxic atmospheres (Figure 5.26*b*).

Why Is Oxygen Toxic?

Why are anaerobic microorganisms inhibited in their growth or even killed by oxygen? Molecular oxygen (O_2), per se, is not toxic, but O_2 can be converted to toxic oxygen by-products, and it is these that can harm or kill cells not able to deal with them. These include *superoxide anion* (O_2^-), *hydrogen peroxide* (H_2O_2), and *hydroxyl radical* (OH·). All of these are by-products of the reduction of O_2 to H_2O in respiration (Figure 5.27). Flavoproteins, quinones, and iron–sulfur proteins, electron carriers found in virtually all cells (Section 3.10), also catalyze some of these reductions. Thus, regardless of whether it can respire O_2, an organism exposed to O_2 will experience toxic forms of oxygen, and if not destroyed, these molecules can wreak havoc in cells. For example, superoxide anion and OH· are strong oxidizing agents that can oxidize macromolecules and any other organic compounds in the cell. Peroxides such as H_2O_2 can also damage cell components but are not as toxic as O_2^- or OH·.

Superoxide Dismutase and Other Enzymes That Destroy Toxic Oxygen

A major requirement for inhabiting an oxic world is to keep toxic oxygen molecules under control. Microbes accomplish this in much the same way as plants and animals do. Superoxide anion and H_2O_2 are the most abundant toxic oxygen species, and all cells have enzymes that destroy these compounds (Figure 5.28). The enzymes catalase and peroxidase attack H_2O_2, forming O_2 and H_2O, respectively (Figure 5.28 and Figure 5.29). Superoxide anion is destroyed by the enzyme *superoxide dismutase*, an enzyme that generates H_2O_2 and O_2 from two molecules of O_2^- (Figure 5.28*c*). Superoxide dismutase and catalase (or peroxidase) thus work in series to convert O_2^- to harmless products (Figure 5.28*d*).

Aerobes and facultative aerobes typically contain both superoxide dismutase and catalase. Superoxide dismutase is an essential enzyme for aerobes. Some aerotolerant anaerobes lack superoxide dismutase and use protein-free manganese complexes instead to carry out the dismutation of O_2^- to H_2O_2 and O_2. Such a system is not as efficient as superoxide dismutase, but it is sufficient to protect the cells from O_2^- damage. In some strictly anaerobic *Archaea* and *Bacteria*, superoxide dismutase is absent and instead the enzyme *superoxide reductase* functions to remove O_2^-. Unlike superoxide dismutase, superoxide reductase reduces O_2^- to H_2O_2 without the production of O_2 (Figure 5.28*e*), thus avoiding exposure of the organism to O_2.

Reactants		Products	
$O_2 + e^-$	→	O_2^-	(superoxide)
$O_2^- + e^- + 2\,H^+$	→	H_2O_2	(hydrogen peroxide)
$H_2O_2 + e^- + H^+$	→	$H_2O + OH^\bullet$	(hydroxyl radical)
$OH^\bullet + e^- + H^+$	→	H_2O	(water)

Outcome:

$$O_2 + 4\,e^- + 4\,H^+ \rightarrow 2\,H_2O$$

Figure 5.27 Four-electron reduction of O_2 to H_2O by stepwise addition of electrons. All the intermediates formed are reactive and toxic to cells; water is not.

$$H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$$

(a) **Catalase**

$$H_2O_2 + \text{NADH} + H^+ \rightarrow 2\,H_2O + NAD^+$$

(b) **Peroxidase**

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

(c) **Superoxide dismutase**

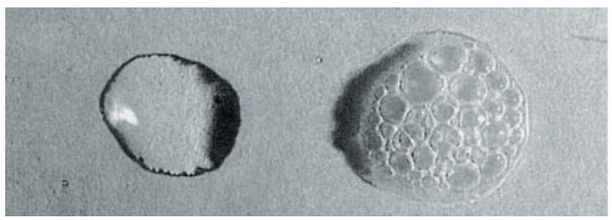
$$4\,O_2^- + 4\,H^+ \rightarrow 2\,H_2O + 3\,O_2$$

(d) **Superoxide dismutase/catalase in combination**

$$O_2^- + 2\,H^+ + \text{rubredoxin}_{\text{reduced}} \rightarrow H_2O_2 + \text{rubredoxin}_{\text{oxidized}}$$

(e) **Superoxide reductase**

Figure 5.28 Enzymes that destroy toxic oxygen species. *(a)* Catalases and *(b)* peroxidases are porphyrin-containing proteins, although some flavoproteins may consume toxic oxygen species as well. *(c)* Superoxide dismutases are metal-containing proteins, the metals being copper and zinc, manganese, or iron. *(d)* Combined reaction of superoxide dismutase and catalase. *(e)* Superoxide reductase catalyzes the one-electron reduction of O_2^- to H_2O_2.



T. D. Brock

Figure 5.29 Method for testing a microbial culture for the presence of catalase. A heavy loopful of cells from an agar culture was mixed on a slide (right) with a drop of 30% hydrogen peroxide. The immediate appearance of bubbles is indicative of the presence of catalase. The bubbles are O_2 produced by the reaction $H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$.

MINIQUIZ

- How does an obligate aerobe differ from a facultative aerobe?
- How does a reducing agent work? Give an example of a reducing agent.
- How does superoxide dismutase or superoxide reductase protect a cell?

V • Controlling Microbial Growth

Thus far in this chapter we have discussed microbial growth from the perspective of conditions that *promote* their growth. We close by considering the opposite side of the coin, microbial growth *control*.

Many aspects of microbial growth control have significant practical applications. For example, we wash fresh produce to remove attached microorganisms and we inhibit microbial growth on body surfaces by washing. However, neither of these processes kills or removes all microorganisms. Only **sterilization**—the killing or removal of all microorganisms (including viruses)—ensures that this is the case. In many circumstances, sterility is not required. In others, however, sterilization is absolutely essential.

5.15 General Principles and Growth Control by Heat

The effects of microorganisms can often be controlled by simply limiting or inhibiting growth. Methods for inhibiting microbial growth include *decontamination*, the treatment of an object or surface to make it safe to handle, and **disinfection**, a process that directly targets pathogens although it may not eliminate all microorganisms. Decontamination can be as simple as wiping off food utensils to remove food fragments (and their attached organisms) before using them, while disinfection requires agents called *disinfectants* that actually kill microorganisms or severely inhibit their growth. Physical methods of microbial growth control are used extensively in industry, medicine, and the home, and we consider three classes of physical controls in this section and the next: heat, radiation, and filtration. Of these three, heat is the most widely used method of physically treating an object or substance to render it sterile.

Heat Sterilization

The effectiveness of heat as a sterilant is quantified by the time required for a 10-fold reduction in the viability of a microbial population at a given temperature. This is called the *decimal reduction time* (*D*). The relationship between *D* and temperature is exponential, as the logarithm of *D* plotted against temperature yields a straight line (**Figure 5.30**). Moreover, heat killing proceeds more rapidly as the temperature rises. The type of heat is also important: Moist heat has better penetrating power than dry heat and, at a given temperature, inhibits growth or kills cells more quickly than does dry heat.

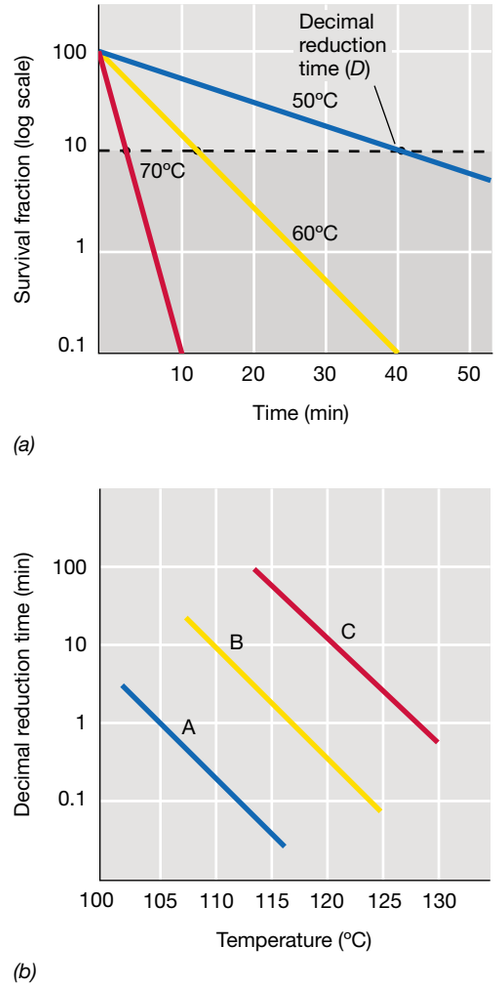
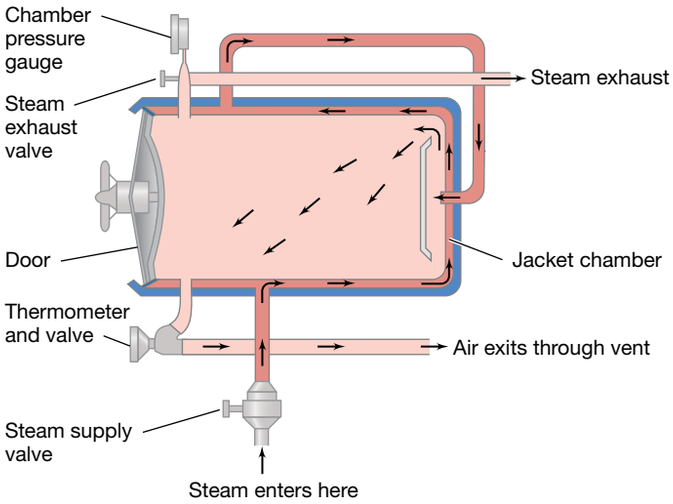


Figure 5.30 The effect of temperature on the heat killing of microorganisms. *(a)* The decimal reduction time (*D*) is the time at which only 10% of the original population of a given organism (in this case, a mesophile) remains viable at a given temperature. For 70°C, $D = 3$ min; for 60°C, $D = 12$ min; for 50°C, $D = 42$ min. *(b)* *D* values for model organisms of different temperature classes: A, mesophile; B, thermophile; C, hyperthermophile.

Another way to characterize the heat sensitivity of an organism is to measure its *thermal death time*, the time it takes to kill all cells at a given temperature. To determine the thermal death time, samples of a cell suspension are heated for different times, mixed with culture medium, and incubated. If all the cells have been killed, no growth is observed in the incubated samples. However, unlike a decimal reduction time measurement that is independent of the original cell number, the thermal death time is greatly affected by population size; a longer time is required to kill all cells in a large population than in a small one.

The presence of endospore-forming bacteria in a heat-treated sample can influence both the decimal reduction and thermal death times. Recall that the mature endospore is very dehydrated and contains calcium dipicolinate and small acid-soluble spore proteins (SASPs) that help confer heat stability on the structure (Section 2.10). The medium in which heating takes place also



(a)

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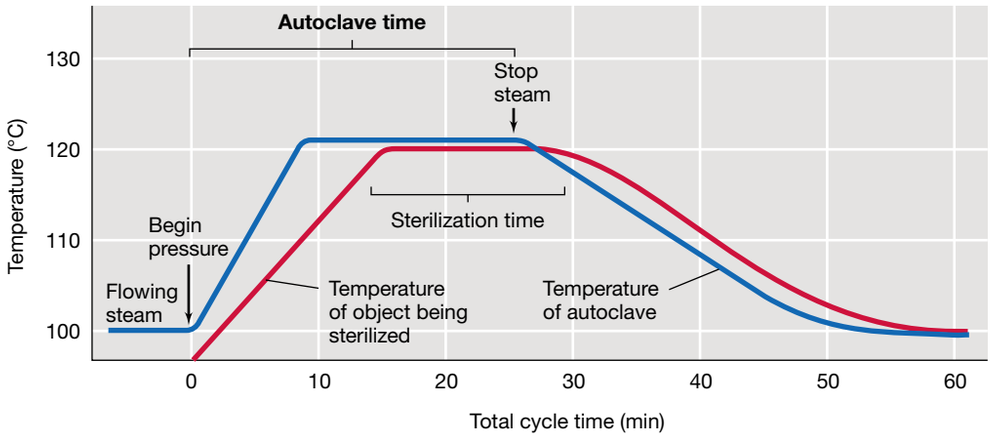


(b)

Figure 5.31 The autoclave and moist heat sterilization. *(a)* The flow of steam through an autoclave. *(b)* A typical autoclave cycle. The temporal heating profile of a fairly bulky object is shown. The temperature of the object rises and falls more slowly than the temperature of the autoclave. The temperature of the object must reach the target temperature and be held for 10–15 min to ensure sterility, regardless of the temperature and time recorded in the autoclave. *(c)* A modern research autoclave. Note the pressure-lock door and the automatic cycle controls on the right panel. The steam inlet and exhaust fittings are on the right side of the autoclave.

influences the rate of killing, and this is especially relevant in food canning procedures. Microbial death is more rapid at acidic pH, and acid foods such as tomatoes, fruits, and pickles are easier to sterilize than neutral-pH foods such as corn and beans. Moreover, high concentrations of sugars, proteins, and fats decrease heat penetration and usually increase the resistance of organisms to heat, whereas high salt concentrations may either increase or decrease heat resistance, depending on the organism.

The Autoclave and Pasteurization

The **autoclave** is a sealed heating device that uses steam under pressure to kill microorganisms (**Figure 5.31**). Killing of heat-resistant endospores requires heating at temperatures above the boiling point of water at 1 atm. The autoclave places steam under a pressure of 1.1 kg/cm^2 (15 lb/in^2), which yields a temperature of 121°C. At 121°C, the time to achieve sterilization of small amounts of endospore-containing material is about 15 min (Figure 5.31*b*). If the object to be autoclaved is bulky or large volumes of liquids are to be sterilized, heat transfer to the interior is retarded, and thus the total heating time must be extended. Note that it is not the *pressure* inside the autoclave that kills the microorganisms but the high *temperatures* that are achieved when steam is placed under pressure.

Pasteurization, a process named for Louis Pasteur (Section 1.9), is not the same as sterilization. Pasteurization uses heat to significantly reduce rather than totally eliminate the microorganisms found in liquids, such as milk. At the temperatures and times standardized for the pasteurization of food products, all known pathogenic bacteria are killed. In addition, however, by decreasing the overall microbial load, pasteurization increases the shelf life of perishable liquids.

To pasteurize milk, the liquid is passed through a tubular heat exchanger. Careful control of flow rate and the size and temperature of the heat source raises the temperature of the milk to 71°C for 15 seconds (or even higher temperatures for shorter time periods; see Figure 5.30), after which it is rapidly cooled. This process is called *flash pasteurization*. *Ultrahigh-temperature (UHT) pasteurization* of milk requires heat treatment at 135°C for 1–2 sec and actually sterilizes the milk such that it can be stored at room temperature for long periods without spoilage.

MINIQUIZ

- Why is heat an effective sterilizing agent?
- What steps are necessary to ensure the sterility of material contaminated with bacterial endospores?
- Distinguish between the sterilization of microbiological media and the pasteurization of dairy products.

5.16 Other Physical Control Methods: Radiation and Filtration

In addition to heat, radiation, in particular ultraviolet (UV) light, X-rays, and gamma rays, are also effective microbial killing agents. However, each type of energy has a different mode of action and killing efficacy and thus their applications vary widely.

Ultraviolet and Ionizing Radiation

Ultraviolet radiation between 220 and 300 nm is absorbed by DNA and can cause mutations or have other serious effects on DNA that lead to death of the exposed organism (↬ Section 11.4). UV radiation is useful for disinfecting surfaces and air, and is widely used to decontaminate and disinfect the work surface of laboratory laminar flow hoods equipped with a "germicidal" UV light (**Figure 5.32**) and air circulating in hospital and food preparation rooms. However, UV radiation has very poor penetrating power, limiting its use to the disinfection of exposed surfaces or air rather than bulk objects such as canned foods or surgical clothing.

Ionizing radiation is electromagnetic radiation of sufficient energy to produce ions and other reactive molecular species from molecules with which the radiation particles collide. The unit of ionizing radiation is the *roentgen*, and the standard for sterilization is the *absorbed radiation dose*, measured in *rads* (100 erg/g) or *grays* (1 Gy = 100 rad). Ionizing radiation is typically generated from X-ray sources or the radioactive nuclides ^{60}Co and ^{137}Cs. These nuclides produce X-rays or gamma rays, both of which have sufficient energy and penetrating power to kill microorganisms in bulk items such as food products and medical supplies.

Table 5.7 shows the dose necessary for a 10-fold reduction (*D*10) in number of selected microorganisms. The *D*10 value is analogous to the decimal reduction time for heat sterilization, and the killing curve of ionizing radiation yields a similar plot (**Figure 5.33**; compare with Figure 5.30). As is also true of heat treatments, killing endospores with ionizing radiation is more difficult than killing vegetative cells, and viruses are more difficult to kill than bacteria (Table 5.7). In addition, microorganisms in general are much more resistant to ionizing radiation than are multicellular organisms. For example, the lethal radiation dose for humans can be as low as 10 Gy if delivered over a short time period.

In the United States, radiation is used for the sterilization of such diverse items as surgical supplies, plastic labware, drugs, and even tissue grafts. Certain foods and food products such as fresh produce, poultry, meat products, and spices are also routinely irradiated to ensure that they are sterile or at least free of pathogens and insects.

TABLE 5.7 Radiation sensitivity of some representative microorganisms

Type of microorganism	*Characteristics*	*D10[a] (Gy)*
Bacteria		
Clostridium botulinum	Gram-positive anaerobe; forms endospores	3,300
Deinococcus radiodurans	Gram-negative, radiation-resistant coccus	2,200
Lactobacillus brevis	Gram-positive, rod-shaped	1,200
Bacillus subtilis	Gram-positive aerobe; forms endospores	600
Escherichia coli	Gram-negative, rod-shaped	300
Salmonella enterica (typhimurium)	Gram-negative, rod-shaped	200
Fungi		
Aspergillus niger	Common mold	500
Saccharomyces cerevisiae	Baker's and brewer's yeast	500
Viruses		
Foot-and-mouth	Pathogen of cloven-hoofed animals	13,000
Coxsackie	Human pathogen	4,500

[a]*D*10 is the amount of radiation necessary to reduce the initial population or activity level 10-fold (1 logarithm, see Figure 5.33). Gy, grays. 1 Gy is equivalent to 100 rads. The lethal dose for humans is 10 Gy.

J. Martinko

Figure 5.32 A laminar flow hood. An ultraviolet light source prevents contamination of the hood when it is not in use. When the hood is in use, air is drawn into the cabinet through a HEPA filter. The filtered air inside the cabinet is exhausted out of the cabinet, preventing contamination of the inside of the hood. The cabinet provides a contaminant-free workspace for microbial and tissue culture manipulations.

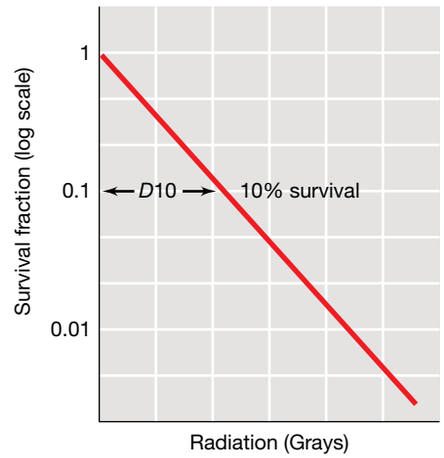


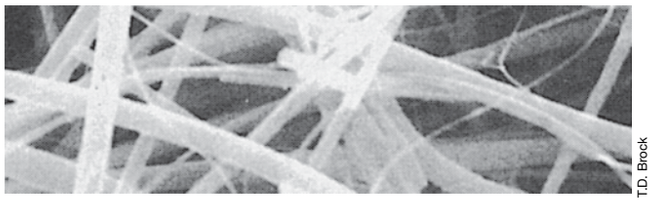
Figure 5.33 Relationship between the survival fraction and the radiation dose of a microorganism. The *D*10, which is the decimal reduction dose, can be interpolated from the data as shown.

Filter Sterilization

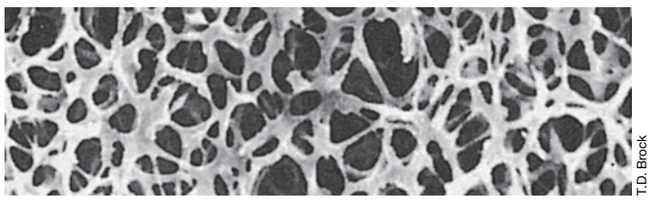
Heat is an effective way to decontaminate most liquids, but heat-sensitive liquids not subject to ionizing radiation are typically sterilized by filtration. For sterilization, a filter with pores of average size 0.2 µm is a minimum requirement; however, even such tiny holes will not trap most viruses. Commonly used filter pore sizes for the filter sterilization of small volumes, such as laboratory solutions, are 0.45 µm and 0.2 µm.

Several types of filters are in routine use in microbiology, including depth filters, membrane filters, and nucleopore filters. A depth filter is a fibrous sheet made from an array of overlapping paper or glass fibers that traps particles in the network of fibers (**Figure 5.34*a***). Depth filters are important in biosafety applications such as in a biological safety cabinet where air, both into and out of the cabinet, flows through a depth filter called a *HEPA filter*, or *h*igh-*e*fficiency *p*articulate *a*ir filter (Figure 5.34*a*). HEPA filters typically remove 0.3-µm or larger particles from an airstream with an efficiency of greater than 99.9%. This does not ensure sterilization, however.

Membrane filters are the most common filters used for liquid sterilization in the microbiology laboratory (Figure 5.34*b* and **Figure 5.35**). Membrane filters are composed of high-tensile-strength polymers manufactured in such a way as to contain a large number of tiny pores. Filtration is accomplished by using a syringe or a pump to force the liquid through the filtration apparatus into a sterile collection vessel (Figure 5.35). Another type of membrane filter is the nucleopore filter (Figure 5.34*c*). Nucleopore filters are made from a thin polycarbonate film that is treated with radiation and then etched with a chemical, yielding very uniform holes (Figure 5.34*c*). Nucleopore filters are commonly used to isolate specimens for scanning electron microscopy. Microorganisms removed by filtration from a liquid or a natural sample, such as lake water, can then be observed directly on the filter (Figure 5.34*d*).



(a)



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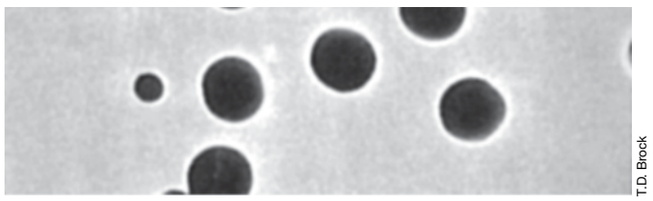
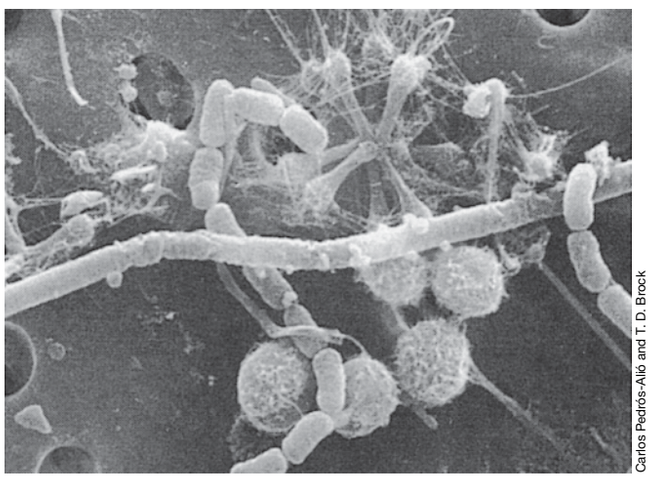


(c)



(d)

Figure 5.34 Microbiological filters. Scanning electron micrograph showing the structure of *(a)* a depth filter, *(b)* a conventional membrane filter, and *(c)* a nucleopore filter. *(d)* Scanning electron micrograph of various aquatic microbes trapped on a nucleopore 0.2-µm-pore-size membrane filter.

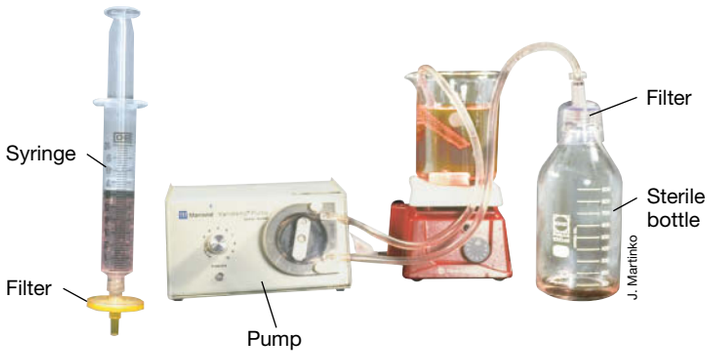


Figure 5.35 Membrane filters. Disposable, presterilized, and assembled membrane filter units. Left: a filter system designed for small volumes forced through the filter by a syringe. Right: a system employing a peristaltic pump for filtering larger volumes.

MINIQUIZ

- Define *D*10 and explain why the killing dose for radiation (Table 5.7) is not the same for all bacteria.
- Why is ionizing radiation more effective than UV radiation for sterilization of food products?
- Distinguish between the major types of sterilization filters used in the microbiology laboratory.

5.17 Chemical Control of Microbial Growth

Chemicals are routinely used to control microbial growth, and an **antimicrobial agent** is a natural or synthetic chemical that kills or inhibits the growth of microorganisms. Agents that actually kill are called *-cidal* agents, with a prefix indicating the type of microorganism killed. Thus, **bactericidal**, *fungicidal*, and *viricidal* agents kill bacteria, fungi, and viruses, respectively. Agents that do not kill but only inhibit growth are called *-static* agents, and include **bacteriostatic**, *fungistatic*, and *viristatic* compounds. We focus

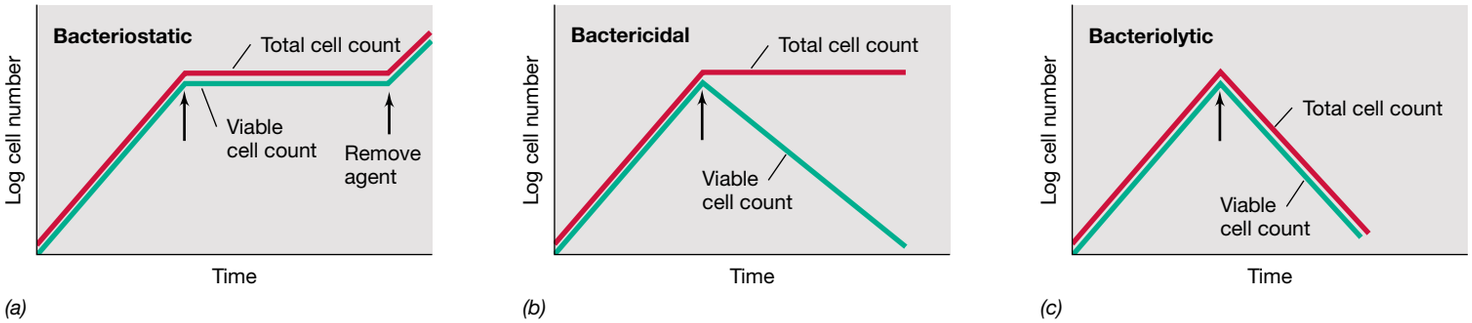


Figure 5.36 Different types of antimicrobial agents. *(a)* Bacteriostatic agents inhibit but do not kill. *(b)* Bactericidal agents kill. *(c)* Bacteriolytic agents lyse cells. At the time indicated by the arrow, a growth-inhibitory concentration of each antimicrobial agent was added to an exponentially growing culture. The turbidity and viable counts shown are characteristic of each type of agent.

here on chemicals commonly used as disinfectants and reserve discussion of the activities of a very important class of chemicals—the antibiotics—for later (Sections 7.10, 24.10, and 28.10–28.12).

Effect of Antimicrobial Agents on Growth

Antibacterial agents are classified as -static, -cidal, or -lytic (cell lysing) by observing their effects on cultures using viable and turbidimetric growth assays (**Figure 5.36**). Bacteriostatic agents are typically inhibitors of some important biochemical process, such as protein synthesis, and bind relatively weakly; if the agent is removed, the cells can resume growing. Some antibiotics work in this way. By contrast, bactericidal agents, for example formaldehyde, bind tightly to their cellular targets and by definition kill the cell. However, the dead cells are not lysed, and total cell numbers, reflected in the turbidity of the culture, remain constant (Figure 5.36*b*). Bacteriolytic agents kill cells by lysing them, and this affects both viable and total cell numbers (Figure 5.36*c*). An example of a bacteriolytic agent would be a detergent that ruptures the cytoplasmic membrane.

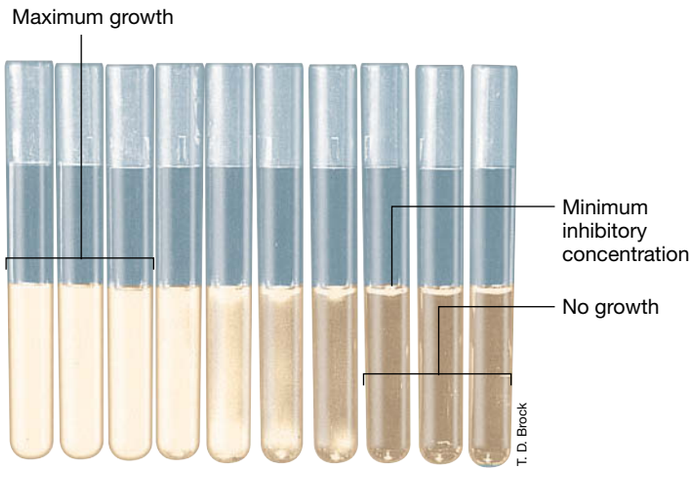


Figure 5.37 Antimicrobial agent susceptibility assay using dilution methods. The assay defines the minimum inhibitory concentration (MIC). A series of increasing concentrations of antimicrobial agent is prepared in the culture medium. Each tube is inoculated with a specific concentration of a test organism, followed by a defined incubation period. Growth, measured as turbidity, occurs in those tubes with antimicrobial agent concentrations below the MIC.

Assaying Antimicrobial Activity

Antimicrobial activity can be measured by determining the smallest amount of the agent needed to inhibit the growth of a test organism, a value called the **minimum inhibitory concentration (MIC)**. One way to determine the MIC of a given agent is to inoculate a series of tubes of liquid growth medium (**Figure 5.37**) containing a test organism and dilutions of the agent. Following incubation, the tubes are scored for growth (turbidimetrically), and the MIC is revealed as the lowest concentration of antimicrobial agent that completely inhibits growth.

Antimicrobial activity can also be assessed using solid media (**Figure 5.38**). Known amounts of an antimicrobial agent are

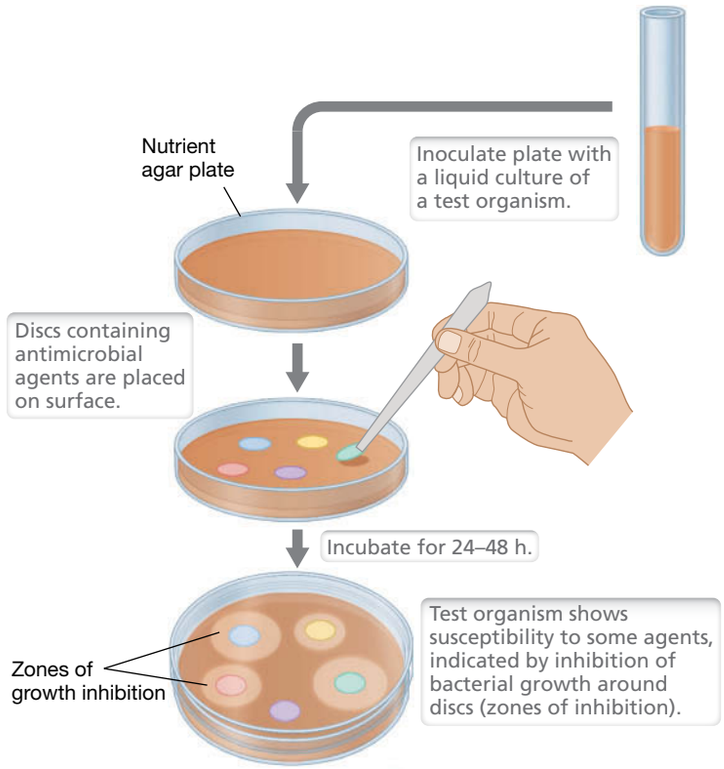


Figure 5.38 Antimicrobial agent susceptibility assay using diffusion methods. The antimicrobial agent diffuses from paper discs into the surrounding agar, inhibiting growth of susceptible microorganisms.

added to filter-paper discs and the discs are arranged on the surface of a uniformly inoculated agar plate. During incubation, the agent diffuses from the disc into the agar, establishing a gradient; the farther a chemical diffuses away from a disc, the lower its concentration. Following an incubation period, a *zone of growth inhibition* forms around discs that released effective chemicals; the zone is a function of several factors, including the amount of antimicrobial agent added to the disc, its solubility and diffusion coefficient, and, most importantly, its overall effectiveness. The disc diffusion assay is routinely used to test clinically isolated pathogenic bacteria for their antibiotic susceptibility (Section 28.4).

Chemical Antimicrobial Agents

Several antimicrobial agents are used to prevent the growth of human pathogens on inanimate surfaces and on external body surfaces. These include sterilants, disinfectants, sanitizers, and antiseptics (Table 5.8).

Sterilants destroy all microorganisms, including endospores. Chemical sterilants are used for decontamination or sterilization in situations where it is impractical or impossible to use heat or radiation. Hospitals, clinics, and laboratories, for example, must routinely decontaminate and sterilize heat-sensitive materials such as thermometers, lensed instruments, polyethylene tubing, catheters, and reusable medical and dental equipment. This process of *cold sterilization*, as it is called, employs gases such as ethylene oxide or aldehydes such as formaldehyde or glutaraldehyde to sterilize the devices.

Disinfectants are chemicals that kill microorganisms but not necessarily endospores and are primarily used on surfaces. For example, phenol and cationic detergents are used to disinfect floors, tables, bench tops, walls, and so on (Table 5.8) and are important agents of infection control in hospitals and other medical settings. **Sanitizers**, by contrast, are less harsh than disinfectants and reduce microbial numbers but do not sterilize. Sanitizers are widely used in the food industry to treat surfaces such as mixing and cooking

TABLE 5.8 Antiseptics, sterilants, disinfectants, and sanitizers[a]

Agent	Mode of action	Use
Antiseptics (germicides)		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	Topical antiseptic
Phenol-containing compounds (hexachlorophene, triclosan, chloroxylenol, chlorhexidine)	Disrupts cytoplasmic membrane	Soaps, lotions, cosmetics, deodorants, topical disinfectants; paper, leather, and textile industries
Cationic detergents, especially quaternary ammonium compounds (benzalkonium chloride)	Disrupts cytoplasmic membrane	Soaps, lotions, topical disinfectants; metal and petroleum industries
Hydrogen peroxide (3% solution)	Oxidizing agent	Topical antiseptic
Iodophors (Betadine®)	Iodinates proteins, rendering them nonfunctional; oxidizing agent	Topical antiseptic
Octenidine	Cationic surfactant, disrupts cytoplasmic membrane	Topical antiseptic
Sterilants, disinfectants, and sanitizers		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	General-purpose disinfectant for virtually any surface
Cationic detergents (quaternary ammonium compounds, Lysol® and many related disinfectants)	Interact with phospholipids	Disinfectant/sanitizer for medical instruments, food and dairy equipment
Chlorine gas	Oxidizing agent	Disinfectant for drinking water and electrical/nuclear cooling towers
Chlorine compounds (chloramines, sodium hypochlorite, sodium chlorite, chlorine dioxide)	Oxidizing agent	Disinfectant/sanitizer for medical instruments, food/dairy equipment, and in water purification
Copper sulfate	Protein precipitant	Algicide in swimming pools
Ethylene oxide (gas)	Alkylating agent	Sterilant for temperature-sensitive materials such as plastics
Formaldehyde	Alkylating agent	Diluted (3% solution) as surface disinfectant/sterilant; concentrated (37% solution) as sterilant
Glutaraldehyde	Alkylating agent	Disinfectant or sterilant as 2% solution
Hydrogen peroxide	Oxidizing agent	Vapor used as sterilant
Iodophors (Wescodyne®)	Iodinates proteins; oxidizing agent	General disinfectant
OPA (ortho-phthalaldehyde)	Alkylating agent	Powerful disinfectant used for sterilizing medical instruments
Ozone	Strong oxidizing agent	Disinfectant for drinking water
Peroxyacetic acid	Strong oxidizing agent	Disinfectant/sterilant
Phenolic compounds	Protein denaturant	General-purpose disinfectant
Pine oils (Pine-Sol®) (contains phenolics and detergents)	Protein denaturant	General-purpose disinfectant for household surfaces

[a]Alcohols, hydrogen peroxide, and iodophors can be antiseptics, disinfectants, sanitizers, or sterilants depending on concentration, length of exposure, and form of delivery.

equipment, dishes, and utensils, and are also used for dry hand washing when water is unavailable. **Antiseptics**, often called **germicides**, are chemicals that kill or inhibit the growth of microorganisms but are sufficiently nontoxic to animals to be applied to living tissues. Most germicides are used for hand washing or for treating surface wounds (Table 5.8). Certain antiseptics are also effective disinfectants. Ethanol, for example, can be both an antiseptic and a disinfectant, depending on the concentration and exposure time employed.

Several factors affect the efficacy of any chemical antimicrobial agent. For example, many antimicrobial agents are bound and inactivated by organic matter; thus, disinfecting a kitchen countertop littered with spilled foods is more difficult than disinfecting a clean countertop. Furthermore, bacteria often form biofilms (Section 5.1), covering surfaces of tissue or soiled medical devices with microbial cells embedded in polysaccharides. Biofilms may slow or even completely prevent penetration of antimicrobial agents, reducing or negating their effectiveness. Thus, the ultimate efficacy of any antimicrobial agent must be determined empirically and under the actual conditions of use. Only by actually testing the chemical and assaying for microbial growth both before and after treatment can one be confident that the agent is working as it should.

MINIQUIZ

- Distinguish between the antimicrobial effects of -static, -cidal, and -lytic agents.
- Describe how the minimum inhibitory concentration of an antibacterial agent is determined.
- Distinguish between a sterilant, a disinfectant, and an antiseptic. What is cold sterilization?

Chapter Review

I • Cell Division and Population Growth

5.1 Microbial growth is defined as an increase in cell numbers and is the final result of the doubling of all cell components prior to actual division that yields two daughter cells. Most microorganisms grow by binary fission but some grow by budding. Biofilms are an alternative growth mode to a suspended (planktonic) lifestyle.

Q What are the advantages of biofilms as a growth mode over a planktonic lifestyle?

5.2 Microbial cells undergo exponential growth, and a semilogarithmic plot of cell numbers with time can reveal the doubling time of the population. Various growth expressions can be calculated from cell number data obtained from an exponentially growing culture. Key expressions here are n, the number of generations; t, time; g, generation time, and k, the instantaneous growth rate constant.

Q How is the generation time (g) of an exponentially growing culture determined?

5.3 Microorganisms show a characteristic growth pattern when inoculated into a fresh culture medium. There is usually a lag phase and then growth commences in an exponential fashion. As essential nutrients are depleted and/or toxic products build up, growth ceases and the population enters the stationary phase. Further incubation can lead to cell death.

Q How does the growth pattern of a population of bacteria inoculated into a fresh medium from an old culture differ from the growth pattern of a population inoculated into a fresh medium from a culture in the mid-exponential phase of growth?

5.4 The chemostat is an open system used to maintain cell populations in exponential growth for extended periods. In a chemostat, the rate at which a culture is diluted with fresh growth medium controls the doubling time of the population, while the cell density (cells/ml) is controlled by the concentration of a growth-limiting nutrient dissolved in the fresh medium.

Q How does a chemostat regulate growth rate and cell density independently?

II • Culturing Microbes and Measuring Their Growth

5.5 Culture media supply the nutritional needs of microorganisms and are either defined or complex. Other media, such as selective, differential, and enriched media, are used for specific purposes. Many microorganisms can be grown in liquid or solid culture media, and pure cultures can be maintained if aseptic technique is practiced.

Q Why would the following medium not be considered a chemically defined medium: glucose, 5 grams (g); NH_4Cl, 1 g; KH_2PO_4, 1 g; $MgSO_4$, 0.3 g; yeast extract, 5 g; distilled water, 1 liter? What is aseptic technique and why is it necessary?

5.6 Total cell counts can be done under the microscope using counting chambers and are useful for assessing the total cell numbers in a microbial habitat or laboratory culture. Certain stains can be used to target specific cell populations in a sample.

Q When assessing a culture's performance, in which growth phase would total cell counting be suitable, and

in which growth phase would knowing the viability of the culture or sample become necessary?

5.7 Viable cell counts measure only the living population present in the sample with the assumption that each colony originates from the growth and division of a single cell. Depending on the growth medium and conditions employed, and the nature of the sample, viable counts can be fairly accurate assessments or highly unreliable.

Q How does a viable count differ from a total count?

5.8 Turbidity measurements are a rapid and useful method of measuring microbial growth and are based on the fact that cells in suspension scatter light. However, in order to relate a turbidity value to a cell number, a standard curve plotting these two parameters against one another must first be established.

Q How can turbidity be used as a measure of cell numbers?

III • Environmental Effects on Growth: Temperature

5.9 Temperature is a major environmental factor controlling microbial growth. An organism's cardinal temperatures describe the minimum, optimum, and maximum temperatures at which it grows. Microorganisms can be grouped by their cardinal temperature from cold-loving to heat-loving as psychrophiles, mesophiles, thermophiles, and hyperthermophiles.

Q Examine the graph in Figure 5.17. Why is the optimum temperature for an organism usually closer to its maximum than its minimum?

5.10 Organisms with temperature optima below 15°C are called psychrophiles, and the most extreme representatives inhabit constantly cold environments. Psychrophiles synthesize macromolecules that remain flexible and functional at cold temperatures but that can be unusually sensitive to warm temperatures.

Q How do the proteins and lipids of psychrophiles differ from those of mesophiles?

5.11 Organisms with growth temperature optima between 45 and 80°C are called thermophiles while those with optima greater than 80°C are called hyperthermophiles. These organisms inhabit hot environments that can have temperatures even above 100°C. Thermophiles and hyperthermophiles produce heat-stable macromolecules.

Q What are some important biotechnological uses from hyperthermophiles?

IV • Environmental Effects on Growth: pH, Osmolarity, and Oxygen

5.12 The acidity or alkalinity of an environment can greatly affect microbial growth. Some organisms grow best at low or high pH (acidophiles and alkaliphiles, respectively), but most organisms grow best between pH 5.5 and 8. The internal pH of a cell must stay relatively close to neutral to prevent the destruction of macromolecules.

Q Concerning the pH of the environment and of the cell cytoplasm, in what ways are acidophiles and alkaliphiles different? In what ways are they similar?

5.13 The water activity of an aqueous environment is a function of its solute concentration. To survive in high-solute environments, organisms produce or accumulate compatible solutes to maintain positive water balance. Some microorganisms grow best at reduced water potential and some even require high levels of salts for growth.

Q How does a halophile maintain positive water balance while growing in a solution high in NaCl?

5.14 Aerobes require O_2 while anaerobes do not and may even be killed by O_2. Facultative organisms can live with or without O_2. Special techniques are needed to grow aerobic and anaerobic microorganisms. Several toxic forms of oxygen can form in the cell, but enzymes are present that neutralize most of them. Superoxide is a major toxic form of oxygen.

Q Contrast an aerotolerant and an obligate anaerobe in terms of sensitivity to O_2 and ability to grow in the presence of O_2. Compare and contrast the enzymes catalase, superoxide dismutase, and superoxide reductase in regard to their substrates and products.

V • Controlling Microbial Growth

5.15 Sterilization is the killing of all microbes including viruses, and heat is the most widely used method of sterilization. An autoclave employs moist heat under pressure to achieve temperatures above the boiling point of water. Pasteurization does not sterilize liquids, but it reduces the microbial load and kills pathogens.

Q Contrast the terms thermal death time and decimal reduction time. How would the presence of bacterial endospores affect either value? What time and temperature is necessary to ensure sterility in the autoclave?

5.16 Radiation can effectively inhibit or kill microorganisms. Ultraviolet radiation is used for decontaminating surfaces and air, whereas ionizing radiation is used for sterilization where penetration is required. Filters remove microorganisms from air or liquids. Membrane and nucleopore filters are used for sterilization of heat-sensitive liquids and to examine the contents of filtration by microscopy.

Q Describe the effects of a lethal dose of ionizing radiation at the molecular level. What type of filter would be used to filter sterilize a heat-sensitive liquid?

5.17 Chemicals that kill organisms are called -cidal agents while those that arrest growth but do not kill are called -static agents. Antimicrobial agents are tested for efficacy by determining their ability to inhibit growth. Sterilants, disinfectants, and sanitizers are used to decontaminate nonliving material, while antiseptics (germicides) are used to reduce the microbial load on living tissues.

Q Describe the procedure for obtaining the minimum inhibitory concentration (MIC) for a chemical that is bactericidal for *Escherichia coli*. Contrast the action of disinfectants and antiseptics.

Application Questions

1. A medium was inoculated with 5×10^6 cells/ml of *Escherichia coli* cells. Following a 1-h lag, the population grew exponentially for 5 h, after which the population was 5.4×10^9 cells/ml. Calculate *g* and *k* for this growth experiment.
2. *Escherichia coli* but not *Pyrolobus fumarii* will grow at 40°C, while *P. fumarii* but not *E. coli* will grow at 110°C. What is happening (or not happening) to prevent growth of each organism at the nonpermissive temperature?
3. In which direction (into or out of the cell) will water flow in cells of *Escherichia coli* (an organism found in your large intestine) suddenly suspended in a solution of 20% NaCl? What if the cells were suspended in distilled water? If growth nutrients were added to each cell suspension, which (if either) would support growth, and why?

Chapter Glossary

Acidophile an organism that grows best at low pH, typically below pH 5.5

Aerobe an organism that can use O_2 in respiration; some require O_2

Aerotolerant anaerobe a microorganism unable to respire O_2 but whose growth is unaffected by it

Alkaliphile an organism that has a growth pH optimum of 8 or higher

Anaerobe an organism that cannot use O_2 in respiration and whose growth is typically inhibited by O_2

Antimicrobial agent a chemical compound that kills or inhibits the growth of microorganisms

Antiseptic (germicide) a chemical agent that kills or inhibits growth of microorganisms and is sufficiently nontoxic to be applied to living tissues

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

Autoclave a sealed heating device that destroys microorganisms with temperature and steam under pressure

Bactericidal agent an agent that kills bacteria

Bacteriostatic agent an agent that inhibits bacterial growth

Batch culture a closed-system microbial culture of fixed volume

Binary fission cell division following enlargement of a cell to twice its minimum size

Biofilm an attached polysaccharide matrix containing bacterial cells

Budding division a cell division process whereby new cell material is produced from a single point instead of along the entire cell

Cardinal temperatures the minimum, maximum, and optimum growth temperatures for a given organism

Chemostat a device that allows for the continuous culture of microorganisms with independent control of both growth rate and cell number

Compatible solute a molecule that is accumulated in the cytoplasm of a cell for adjustment of water activity but that does not inhibit biochemical processes

Complex medium a culture medium of highly nutritious substances for which the exact composition is unknown

Culture medium a nutrient solution required for growing a laboratory culture of a specific microorganism

Defined medium a culture medium for which the exact composition is known

Disinfectant an antimicrobial agent used only on inanimate objects

Disinfection rendering a surface or object free of all pathogenic microorganisms

Exponential growth growth of a microbial population in which cell numbers double within a specific time interval

Extreme halophile a microorganism that requires very large amounts of NaCl, usually greater than 10% and in some cases near saturation, for growth

Facultative with respect to O_2, an organism that can grow in either its presence or absence

Generation time the time required for a population of microbial cells to double

Germicide (antiseptic) a chemical agent that kills or inhibits growth of microorganisms and is sufficiently nontoxic to be applied to living tissues

Growth in microbiology, an increase in cell number

Halophile a microorganism that requires NaCl for growth

Halotolerant a microorganism that does not require NaCl for growth but can grow in the presence of NaCl, in some cases, substantial levels of NaCl

Hyperthermophile a species of *Bacteria* or *Archaea* whose growth temperature optimum is 80°C or greater

Mesophile an organism that grows best at temperatures between 20 and 40°C

Microaerophile an aerobic organism that can grow only when O_2 tensions are reduced from that present in air

Minimum inhibitory concentration (MIC) the minimum concentration of a substance necessary to prevent microbial growth

Neutrophile an organism that grows best at neutral pH, between pH 5.5 and 8

Obligate anaerobe an organism that cannot grow in the presence of O_2

Osmophile an organism that grows best in the presence of high levels of solute, typically a sugar

Pasteurization the heat treatment of milk or another liquid to reduce its total number of microorganisms

pH the negative logarithm of the hydrogen ion (H^+) concentration of a solution

Plate count a method for counting viable cells; the number of colonies on a plate is used as a measure of cell numbers

Psychrophile an organism with a growth temperature optimum of 15°C or lower and a maximum growth temperature below 20°C

Psychrotolerant capable of growing at low temperatures but having an optimum above 20°C

Sanitizer an agent that reduces microorganisms to a safe level, but may not eliminate them

Sterilant a chemical agent that destroys all forms of microbial life

Sterilization the killing or removal of all living organisms and viruses

Thermophile an organism whose growth temperature optimum lies between 45 and 80°C

Viable capable of reproducing

Viable count a measurement of the concentration of live cells in a population

Water activity the ratio of the vapor pressure of air in equilibrium with a solution to the vapor pressure of pure water

Xerophile an organism that is able to live, or that lives best, in very dry environments

Microbial Regulatory Systems

6

microbiologynow

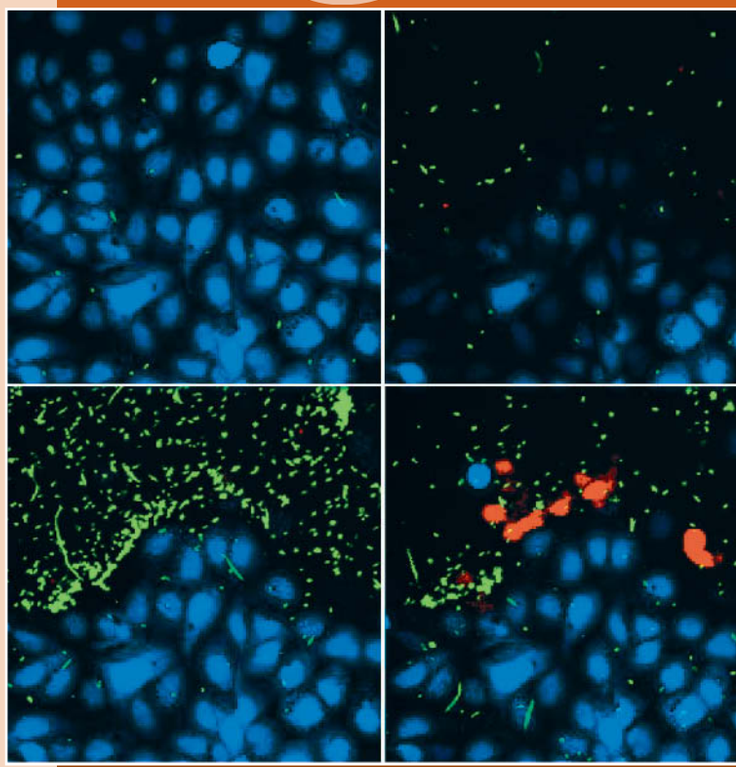
A Microbial Hunter: *Pseudomonas aeruginosa* Senses and Scavenges Nutrients from Damaged Tissues

Despite their single-cell nature, many microbes can sense and respond to their surroundings. Motile bacteria are able to move toward nutrients and away from poisons through a process called chemotaxis. Using special chemoreceptors that extend like antennae to the exterior of the cell, bacteria can sense and respond to a wide range of signals including attractants (such as sugars and amino acids) and repellents (such as certain metals and toxins). Binding of sensory molecules to chemoreceptors sends a signal to the cell's flagellar motor to steer the cell toward or away from the molecule.

This internal sense of direction not only enhances a microbe's ability to hunt down food and avoid toxins, but also plays a critical role in the infection process for some pathogens. For example, the opportunistic human pathogen *Pseudomonas aeruginosa* can respond by chemotaxis to over 25 different molecules and is a major factor in the symptoms observed in the disease cystic fibrosis. Cystic fibrosis patients are unable to clear mucus from their lungs, and this mucus provides nutrients to bacteria. Microbiologists have shown that *P. aeruginosa* cells use chemotaxis to scavenge nutrients from damaged lung tissues by sensing amino acids released from injured cells.

The images here show the remarkable time course of live human epithelial cells (blue) and the successful hunt for nutrients by *P. aeruginosa* cells (green) following host cell damage. Before damage (top left photo) and two minutes after scratching the epithelial monolayer (top right photo), very few bacterial cells are present. However, within 5 minutes of wound induction (bottom left photo), *P. aeruginosa* cells migrate to the damaged epithelial cells to devour nutrients released from the cells. Over time, some of the epithelial cells die (red) and the bacterial hunters move on to scavenge more nutrients from living cells (bottom right photo).

It is truly remarkable that all of these events can occur within half an hour, considering that the directed movement of bacterial cells is an orchestrated process that requires a complex regulatory cascade. But this is the pace at which bacterial regulatory events can occur, as we will see many times in this chapter.



Source: Schwarzer, C., H. Fischer, and T. E. Machen. 2016. Chemotaxis and binding of *Pseudomonas aeruginosa* to scratch-wounded human cystic fibrosis airway epithelial cells. *PLoS ONE 11(3):* e0150109.

To efficiently orchestrate the numerous reactions that occur in a cell and to maximally use available resources, cells must *regulate* the types, amounts, and activities of proteins and other macromolecules they make. Because some proteins and RNA molecules are needed in the cell at about the same level under all growth conditions, the expression of these molecules is said to be *constitutive*. However, more often a particular protein or RNA is needed under some conditions but not others. For instance, enzymes required for using the sugar lactose are useful only if lactose is available to the cell. Ultimately, regulatory systems work to improve the fitness of the organism and its ability to produce the maximum number of offspring that available resources will allow. In this way, regulation and microbial growth go hand in hand.

Overall, cells use two major approaches to regulate protein function. One controls the *amount* of an enzyme or other protein and the second controls the *activity* of a preformed enzyme or other protein. Figure 6.1 gives an overview of the regulatory mechanisms a cell has at its disposal. The amount of protein synthesized can be regulated at either the level of transcription, by varying the amount of mRNA made, or at the level of translation, by translating or not translating the mRNA. Collectively, these processes are called **gene expression**. After the protein has been synthesized, post-translational regulatory processes can further regulate the activity of some proteins. In this chapter we discuss how the cell efficiently controls its metabolism by regulating both enzyme synthesis and enzyme activity.

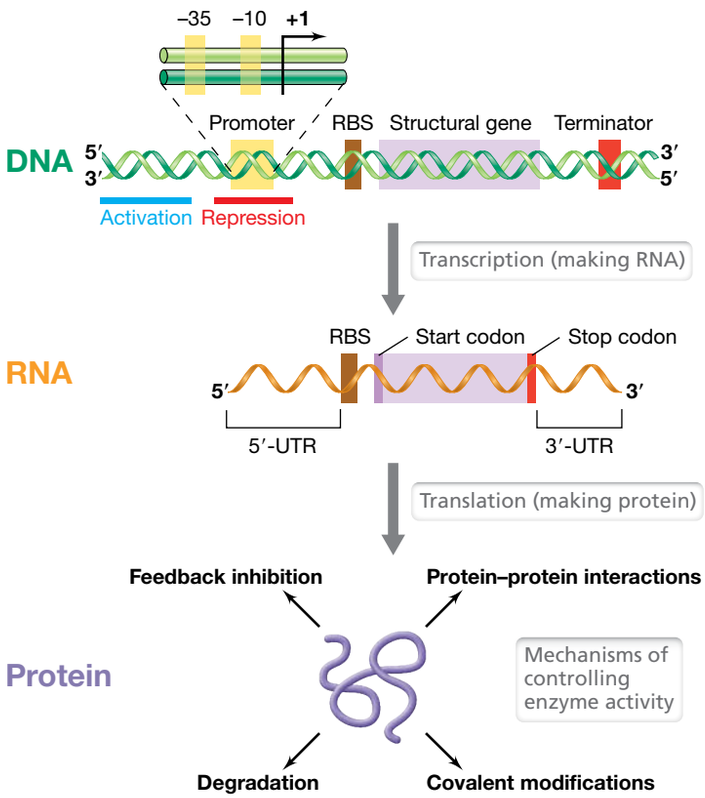


Figure 6.1 Gene expression and regulation of protein activity. For DNA, the promoter and terminator as well as regions related to transcriptional activation and repression are indicated. The 5′ untranslated region (5′-UTR) is a short region between the start of transcription and the start of translation, including the ribosome-binding site (RBS). The 3′ untranslated region (3′-UTR) is a short region between the stop codon and the transcription terminator. These are the regions where translational regulation often occurs. Mechanisms for regulating protein activity after translation are shown in the bottom part of the figure.

I • DNA-Binding Proteins and Transcriptional Regulation

Although several microbial regulatory mechanisms are possible, our discussion begins with control at the level of *transcription* because this is the major means of regulation in *Bacteria* and *Archaea*.

6.1 DNA-Binding Proteins

For a gene to be transcribed, RNA polymerase must recognize a specific promoter on the DNA (Section 4.5), and small molecules often take part in regulating this process. However, they rarely do so directly. Instead, they typically influence the binding of certain proteins, called *regulatory proteins*, to specific sites on the DNA. This event regulates gene expression by turning transcription either on or off.

Interaction of Proteins with Nucleic Acids

Interactions between proteins and nucleic acids are central to replication, transcription, and translation, and also to the regulation of these processes. Protein–nucleic acid interactions may be specific or nonspecific, depending on whether the protein attaches anywhere along the nucleic acid or binds to a specific site. Most DNA-binding proteins interact with DNA in a sequence-specific manner. Specificity is provided by interactions between specific amino acid side chains of the proteins and specific chemical groups on the nitrogenous bases and the sugar–phosphate backbone of the DNA. Because of its size, the *major groove* of DNA (Figure 4.3) is the main site of protein binding, and Figure 4.1*c* identified atoms of the bases in the major groove that are known to interact with proteins. To achieve high specificity, the binding protein must interact simultaneously with several nucleotides.

We have already described a structure in DNA called an *inverted repeat* (Figure 4.24). Such inverted repeats are frequently the locations at which regulatory proteins bind specifically to DNA (Figure 6.2). Note that this interaction does not require the formation of stem–loop structures in the DNA. DNA-binding proteins are often homodimeric, meaning they are composed of two identical polypeptide subunits, each subdivided into **domains**—regions of the protein with a specific structure and function. Each subunit has a domain that interacts specifically with a region of DNA in the major groove. When protein dimers interact with inverted repeats on DNA, each subunit binds to one of the inverted repeats. The dimer as a whole thus binds to both DNA strands (Figure 6.2).

Structure of DNA-Binding Proteins

DNA-binding proteins in both *Bacteria* and *Archaea* as well as eukaryotes possess several classes of protein domains that are

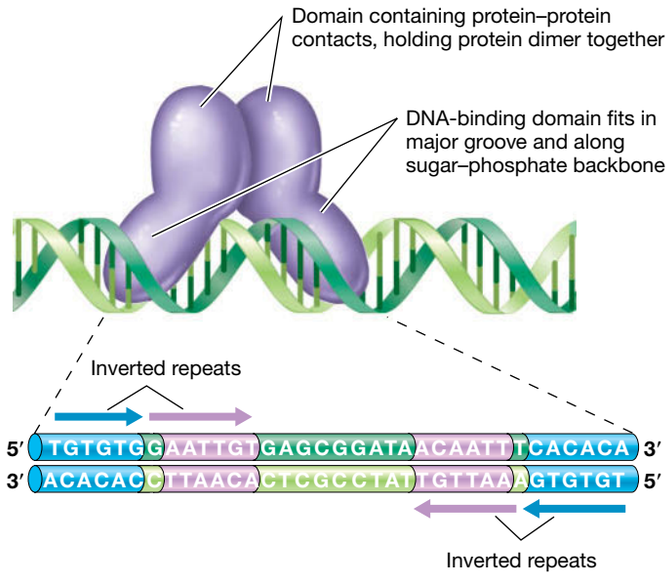


Figure 6.2 DNA-binding proteins. Many DNA-binding proteins are dimers that combine specifically with two sites on the DNA. The specific DNA sequences that interact with the protein are inverted repeats. The nucleotide sequence of the operator gene of the lactose operon (Section 6.2) is shown, and the inverted repeats, which are sites at which the *lac* repressor makes contact with the DNA, are shown in purple and blue boxes.

critical for proper binding to DNA. One of the most common is the *helix-turn-helix* structure (**Figure 6.3*a***). This consists of two segments of polypeptide chain that have an α-helix secondary structure connected by a short sequence forming the "turn." The first helix is the *recognition helix*, which interacts specifically with DNA. The second helix, the *stabilizing helix*, stabilizes the first helix by interacting with it by way of hydrophobic interactions. The turn linking the two helices consists of three amino acid residues, the first of which is typically a glycine. Sequences are recognized by noncovalent interactions, including hydrogen bonds and van der Waals contacts, between the recognition helix of the protein and specific chemical groups in the sequence of base pairs on the DNA.

Many different DNA-binding proteins from *Bacteria* contain the helix-turn-helix structure. These include many repressor proteins, such as the *lac* and *trp* repressors of *Escherichia coli* (Section 6.2 and see Figure 6.3 inset), and some proteins of bacterial viruses, such as the bacteriophage lambda repressor (Figure 6.3*b*). Indeed, over 250 different proteins with this motif bind to DNA to regulate transcription in *E. coli*. Two other types of protein domains are commonly found in DNA-binding proteins. One of these, the *zinc finger*, is frequently found in regulatory proteins in eukaryotes and, as its name implies, binds a zinc ion. The other protein domain commonly found in DNA-binding proteins is the

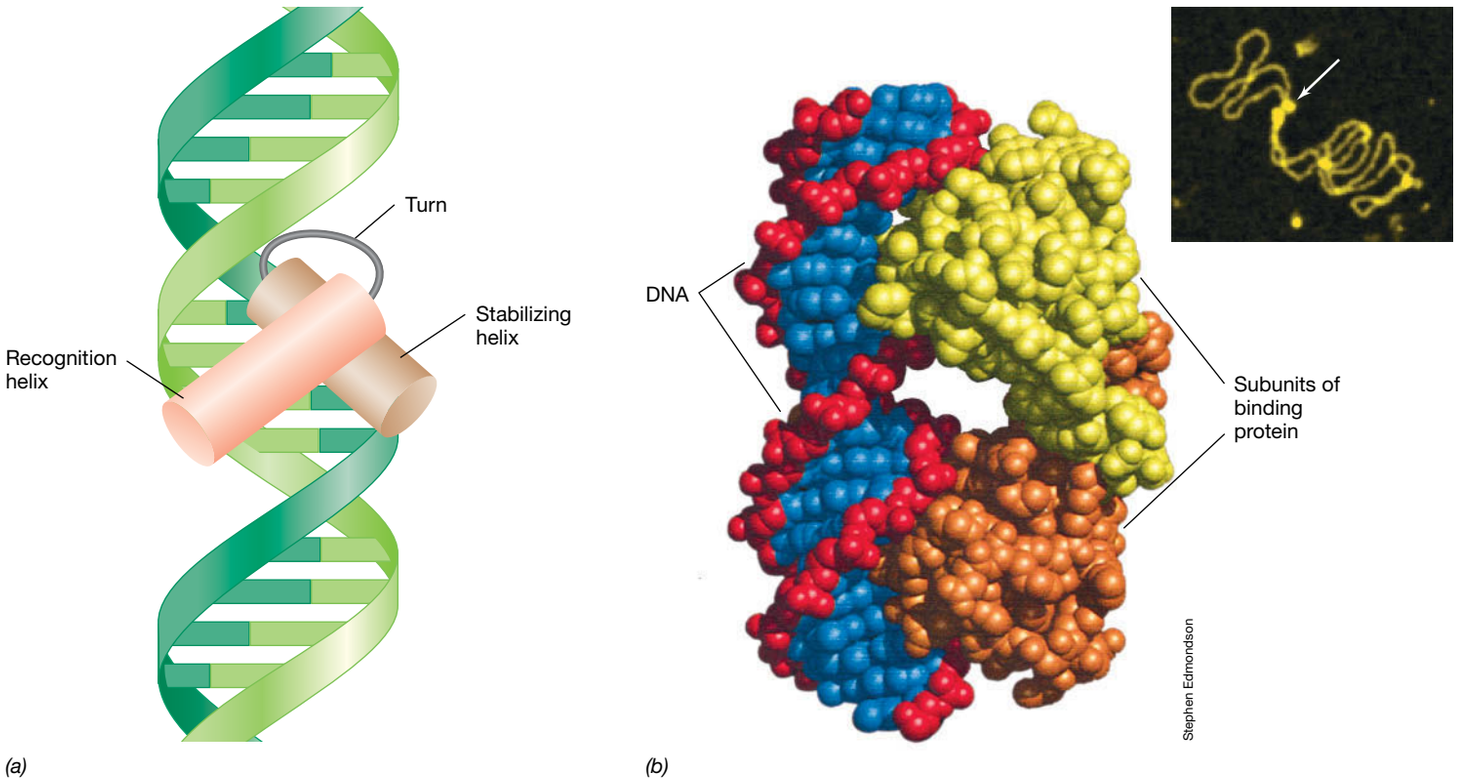


Figure 6.3 The helix-turn-helix structure of some DNA-binding proteins. *(a)* A simple model of the helix-turn-helix structure within a single protein subunit. *(b)* A computer model of both subunits of the bacteriophage lambda repressor bound to its operator. The DNA is red and blue. One subunit of the dimeric repressor is shown in brown and the other in yellow. Each subunit contains a helix-turn-helix structure. The coordinates used to generate this image were downloaded from the Protein Data Bank (http://www.rcsb.org). Inset: Atomic force microscopy showing copies of the LacI repressor protein (arrow) bound to multiple operator sites on a DNA molecule.

leucine zipper, which contains regularly spaced leucine residues that function to hold two recognition helices in the correct orientation to bind DNA.

Once a protein binds at a specific site on the DNA, various outcomes are possible. Some DNA-binding proteins are enzymes that catalyze a specific reaction on the DNA, such as transcription. In other cases, however, the binding event either blocks transcription (*negative regulation*, Section 6.2) or activates it (*positive regulation*, Section 6.3).

MINIQUIZ

- What is a protein domain?
- Why are most DNA-binding proteins specific to certain chemical groups within the DNA?

6.2 Negative Control: Repression and Induction

Transcription is the first step in biological information flow; because of this, it is simple and efficient to control gene expression at this point. If one gene is transcribed more frequently than another, there will be more of its mRNA available for translation and therefore a greater amount of its protein product in the cell. We begin with the processes of repression and induction, simple forms of regulation that govern gene expression at the level of transcription. Here we deal with **negative control** of transcription, control that *prevents* transcription.

Enzyme Repression and Induction

Often the enzymes that catalyze the synthesis of a specific product are not made if the product is already present in the medium in sufficient amounts. For example, in *Escherichia coli* and many other *Bacteria*, the enzymes needed to synthesize the amino acid arginine are made only when arginine is absent from the culture medium; an excess of arginine decreases the synthesis of these enzymes. This is called enzyme **repression**.

As can be seen in Figure 6.4, if arginine is added to a culture growing exponentially in a medium devoid of arginine, growth continues at the previous rate, but production of the enzymes for arginine synthesis stops. Note that this is a *specific* effect, as the synthesis of all other enzymes in the cell continues at the previous rate. This is because the enzymes affected by a particular repression event make up only a tiny fraction of the entire complement of proteins in the cell. Enzyme repression is widespread in bacteria as a means of controlling the synthesis of enzymes required for the production of amino acids and the nucleotide precursors purines and pyrimidines. In most cases, the final product of a particular biosynthetic pathway represses the enzymes of the pathway. This ensures that the organism does not waste energy and nutrients synthesizing unneeded enzymes.

Enzyme **induction** is conceptually the opposite of enzyme repression. In enzyme induction, an enzyme is made only when its substrate is *present*. Enzyme repression typically affects biosynthetic (anabolic) enzymes. In contrast, enzyme induction usually affects degradative (catabolic) enzymes. To illustrate induction, consider

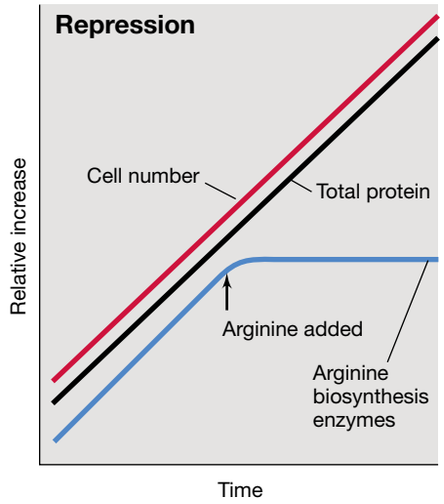


Figure 6.4 Enzyme repression. In a growing bacterial culture, the addition of arginine to the medium specifically represses production of enzymes needed to make arginine. Net protein synthesis is unaffected.

the utilization of the sugar lactose as a carbon and energy source by *E. coli*, the enzymes for which are encoded by the *lac* operon (Section 4.2). Figure 6.5 shows the induction of β-galactosidase, the enzyme that cleaves lactose into glucose and galactose. This enzyme is required for *E. coli* to grow on lactose. If lactose is absent, the enzyme is not made, but synthesis begins almost immediately after lactose is added. The three genes in the *lac* operon encode three proteins, including β-galactosidase, that are induced simultaneously upon adding lactose. This type of control mechanism ensures that specific enzymes are synthesized only when needed.

Inducers and Corepressors

A substance that induces enzyme synthesis is called an *inducer* and a substance that represses enzyme synthesis is called a *corepressor*.

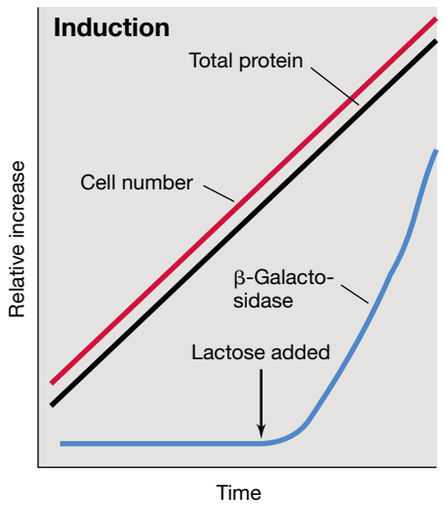


Figure 6.5 Enzyme induction. In a growing bacterial culture, the addition of lactose to the medium specifically induces synthesis of the enzyme β-galactosidase. Net protein synthesis is unaffected.

These substances, which are typically small molecules, are collectively called *effectors*. Interestingly, not all inducers and corepressors are actual substrates or end products of the enzymes involved. For example, structural analogs may induce or repress even though they are not substrates of the enzyme. Isopropylthiogalactoside (IPTG), for instance, is an inducer of β-galactosidase even though IPTG cannot be hydrolyzed by this enzyme. In nature, however, inducers and corepressors are probably normal cell metabolites. Detailed studies of lactose utilization in *E. coli* have shown that the actual inducer of β-galactosidase is not lactose but its isomer allolactose, which is made from lactose.

Mechanism of Repression and Induction

How can inducers and corepressors affect transcription in such a specific manner? They do this indirectly by binding to specific DNA-binding proteins, which, in turn, affect transcription. For an example of a repressible enzyme, we consider the arginine operon (Figure 6.4). **Figure 6.6***a* shows transcription of the arginine genes, which proceeds when the cell needs arginine. However, when arginine is plentiful, it becomes a corepressor. As Figure 6.6*b* shows, arginine binds to a specific **repressor protein**, the *arginine repressor*, present in the cell. The repressor protein is **allosteric**; that is, its conformation is altered when the effector molecule binds to it (Section 6.14).

By binding its effector, the repressor protein is *activated* and can then bind to a specific region of the DNA near the promoter of the gene called the *operator*. This region gave its name to the **operon**, a cluster of consecutive genes whose expression is under the control of a single operator (↔ Section 4.2). All of the genes in an operon are transcribed as a single unit yielding a single mRNA (↔ Section 4.2). The operator is located downstream of the promoter where synthesis of mRNA is initiated (Figure 6.6). If the repressor binds to the operator, transcription is physically blocked because RNA polymerase can neither bind nor proceed. Hence, the polypeptides encoded by the genes in the operon cannot be synthesized. If the mRNA is polycistronic (↔ Section 4.5), all the polypeptides encoded by this mRNA will be repressed.

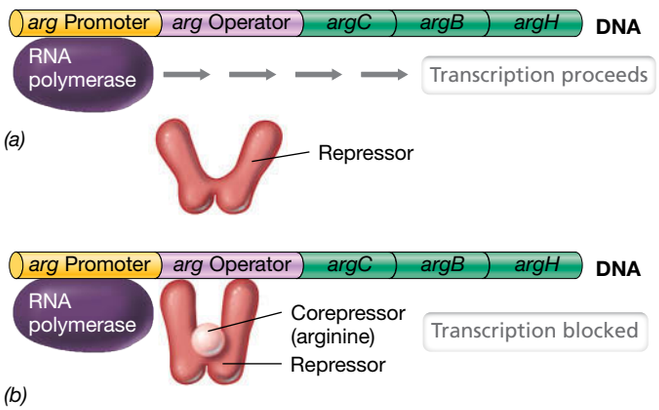


Figure 6.6 Enzyme repression in the arginine operon. *(a)* The operon is transcribed because the repressor is unable to bind to the operator. *(b)* After a corepressor (small molecule) binds to the repressor, the repressor binds to the operator and blocks transcription; mRNA and the proteins it encodes are not made. For the *argCBH* operon, the amino acid arginine is the corepressor that binds to the arginine repressor.

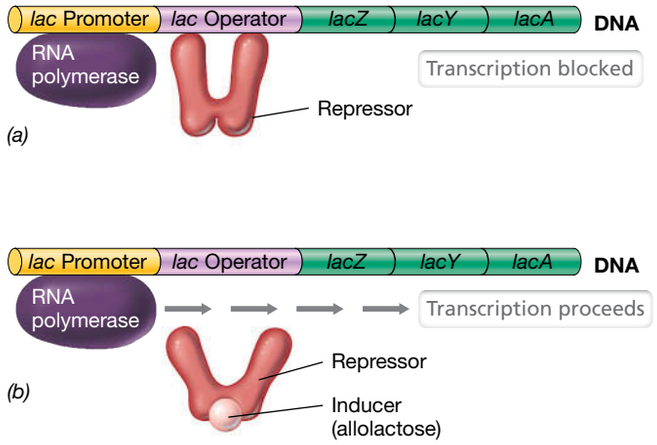


Figure 6.7 Enzyme induction in the lactose operon. *(a)* A repressor protein bound to the operator blocks the binding of RNA polymerase. *(b)* An inducer molecule binds to the repressor and inactivates it so that it no longer can bind to the operator. RNA polymerase then transcribes the DNA and makes an mRNA for that operon. For the *lac* operon, the sugar allolactose is the inducer that binds to the lactose repressor.

Enzyme induction may also be controlled by a repressor. In this case, the repressor protein is *active* in the absence of the inducer, completely blocking transcription. When the inducer is added, it combines with the repressor protein and inactivates it; inhibition is overcome and transcription can proceed (**Figure 6.7**).

All regulatory systems employing repressors have the same underlying mechanism: prevention of mRNA synthesis by the activity of specific repressor proteins that are themselves under the control of specific small effector molecules. And, as previously noted, because the repressor's role is to stop transcription, regulation by repressors is called *negative control*. One point to note is that genes are not turned on and off completely like light switches. DNA-binding proteins vary in concentration and affinity and thus control is quantitative. Even when a gene is "fully repressed" there is often a very low level of basal transcription.

MINIQUIZ

- Why is "negative control" so named?
- How does a repressor inhibit the synthesis of a specific mRNA?

6.3 Positive Control: Activation

Negative control relies on a protein (the repressor) to repress mRNA synthesis. By contrast, in **positive control** of transcription, the regulatory protein is an *activator* that activates the binding of RNA polymerase to DNA. An example of positive regulation is the catabolism of the disaccharide sugar maltose in *Escherichia coli*.

Maltose Catabolism in *Escherichia coli*

The enzymes for maltose catabolism in *E. coli* are synthesized only after the addition of maltose to the medium. The expression of these enzymes thus follows the pattern shown for β-galactosidase in Figure 6.5 except that maltose rather than lactose is required to induce gene expression. However, the synthesis of

maltose-degrading enzymes is not under negative control, as in the *lac* operon, but under positive control; transcription requires the binding of an **activator protein** to the DNA.

The maltose activator protein cannot bind to the DNA unless it first binds maltose, the inducer. When the maltose activator protein binds to DNA, it allows RNA polymerase to begin transcription (Figure 6.8). Like repressor proteins, activator proteins bind specifically to certain chemical groups within the DNA. However, the region on the DNA that is the binding site of the activator is not called an operator (Figures 6.6 and 6.7) but instead an *activator-binding site* (Figure 6.8). Nevertheless, the genes controlled by this activator-binding site are still called an operon.

Binding of Activator Proteins

The promoters of positively controlled operons have nucleotide sequences that bind RNA polymerase weakly and are poor matches to the consensus sequence (↬ Section 4.5). Thus, even with the correct sigma (σ) factor, the RNA polymerase has difficulty binding to these promoters. The role of the activator protein is to help the RNA polymerase recognize the promoter and begin transcription. For example, the activator protein may modify the structure of the DNA by bending it (Figure 6.9), allowing the RNA polymerase to make necessary contacts with nucleotides in the promoter region to begin transcription. Alternatively, the activator protein may interact directly with the RNA polymerase. This can happen either when the activator-binding site is close to the promoter (Figure 6.10*a*) or when it is several hundred base pairs away from the promoter, a situation in which DNA looping is required to make the necessary contacts between protein and nucleic acid (Figure 6.10*b*).

Many genes in *E. coli* have promoters under positive control and many have promoters under negative control. In addition, many

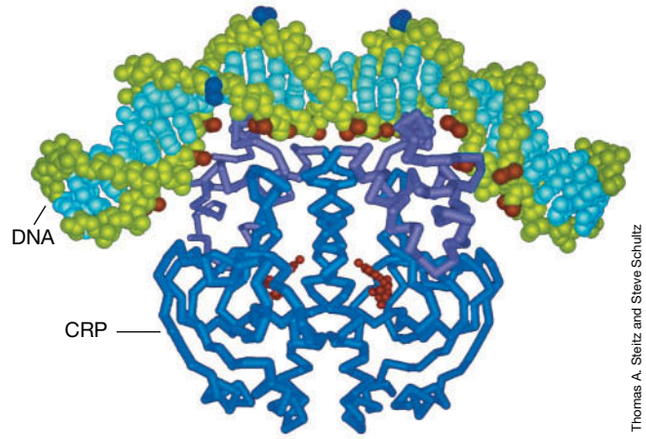


Figure 6.9 Computer model of a positive regulatory protein interacting with DNA. This model shows the cyclic AMP receptor protein (CRP), a regulatory protein that controls several operons. The α-carbon backbone of this protein is shown in blue and purple. The protein is binding to a DNA double helix (green and light blue). Note that binding of the CRP protein to DNA has bent the DNA.

operons have promoters with multiple types of control and some have more than one promoter, each with its own control system! Thus, the simple picture outlined above does not hold for all operons. Multiple control features are common in the operons of virtually all *Bacteria* and *Archaea*, and thus their overall regulation may require a network of interactions.

Operons versus Regulons

In *E. coli*, the genes required for maltose utilization are spread out over the chromosome in several operons, each of which has an activator-binding site to which a copy of the maltose activator protein can bind (Figure 6.11). Therefore, the maltose activator

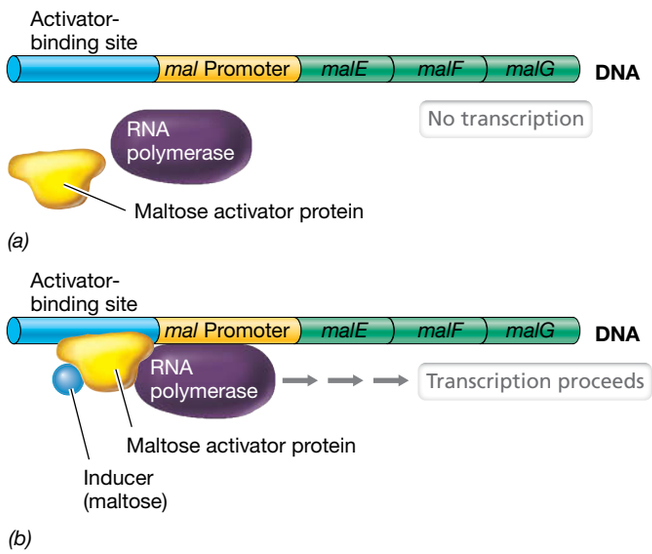


Figure 6.8 Positive control of enzyme induction in the maltose operon. *(a)* In the absence of an inducer, neither the activator protein nor the RNA polymerase can bind to the DNA. *(b)* An inducer molecule (for the *malEFG* operon it is the sugar maltose) binds to the activator protein (MalT), which in turn binds to the activator-binding site. This recruits RNA polymerase to bind to the promoter and begin transcription.

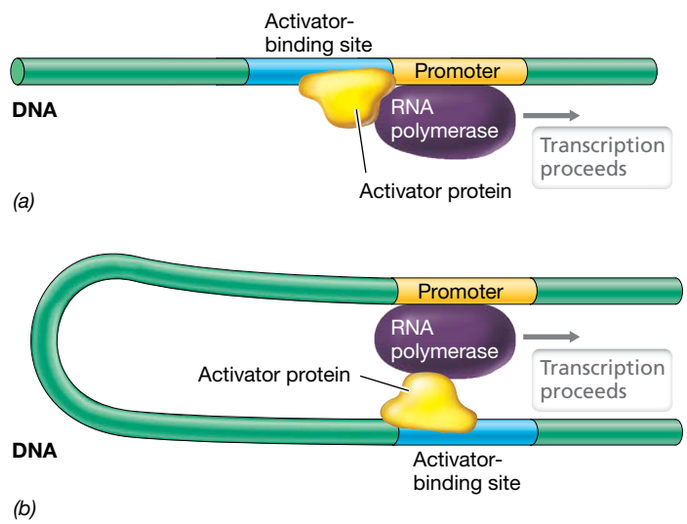


Figure 6.10 Activator protein interactions with RNA polymerase. *(a)* The activator-binding site is near the promoter. *(b)* The activator-binding site is several hundred base pairs from the promoter. In this case, the DNA must be looped to allow the activator and the RNA polymerase to contact.

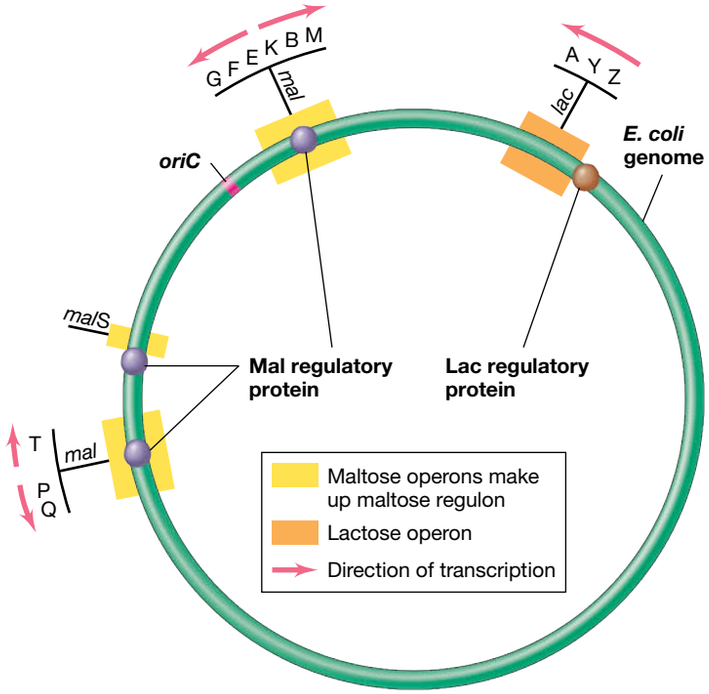


Figure 6.11 Maltose regulon of *Escherichia coli*. The genes and operons required for maltose utilization (*mal*) are dispersed throughout the *E. coli* genome and regulated by the same maltose regulatory protein. Note that the Lac repressor protein binds only to the *lac* operon, which is only located at one position on the chromosome, while the Mal repressor protein binds to multiple operons (the *mal* regulon).

protein actually controls the transcription of more than one operon. When more than one operon is under the control of a single regulatory protein, these operons are collectively called a **regulon**. The enzymes for maltose utilization are encoded by the maltose regulon.

Regulons are known for operons under negative control as well. For example, the arginine biosynthetic enzymes (Section 6.2) are encoded by the arginine regulon, whose operons are all under the control of the arginine repressor protein (only one of the arginine operons was shown in Figure 6.6). In regulon control, a specific DNA-binding protein binds only at those operons it controls regardless of whether it is functioning as an activator or repressor; other operons are not affected.

MINIQUIZ

- Compare and contrast the activities of an activator protein and a repressor protein.
- Distinguish between an operon and a regulon.

6.4 Global Control and the *lac* Operon

An organism often needs to regulate many unrelated genes simultaneously in response to a change in its environment. Regulatory mechanisms that respond to environmental signals by regulating the transcription of many different genes are called *global control systems*. Both the lactose operon and the maltose regulon respond to global controls in addition to their own controls discussed in Sections 6.2 and 6.3. We begin our consideration of global regulation by revisiting the *lac* operon and seeing how cells respond when given more than one sugar.

Catabolite Repression

We have not yet considered the possibility that bacteria might be confronted with several different utilizable carbon sources. For example, *Escherichia coli* can use many different sugars. When given several sugars, including glucose, do cells of *E. coli* use them simultaneously or one at a time? The answer is that *glucose is always used first*. It would be wasteful to induce enzymes for using other sugars when glucose is available, because *E. coli* grows faster on glucose than on other carbon sources. **Catabolite repression** is a mechanism of global control that controls the use of carbon sources if more than one is present.

When cells of *E. coli* are grown in a medium that contains glucose, the synthesis of enzymes needed for the breakdown of other carbon sources (such as lactose or maltose) is repressed, even if those other carbon sources are present. Thus, the presence of a favored carbon source represses the induction of pathways that catabolize other carbon sources. Catabolite repression is sometimes called the "glucose effect" because glucose was the first substance shown to cause this response. But catabolite repression is not always linked to glucose; the key point is that the favored substrate is a better carbon and energy source than other available carbon sources. Thus, catabolite repression ensures that the organism uses the *best* carbon and energy source first.

Why is catabolite repression called *global* control? In *E. coli* and other organisms for which glucose is the best energy source, catabolite repression prevents expression of most other catabolic operons as long as glucose is present. Dozens of catabolic operons are affected, including those for lactose, maltose, a host of other sugars, and most other commonly used carbon and energy sources for *E. coli*. In addition, genes for the synthesis of flagella are controlled by catabolite repression because if bacteria have a good carbon source available, there is no need to swim around in search of nutrients.

One consequence of catabolite repression is that it may lead to two exponential growth phases, a phenomenon called *diauxic growth*. If two usable energy sources are available, the cells first consume the better energy source. Growth stops when the better source is depleted, but then following a lag period, it resumes on the other energy source. Diauxic growth is illustrated in Figure 6.12 for a culture of *E. coli* grown on a mixture of glucose and lactose. The cells grow more rapidly on glucose than on lactose. Although glucose and lactose are both excellent energy sources for *E. coli*, glucose is superior, and growth is faster.

The proteins of the *lac* operon, including the enzyme β-galactosidase, are required for using lactose and are induced in its presence (Figures 6.5 and 6.7). But the synthesis of these proteins is also subject to catabolite repression. As long as glucose is present, the *lac* operon is not expressed and lactose is not used. However, when glucose is depleted, catabolite repression is abolished and the *lac* operon is expressed; shortly thereafter, cells begin to grow on lactose.

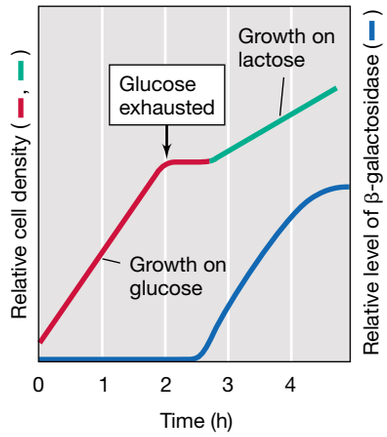


Figure 6.12 Diauxic growth of *Escherichia coli* on a mixture of glucose and lactose. The presence of glucose represses the synthesis of β-galactosidase, the enzyme that cleaves lactose into glucose and galactose. After glucose is depleted, there is a lag during which β-galactosidase is synthesized. Growth then resumes on lactose but at a slower rate, as indicated by the green line.

Cyclic AMP and Cyclic AMP Receptor Protein

Despite its name, catabolite repression relies on an activator protein and is actually a form of positive control (Section 6.3). The activator protein is called the *cyclic AMP receptor protein* (*CRP*). A gene that encodes a catabolite-repressible enzyme is expressed only if CRP binds to DNA in the promoter region. This allows RNA polymerase to bind to the promoter. CRP is an allosteric protein and binds to DNA only if it has first bound a small molecule called *cyclic adenosine monophosphate* (*cyclic AMP* or *cAMP*) (**Figure 6.13**). Like many DNA-binding proteins (Section 6.1), CRP binds to DNA as a dimer.

Cyclic AMP is a key molecule in many metabolic control systems, both in prokaryotic cells and eukaryotes. Because it is derived from a nucleic acid precursor, it is a **regulatory nucleotide**. Other regulatory nucleotides include cyclic guanosine monophosphate (cyclic GMP; important mostly in eukaryotes), cyclic di-GMP (important in biofilm formation; Section 7.9), and guanosine tetraphosphate (ppGpp, important in the stringent response, Section 6.9). Cyclic AMP is synthesized from ATP by an enzyme called *adenylate cyclase* (Figure 6.13). However, glucose inhibits the synthesis of cyclic AMP and also stimulates cyclic AMP transport out of the cell. When glucose enters the cell, the cyclic AMP level is lowered, CRP cannot bind DNA, and RNA polymerase fails to bind to the promoters of operons subject to catabolite repression. Thus, catabolite repression is an indirect result of the presence of a better energy source (glucose); the direct cause of catabolite repression is a low level of cyclic AMP.

Let us return to the *lac* operon and include catabolite repression. The entire regulatory region of the *lac* operon is diagrammed in **Figure 6.14**. For *lac* genes to be transcribed, two requirements must be met: (1) The level of cyclic AMP must be high enough for the CRP protein to bind to the CRP-binding site (positive control), and (2) lactose or another suitable inducer must be present so that

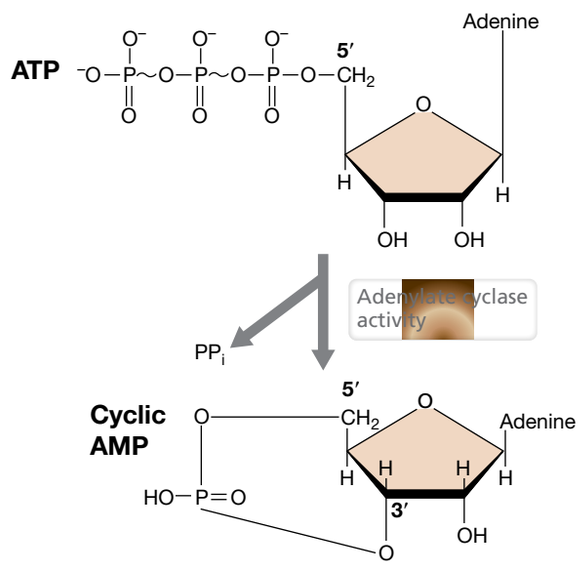


Figure 6.13 Cyclic AMP. Cyclic adenosine monophosphate (cyclic AMP) is made from ATP by the enzyme adenylate cyclase.

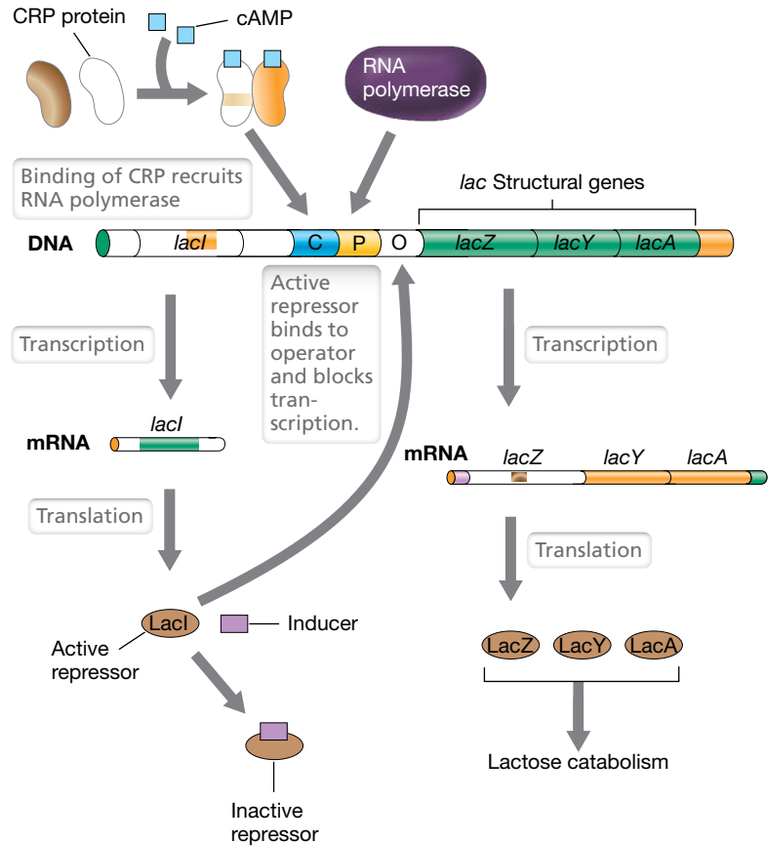


Figure 6.14 Overall regulation of the *lac* system. The *lac* operon consists of *lacZ*, encoding β-galactosidase, *lacY*, encoding lactose permease, and *lacA*, encoding lactose acetylase. The LacI repressor protein is encoded by a separate gene, *lacI*. LacI binds to the operator (O) unless the inducer is present. CRP binds to the C site when activated by cyclic AMP and recruits RNA polymerase to bind to the promoter (P). For the *lac* operon to be transcribed by RNA polymerase, the LacI repressor must be absent (that is, inducer must be present) and cyclic AMP levels must be high (due to the absence of glucose), allowing CRP to bind.

the lactose repressor (LacI protein) does not block transcription by binding to the operator (negative control). If these two conditions are met, the cell is signaled that glucose is absent and lactose is present; then and only then does transcription of the *lac* operon begin.

MINIQUIZ

- Explain how catabolite repression depends on an activator protein.
- What role does cyclic AMP play in glucose regulation?
- Explain how the *lac* operon is both positively and negatively controlled.

6.5 Transcription Controls in *Archaea*

There are two alternative approaches to regulating the activity of RNA polymerase. One strategy, common in *Bacteria*, is to use DNA-binding proteins that either block RNA polymerase activity (repressor proteins) or stimulate RNA polymerase activity (activator proteins). The alternative, common in eukaryotes, is to coordinate numerous DNA-binding proteins known as *transcription factors* to interact with RNA polymerase. Given the greater overall similarity between the mechanism of transcription in *Archaea* and *Eukarya* (Section 4.6), it is perhaps surprising that the regulation of transcription in *Archaea* more closely resembles that of *Bacteria*.

Few repressor or activator proteins from *Archaea* have yet been characterized in detail, but it is clear that *Archaea* have both types of regulatory proteins. Archaeal repressor proteins either block the binding of RNA polymerase itself or block the binding of TBP (TATA-binding protein) and TFB (transcription factor B), proteins that are required for RNA polymerase to bind to the promoter in *Archaea* (Section 4.6). At least some archaeal activator proteins function in just the opposite way, by recruiting TBP to the promoter, thereby facilitating transcription.

Control of Nitrogen Assimilation in *Archaea*

A good example of an archaeal repressor is the NrpR protein from the methanogen *Methanococcus maripaludis* (Section 17.2). NrpR represses genes encoding nitrogen assimilation functions (**Figure 6.15**), such as those for nitrogen fixation and synthesis of the amino acid glutamine. When organic nitrogen is plentiful in the *M. maripaludis* cell, NrpR represses nitrogen assimilation genes. However, if the level of nitrogen becomes limiting, α-ketoglutarate accumulates to high levels. This occurs because α-ketoglutarate, a citric acid cycle intermediate, is also a major acceptor of ammonia during nitrogen assimilation (Section 3.14).

When levels of α-ketoglutarate rise, this signals that ammonia is limiting and that additional pathways need to be activated for obtaining ammonia, such as nitrogen fixation or the high-affinity nitrogen assimilation enzyme glutamine synthetase. Elevated levels of α-ketoglutarate function as an inducer by binding to the NrpR protein. In this state, NrpR loses its affinity for the promoter regions of its target genes and no longer blocks transcription from promoters. In this respect, the NrpR protein resembles the LacI repressor and similar-functioning proteins of *Bacteria* (Section 6.2 and Figure 6.7).

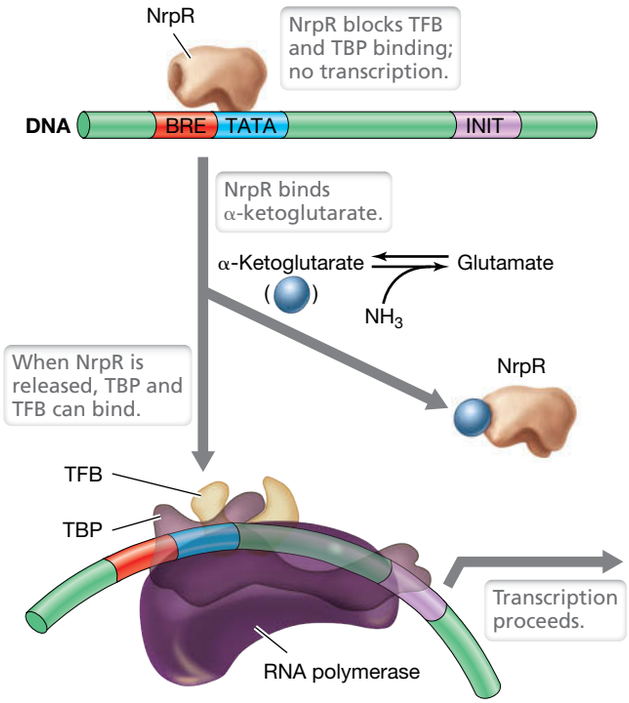


Figure 6.15 Repression of genes for nitrogen metabolism in *Archaea*. The NrpR protein of *Methanococcus maripaludis* acts as a repressor. It blocks the binding of the TFB and TBP proteins, which are required for promoter recognition, to the BRE (B recognition element) site and TATA box, respectively. If there is a shortage of ammonia, α-ketoglutarate is not converted to glutamate. The α-ketoglutarate accumulates and binds to NrpR, releasing it from the DNA. Now TBP and TFB can bind. This in turn allows RNA polymerase to bind and transcribe the operon.

Dual-Acting Transcriptional Regulators in *Archaea*

Some archaeal regulators, such as the TrmB family, can possess *dual functionality* and act as both a repressor and an activator. The TrmB family of transcriptional regulators is widespread in *Archaea,* with more than 250 proteins identified. Members of the TrmB family primarily regulate sugar metabolism and can function as a repressor, an activator, or both. The activity of the allosteric regulator depends on its DNA binding site. In *Pyrococcus furiosus*, a hyperthermophile that does not possess a glucose transporter but can perform glycolysis, TrmBL1 simultaneously functions as a repressor of genes for other sugar transport systems present in this organism and as an activator for gluconeogenesis (glucose synthesis) genes.

As a repressor, TrmBL1 binds to a specific DNA sequence *downstream* of the BRE (B recognition element)/TATA binding sites of maltodextrin and maltose/trehalose uptake genes (**Figure 6.16*a***). This binding blocks the recruitment of RNA polymerase to these sites and thus prevents gene expression. If cellular conditions shift and the inducer molecules maltose, maltotriose, or fructose are present, they bind to TrmBL1, resulting in an allosteric change and an inactive repressor that can no longer bind DNA. Without TrmBL1 bound to the DNA, RNA polymerase is able to bind to the

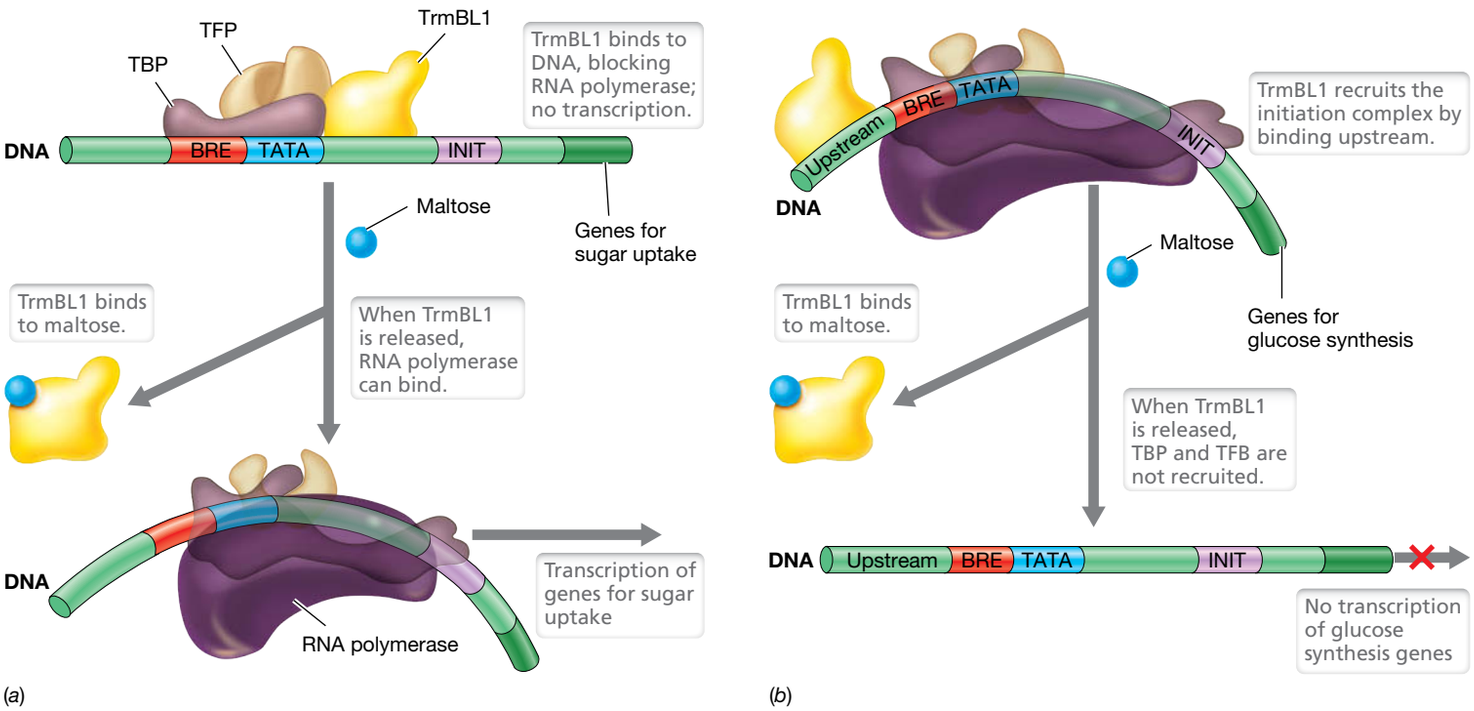


Figure 6.16 Dual functionality of the *Pyrococcus furiosus* TrmBL1 regulator. *Pyrococcus furiosus* is a hyperthermophilic species of the *Euryarchaeota* with a growth temperature optimum of 100°C (Section 17.4). *(a)* The TrmBL1 protein acts as a repressor of genes for sugar uptake. It binds to DNA downstream of the TATA box, blocking the binding of RNA polymerase. Binding of maltose (or another inducer) to TrmBL1 results in release of the regulator from the DNA. Now RNA polymerase can bind and transcribe sugar uptake genes. *(b)* The TrmBL1 protein acts as an activator of genes for glucose synthesis. It binds to a sequence upstream of the promoter region and recruits the transcription initiation complex. This in turn results in transcription of glucose synthesis genes. The presence of maltose results in release of TrmBL1 from the DNA. Without TrmBL1 bound to the DNA, TBP and TFB are not recruited and glucose synthesis genes are not transcribed.

promoter region and derepression of sugar transporter genes occurs (Figure 6.16*a*).

In contrast, TrmBL1 can function as an activator protein by binding to a separate and distinct DNA sequence associated with genes for glucose biosynthesis. This regulatory region differs from the one that TrmBL1 binds to as a repressor as it is located *upstream* of the BRE/TATA binding sites (Figure 6.16*b*). Binding of TrmBL1 to this DNA sequence helps recruit the archaeal transcription initiation complex (TBP, TFB, and RNA polymerase, Section 4.6), thus activating gene expression. However, in the same manner as its role as a repressor, TrmBL1 as an allosteric activator will not bind to DNA if the effector molecules maltose, maltotriose, or fructose are present. Without TrmBL1 binding upstream of the BRE/TATA sequences and activating transcription, RNA polymerase and the other needed initiation factors cannot be recruited for transcription of genes necessary for glucose synthesis. The rationale of this dual-functioning control surrounds energy conservation. That is, from an energetic standpoint, this form of regulation prevents expression of the gluconeogenesis pathway when other sugars that can feed into the glycolytic pathway are present in the cell.

The SurR protein of *P. furiosus* is another example of a regulatory protein that functions as both an activator and a repressor, depending on the location of its binding site within the promoter region. SurR controls a catabolic shift of *P. furiosus* from fermentation (which produces H_2) to sulfur (S^0) reduction, a form of anaerobic respiration that produces H_2S (Sections 3.12 and 14.14). When S^0 is absent, SurR binds to regulatory regions activating the transcription of genes necessary for hydrogenase production so that *P. furiosus* can grow by fermentation. At the same time, SurR functions as a repressor to prevent transcription of genes encoding proteins that participate in sulfur metabolism. However, when S^0 is present, SurR is no longer able to bind to DNA. This inability to bind to DNA is not the result of effector binding to SurR, but is due to the oxidation of cysteine residues within the DNA-binding motif of this regulatory protein that releases it from the DNA. The release of SurR from regulatory regions both promotes expression of genes that participate in S^0 metabolism and represses the expression of hydrogenase genes that encode a hydrogenase enzyme necessary for *P. furiosis* to grow at the expense of fermentation.

MINIQUIZ

- What is the major difference between transcriptional regulation in *Archaea* and eukaryotes?
- How do transcriptional activators in *Archaea* often differ in mechanism from those in *Bacteria*?
- Explain how the *Pyrococcus furiosus* TrmBL1 transcription regulator is able to act as both an activator and a repressor.

II • Sensing and Signal Transduction

Prokaryotic cells regulate cell metabolism in response to many different environmental fluctuations, including changes in temperature, pH, oxygen or nutrient availability, and even to changes in the number of other cells present. To do this, mechanisms exist by which cells receive signals from the environment and transmit them to the specific target to be regulated. Some of these signals are small molecules that enter the cell and function as effectors. However, in many cases the external signal is not transmitted directly to the regulatory protein but instead is detected by a surface sensing system that transmits the signal to the rest of the regulatory machinery, a process called **signal transduction**.

6.6 Two-Component Regulatory Systems

Because most signal transduction systems contain two parts, they are called **two-component regulatory systems**. Characteristically, such systems consist of a specific **sensor kinase protein** usually located in the cytoplasmic membrane, and a **response regulator protein**, present in the cytoplasm.

A kinase is an enzyme that phosphorylates compounds, typically using phosphate from ATP. Sensor kinases detect a signal from the environment and phosphorylate themselves (a process called *autophosphorylation*) at a specific histidine residue on the protein (Figure 6.17). Sensor kinases thus belong to the class of enzymes called *histidine kinases*. The phosphate is then transferred from the sensor to another protein inside the cell, the response regulator. The latter is typically a DNA-binding protein that regulates transcription in either a positive or a negative fashion. In the example shown in Figure 6.17, regulation is negative; the phosphorylated response regulator functions as a repressor that binds DNA, thereby blocking transcription. Once dephosphorylated, the response regulator is released and transcription is permitted.

For a balanced regulatory system to work properly, it must have a *feedback loop*, that is, a way to complete the regulatory circuit and terminate the response. This resets the system for another cycle. This feedback loop employs a *phosphatase*, an enzyme that removes the phosphate from the response regulator at a constant rate. The response regulator itself often catalyzes this reaction, although in some cases separate proteins are needed (Figure 6.17). Phosphatase activity is typically slower than phosphorylation. However, if phosphorylation ceases due to reduced sensor kinase activity, phosphatase activity eventually returns the response regulator to the fully nonphosphorylated state, and the system is reset.

Examples of Two-Component Regulatory Systems

Two-component systems regulate a large number of genes in many different bacteria. Interestingly, two-component systems are either extremely rare or absent in *Archaea* and in *Bacteria* that live as parasites of higher organisms. A few key examples of two-component systems include those that respond to phosphate limitation, nitrogen limitation, and osmotic pressure.

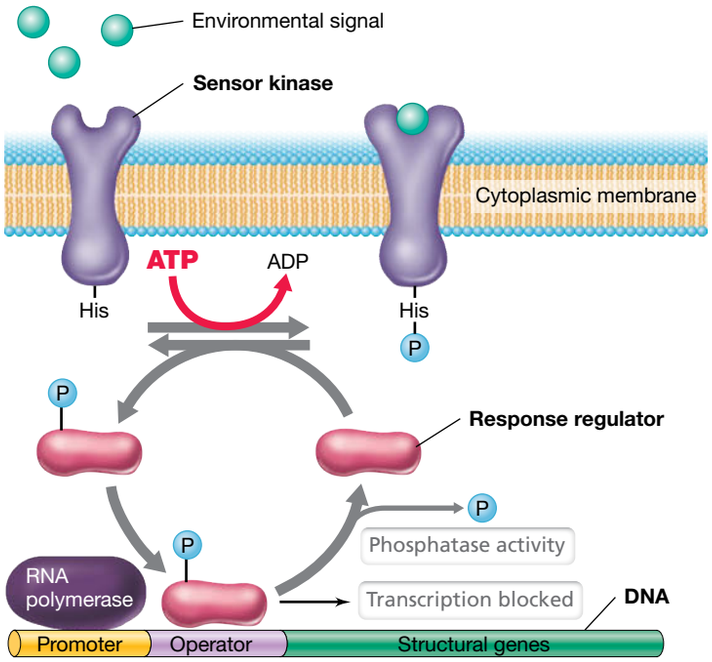


Figure 6.17 The control of gene expression by a two-component regulatory system. One component is a sensor kinase in the cytoplasmic membrane that phosphorylates itself in response to an environmental signal. The phosphoryl group is then transferred to the second component, a response regulator. The phosphorylated form of the response regulator then binds to DNA. In the system shown here, the phosphorylated response regulator is a repressor protein. The phosphatase activity of the response regulator slowly releases the phosphate from the response regulator and resets the system.

In *Escherichia coli* almost 50 different two-component systems are present, and several are listed in Table 6.1. In one example, the osmolarity of the environment controls the relative levels of the proteins OmpC and OmpF in the *E. coli* outer membrane. OmpC and OmpF are *porins*, proteins that allow metabolites to cross the outer membrane of gram-negative bacteria (↔ Section 2.5). If osmotic pressure is *low*, the synthesis of OmpF, a porin with a larger pore, increases; if osmotic pressure is *high*, OmpC, a porin with a smaller pore, is made in larger amounts.

EnvZ, a sensor histidine kinase in the cytoplasmic membrane, detects changes in osmotic pressure. When a shift occurs, EnvZ is autophosphorylated and transfers its phosphate group to OmpR, the response regulator of this system (Figure 6.18). Under conditions of *low* osmotic pressure, phosphorylated OmpR (OmpR-P) *activates* transcription of the *ompF* gene. Conversely, when osmotic pressure is *high*, OmpR-P *represses* transcription of *ompF* gene and activates transcription of *ompC* instead (Figure 6.18). The expression of *ompF* is also regulated by an additional control mechanism: regulatory RNA, and we discuss this in Section 6.11.

Two-Component Systems with Multiple Regulators

Some signal transduction systems have more than one regulatory element and their activities can quickly become quite complex. For instance, in the Ntr regulatory system, which regulates nitrogen assimilation in many *Bacteria*, the response regulator is an activator called *nitrogen regulator I* (*NRI*). NRI activates transcription from

TABLE 6.1 Examples of two-component systems that regulate transcription in *Escherichia coli*

System	*Environmental signal*	*Sensor kinase*	*Response regulator*	*Primary activity of response regulator*[a]
Arc system	Oxygen	ArcB	ArcA	Repressor/activator
Nitrate and nitrite respiration (Nar)	Nitrate and nitrite	NarX	NarL	Activator/repressor
		NarQ	NarP	Activator/repressor
Nitrogen utilization (Ntr)	Shortage of organic nitrogen	NRII (= GlnL)	NRI (= GlnG)	Activator of promoters requiring RpoN/σ^{54}
Pho regulon	Inorganic phosphate	PhoR	PhoB	Activator/repressor
Porin regulation	Osmotic pressure	EnvZ	OmpR	Activator/repressor

[a]Note that many response regulator proteins act as both activators and repressors depending on the genes being regulated. Although ArcA can function as either an activator or a repressor, it functions as a repressor on most operons that it regulates.

promoters recognized by RNA polymerase using the alternative sigma factor σ^{54} (RpoN) (↔ Section 4.5). The sensor kinase in the Ntr system is a protein called *nitrogen regulator II* (*NRII*), which functions as both a kinase and a phosphatase. The activity of NRII is in turn regulated by another protein called *PII*, whose own activity is regulated by the addition or removal of uridine monophosphate (UMP) groups (Section 6.15). Under nitrogen starvation conditions, UMP is added to PII, and the resulting PII–UMP complex promotes the kinase activity of NRII and results in the phosphorylation of NRI. Conversely, removal of UMP from PII promotes the phosphatase activity of NRII.

The *Nar regulatory system* (Table 6.1) is another example of a two-component regulatory system with multiple regulators; this system controls a set of genes that allow the use of nitrate (NO_3^-) or nitrite (NO_2^-) (or both) as alternative electron acceptors during anaerobic respiration (↔ Sections 3.12 and 14.13). The Nar system contains two different sensor kinases and two different response regulators. In addition, all of the genes regulated by this system are themselves controlled by a protein called FNR (*f*umarate *n*itrite *r*egulator); FNR is a global regulator of genes encoding anaerobic respiration functions (see Table 6.2). This type of regulation in which a hierarchy of systems are deployed in a cascading fashion is common for systems of central importance to cellular metabolism.

Two-component systems closely related to those in *Bacteria* are also present in microbial eukaryotes, such as the yeast *Saccharomyces cerevisiae*, and even in plants. However, most eukaryotic signal transduction pathways rely on phosphorylation of serine, threonine, and tyrosine residues of proteins that are unrelated to those of the bacterial two-component systems that phosphorylate histidine residues (Figures 6.17 and 6.18).

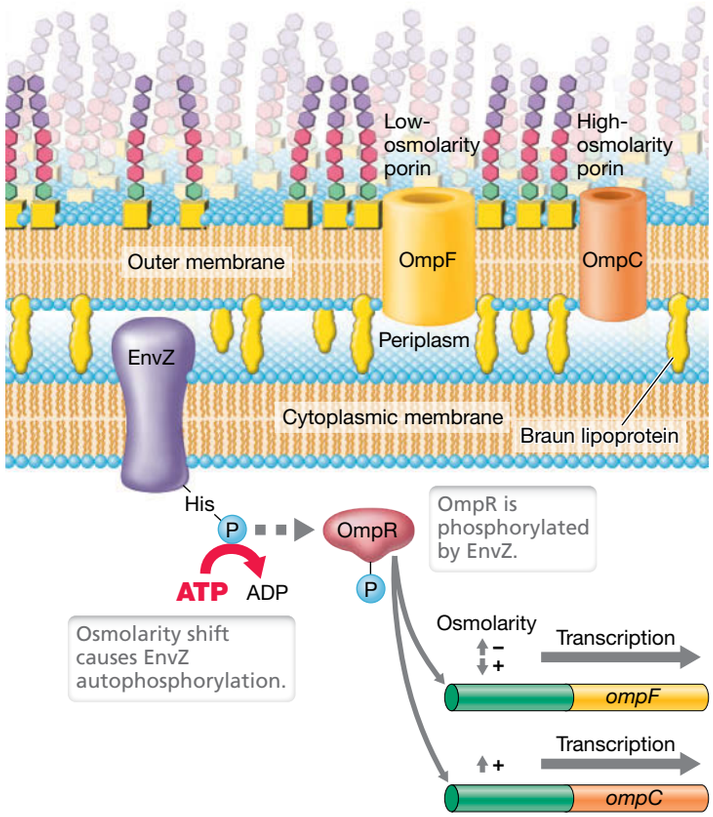


Figure 6.18 Regulation of outer membrane proteins in *Escherichia coli*. The cytoplasmic membrane histidine kinase EnvZ autophosphorylates itself under osmotic pressure changes and then activates the transcriptional regulator OmpR by phosphorylation. OmpR-P binds upstream of the *ompF* gene and activates transcription under low osmotic pressure, but conversely it represses transcription of *ompF* under high osmotic pressure. OmpR-P only activates transcription of the *ompC* gene under conditions of high osmolarity.

MINIQUIZ

- What are kinases and what is their role in two-component regulatory systems?
- What are phosphatases and what is their role in two-component regulatory systems?

6.7 Regulation of Chemotaxis

We have previously seen that some motile cells of *Bacteria* and *Archaea* respond to challenges such as nutrient limitation and toxin accumulation by moving toward attractants and away from repellents, a behavior called *chemotaxis* (↔ Section 2.13). We noted there that prokaryotic cells are too small to sense spatial gradients of a chemical, but they can respond to temporal gradients. That is, they can sense the *change* in concentration of a

chemical over time rather than the absolute concentration of the chemical stimulus.

Chemotaxis has been well studied in *Bacteria*, which use a modified two-component system to sense temporal changes in attractants or repellents and process this information to regulate the direction of flagellar rotation. This differs from what was considered in the previous section in that the two-component system is used to regulate the activity of *preexisting proteins* (the flagellum protein complex) rather than to control the transcription of genes encoding flagellar proteins.

Response to Signal

The mechanism of chemotaxis depends upon a signal cascade of multiple proteins. Several sensory proteins reside in the cytoplasmic membrane and sense attractants or repellents. These sensor proteins are not themselves sensor kinases but interact with cytoplasmic sensor kinases. These sensory proteins allow the cell to monitor the concentration of various substances over time, and are called *methyl-accepting chemotaxis proteins* (*MCPs*). *Escherichia coli* produces five different transmembrane MCPs, each specific for certain compounds. For example, the Tar MCP of *E. coli* senses the attractants aspartate and maltose and the repellents cobalt and nickel. MCPs bind attractants or repellents directly or in some cases indirectly through interactions with periplasmic binding proteins. Binding of an attractant or repellent triggers interactions with cytoplasmic proteins that eventually affect flagellar rotation.

In *E. coli*, thousands of MCPs are often clustered, forming chemoreceptors. These chemoreceptors make contact with the cytoplasmic proteins CheA and CheW (**Figure 6.19**). CheA is the sensor kinase (Section 6.6) for chemotaxis. When an MCP binds a chemical, it changes conformation and, with help from CheW, leads to the autophosphorylation of CheA to form CheA-P. An increase in attractant concentration *decreases* the rate of autophosphorylation, whereas a decrease in attractant or an increase in repellent *increases* the rate of autophosphorylation. CheA-P then passes the phosphate to CheY (forming CheY-P); this is the response regulator (Section 6.6) that controls flagellar rotation. CheA-P can also transfer the phosphate to CheB, which plays a role in adaptation to be described later.

Controlling Flagellar Rotation

CheY is a central protein in the regulatory system because it governs the direction of rotation of the flagellum. Recall that if rotation of the flagellum is counterclockwise, the cell will continue to move in a run (swim smoothly), whereas if the flagellum rotates clockwise, the cell will tumble (move randomly) (Section 2.13 and Figure 2.36). Once CheY is phosphorylated, it interacts with the flagellar motor to induce clockwise flagellar rotation, which causes tumbling (Figure 6.19). When unphosphorylated, CheY cannot bind to the flagellar motor and the flagellum rotates counterclockwise; this causes the cell to run. Another protein, CheZ, dephosphorylates CheY, returning it to the form that allows runs instead of tumbles. Either an increase in repellents or a decrease in attractants leads to an increase in the level of CheY-P and thus tumbling. By contrast, if the cell is swimming toward attractants, the lower level of CheY-P suppresses tumbles and promotes runs.

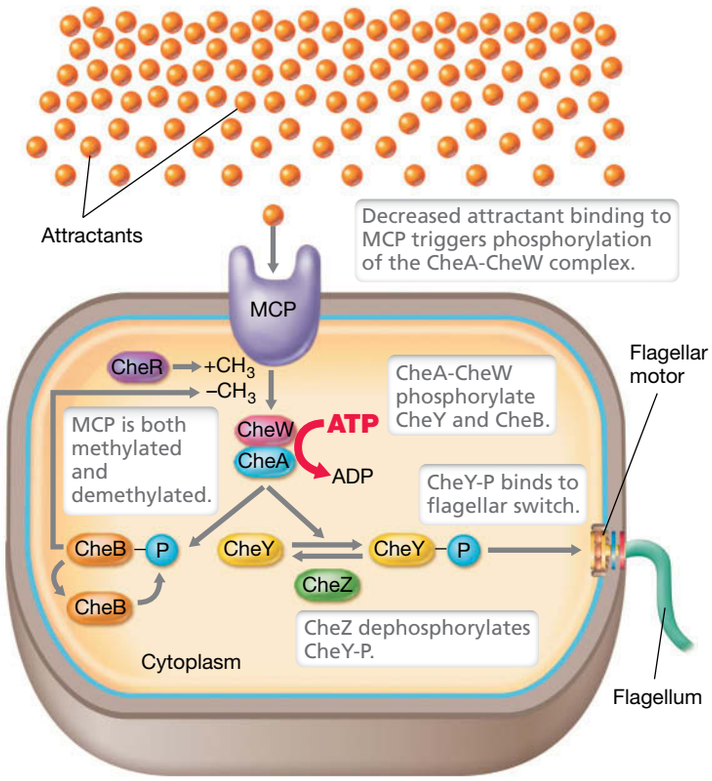


Figure 6.19 Interactions of MCPs, Che proteins, and the flagellar motor in bacterial chemotaxis. The methyl-accepting chemotaxis protein (MCP) forms a complex with the sensor kinase CheA and the coupling protein CheW. This combination triggers autophosphorylation, which can then phosphorylate the response regulators CheB and CheY. Phosphorylated CheY (CheY-P) binds to the flagellar motor switch. CheZ dephosphorylates CheY-P. CheR continually adds methyl groups to the MCP. CheB-P (but not CheB) removes them. The degree of methylation of the MCPs controls their ability to respond to attractants and repellents and leads to adaptation.

Adaptation

Once an organism has successfully responded to a stimulus, it must stop responding and reset the sensory system to await further signals. This is known as *adaptation*. During adaptation of the chemotaxis system, a feedback loop resets the system. This relies on the response regulator CheB, mentioned earlier.

As their name implies, MCPs can be methylated. When MCPs are fully methylated they no longer respond to attractants but are more sensitive to repellents. Conversely, when MCPs are unmethylated they respond strongly to attractants but are insensitive to repellents. Varying the methylation level thus allows adaptation to sensory signals. This is accomplished by methylation and demethylation of the MCPs by CheR and phosphorylated CheB (CheB-P), respectively (Figure 6.19).

If the level of an attractant remains high, the rate of CheA autophosphorylation is low. This leads to unphosphorylated CheY and CheB. Consequently, the cell swims smoothly. Methylation of the MCPs increases during this period because CheB-P is not present to demethylate them. However, MCPs no longer respond to the attractant when they become fully methylated. Therefore, if the level of attractant remains high but constant, the cell begins to tumble. Eventually, CheB becomes phosphorylated and CheB-P

demethylates the MCPs. This resets the receptors and they can once again respond to further increases or decreases in level of attractants. Put another way, the cell stops swimming if the attractant concentration is constant. It only continues to swim if even higher levels of attractant are encountered. The course of events is just the opposite for repellents. Fully methylated MCPs respond best to an increasing gradient of repellents and send a signal for cell tumbling to begin. The cell then moves off in a random direction while MCPs are slowly demethylated. With this mechanism for adaptation, chemotaxis successfully achieves the ability to monitor small changes in the concentrations of both attractants and repellents over time.

Other Taxes

In addition to chemotaxis, several other forms of taxis are known, for example, *phototaxis* (movement toward light) and *aerotaxis* (movement toward oxygen) (Section 2.13). Interestingly, many of the cytoplasmic Che proteins that function to control flagellar activity in chemotaxis also play a role in these other taxes. For example, in phototaxis, a light sensor protein replaces the MCPs of chemotaxis, and in aerotaxis, a redox protein monitors levels of oxygen. These sensors then interact with cytoplasmic Che proteins to begin the cascade of events that direct runs or tumbles. Thus several different kinds of environmental signals converge on the same flagellar control system, and this allows the cell to economize on its regulatory systems.

MINIQUIZ

- What are the primary response regulator and the primary sensor kinase for regulating chemotaxis?
- Why is adaptation during chemotaxis important?
- How does the response of the chemotaxis system to an attractant differ from its response to a repellent?

6.8 Quorum Sensing

Many *Bacteria* respond to the presence in their surroundings of other cells of their own species, and in some species, regulatory pathways are controlled by the density of cells of their own kind. This is a phenomenon called **quorum sensing** (the word "quorum" in this sense means "sufficient numbers"). Quorum-sensing systems have also been detected in a few *Archaea*.

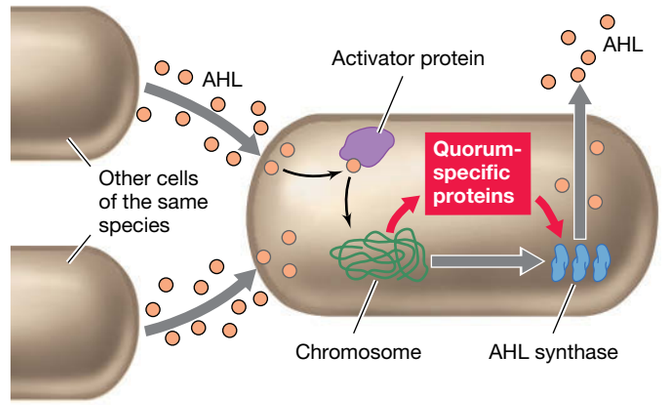
Mechanism of Quorum Sensing

Quorum sensing is a regulatory mechanism that assesses population density. Many bacteria use this approach to ensure that sufficient cell numbers are present before initiating activities that require a certain cell density to work effectively. For example, a pathogenic (disease-causing) bacterium that secretes a toxin will have no effect as a single cell; production of toxin by one cell alone would merely waste resources. However, if a sufficiently large population of cells is present, the coordinated expression of the toxin may successfully cause disease and release resources from the host that can be used to support growth of the pathogen.

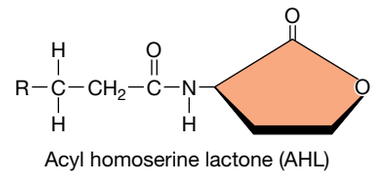
Quorum sensing is widespread among gram-negative *Bacteria* but is also found in many gram-positive species as well. Each species that employs quorum sensing synthesizes a specific signal molecule called an **autoinducer**. This molecule usually diffuses freely across the cell envelope in either direction. Because of this, the autoinducer reaches high concentrations inside the cell only if many cells are nearby, each making the same autoinducer. In the cytoplasm, the autoinducer binds to a specific transcriptional activator protein or a sensor kinase of a two-component system, ultimately triggering transcription of specific genes (**Figure 6.20*a***).

While several different classes of autoinducers exist, the first to be identified were the *acyl homoserine lactones* (AHLs) (Figure 6.20*b*). Several different AHLs, with acyl groups (functional groups containing both carbonyl and alkyl groups) of different lengths, are found in different species of gram-negative bacteria. In addition, many gram-negative bacteria make autoinducer 2 (AI-2; a cyclic furan derivative). This is apparently used as a common autoinducer between many species of bacteria. Gram-positive bacteria generally use certain short peptides as autoinducers.

The phenomenon of quorum sensing was discovered as the mechanism by which light emission in bioluminescent bacteria is regulated (Section 15.18). Several bacterial species can emit light, including the marine bacterium *Aliivibrio fischeri*. **Figure 6.21** shows bioluminescent colonies of *A. fischeri*. The light is generated by an enzyme called *luciferase*. The *lux* operons encode the proteins needed for bioluminescence. They are under control of the activator protein LuxR and are induced when the concentration of the specific *A. fischeri* AHL, *N*-3-oxohexanoyl homoserine lactone, becomes high enough. This AHL is synthesized by the enzyme encoded by the *luxI* gene.



(a)



Acyl homoserine lactone (AHL)

(b)

Figure 6.20 Quorum sensing. *(a)* A cell capable of quorum sensing expresses acyl homoserine lactone (AHL) synthase at basal levels. This enzyme makes the cell's specific AHL. When cells of the same species reach a certain density, the concentration of AHL rises sufficiently to bind to the activator protein, which activates transcription of quorum-specific genes. *(b)* General structure of an AHL. Different AHLs are variants of this parent structure. R = alkyl group (C_1–C_{17}); the carbon next to the R group is often modified to a keto group (C = O).

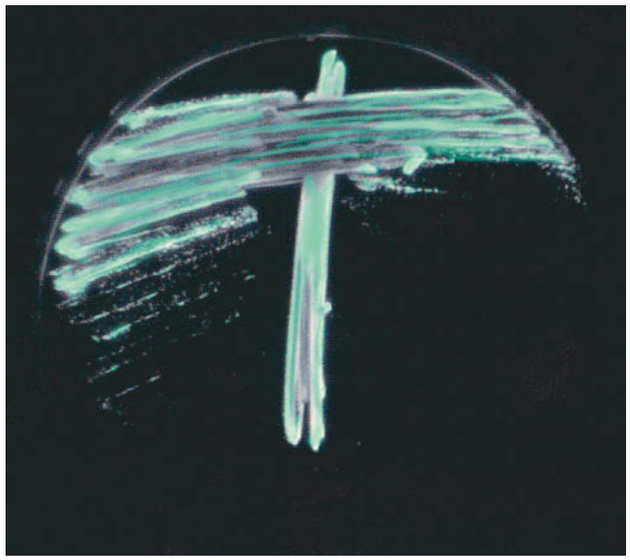


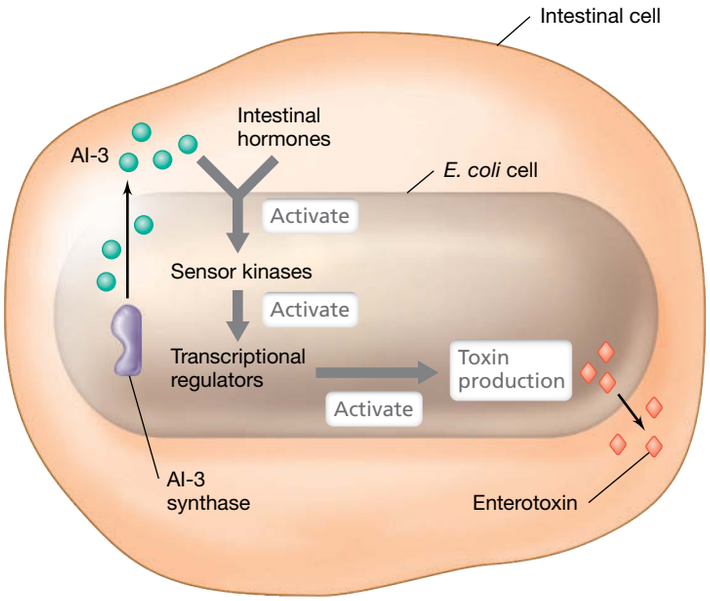
Figure 6.21 Bioluminescent bacteria producing the enzyme luciferase. Cells of the bacterium *Aliivibrio fischeri* were streaked on nutrient agar in a Petri dish and allowed to grow overnight. The photograph was taken in a darkened room using only the light generated by the bacteria.

Quorum sensing also occurs in microbial eukaryotes. For example, in the yeast *Saccharomyces cerevisiae*, specific aromatic alcohols are produced as autoinducers and control the transition between growth of *S. cerevisiae* as single cells and as elongated filaments. Similar transitions are seen in other fungi, some of which cause disease in humans. An example is *Candida*, whose quorum sensing is mediated by the long-chain alcohol farnesol. As the concentration of farnesol increases in this dimorphic fungus, the transition from budding yeast to elongated hyphae is prevented.

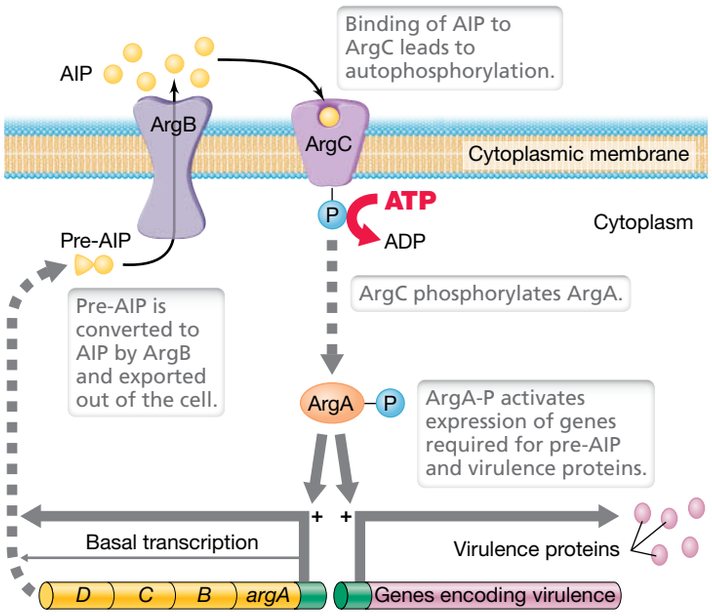
Virulence Factors

Various genes are controlled by quorum sensing, including some in pathogenic bacteria. For example, Shiga toxin–producing *Escherichia coli*, such as the notorious foodborne pathogen *E. coli* O157:H7 (Section 32.7), produces an AHL called AI-3 that induces virulence genes. As the *E. coli* population increases in the intestine, bacterial cells produce AI-3 while host intestinal cells produce the stress hormones epinephrine and norepinephrine. All three of these signal molecules bind to two separate sensor kinases in the *E. coli* cytoplasmic membrane, resulting in the phosphorylation and activation of two transcriptional activator proteins (**Figure 6.22a**). These proteins activate transcription of genes encoding motility functions and secretion of the toxin as well as genes encoding proteins that form lesions on the host intestinal mucosa. This is a rare example of a system that senses both bacterial and eukaryotic chemical signals to regulate gene expression.

The pathogenesis of the gram-positive bacterium *Staphylococcus aureus* (Section 30.9) requires, among many other things, the production and secretion of small extracellular peptides that damage host cells or that interfere with the host's immune system. The genes encoding these virulence factors are under the control of a quorum-sensing system that uses a small peptide called the



(a) **Virulence factor production in Shiga toxin-producing *Escherichia coli***



(b) **Virulence factor production in *Staphylococcus aureus***

Figure 6.22 Quorum sensing regulation of virulence factors. The bacteria *Escherichia coli* and *Staphylococcus aureus* can be harmless saprophytes or potent pathogens, depending on the strain. As pathogens, the production of major virulence factors is controlled by quorum sensing. *(a)* As the bacterial population increases, AI-3 produced by *Escherichia coli* and epinephrine and norepinephrine produced by the intestinal cell accumulate and bind to sensor kinases, initiating a cascade of events necessary for production of virulence factors (for example, enterotoxin). *(b)* Basal level transcription of the *argABCD* operon in *Staphylococcus* leads to production of ArgD, the pre-autoinducing peptide (AIP). ArgB trims ArgD into the functional AIP and exports it out of the cell. As the cell population increases, the AIP concentration increases and binds to ArgC, leading to autophosphorylation of ArgC. ArgC-P then activates the transcriptional activator ArgA by transfer of a phosphate group. ArgA-P increases transcription of the *argABCD* operon as well as activating the transcription of an RNA that leads to the production of virulence proteins.

autoinducing peptide (AIP), encoded by the *argD* gene, as the autoinducer. After synthesis of ArgD (pre-AIP), the membrane-bound ArgB protein trims the peptide into its active AIP form and secretes the small peptide outside of the cell (Figure 6.22*b*). As the cell density of *S. aureus* increases, so does the concentration of AIP. ArgC is a membrane-bound sensor kinase that binds to AIP, resulting in autophosphorylation. ArgC-P transfers its phosphate to the transcriptional activator ArgA. ArgA-P increases transcription of *argABCD* genes that encode the signal transduction system as well as an RNA molecule that controls production of a range of virulence proteins.

Some eukaryotes produce molecules that specifically interfere with bacterial quorum sensing. Most of these found so far have been furanone derivatives containing a halogen atom. These components mimic the AHLs or AI-2 and disrupt bacterial behavior that relies on quorum sensing. Because of this, quorum-sensing disruptors have been proposed as potential drugs to disperse bacterial biofilms (Sections 5.1 and 7.9) and prevent the expression of virulence genes.

MINIQUIZ

- What advantage do quorum-sensing systems confer on bacterial cells?
- What properties are required for a molecule to function as an autoinducer?
- How do the autoinducers used in quorum sensing by gram-negative bacteria differ from those used by gram-positive bacteria?

6.9 Stringent Response

While originally studied as a response to amino acid starvation, the **stringent response** is now recognized as a widely distributed regulatory mechanism used by bacteria to survive nutrient deprivation, environmental stresses, and antibiotic exposure. Triggering of the stringent response ultimately leads to a shutdown of macromolecule synthesis and the activation of *stress survival pathways* to improve the cell's ability to compete in nature. Because most of the work to elucidate the pathway has been done in *Escherichia coli*, we will focus on this organism. Later we will extend what we know to how the stringent response is triggered in other bacteria in response to environmental stresses.

Mechanism of the Stringent Response

Nutrient levels for microbes in nature can change significantly and rapidly. Such changing conditions can easily be simulated in the laboratory, and much work has been done on the regulation of gene expression following a "shift down" or "shift up" in nutrient status. These include, in particular, the regulatory events triggered by starvation for amino acids or energy.

As a result of a shift down from amino acid excess to limitation, as occurs when a culture is transferred from a rich complex medium to a defined medium with a single carbon source (Table 5.1), the synthesis of rRNA and tRNA ceases almost immediately, and no new ribosomes are produced (**Figure 6.23*a***). Protein and DNA

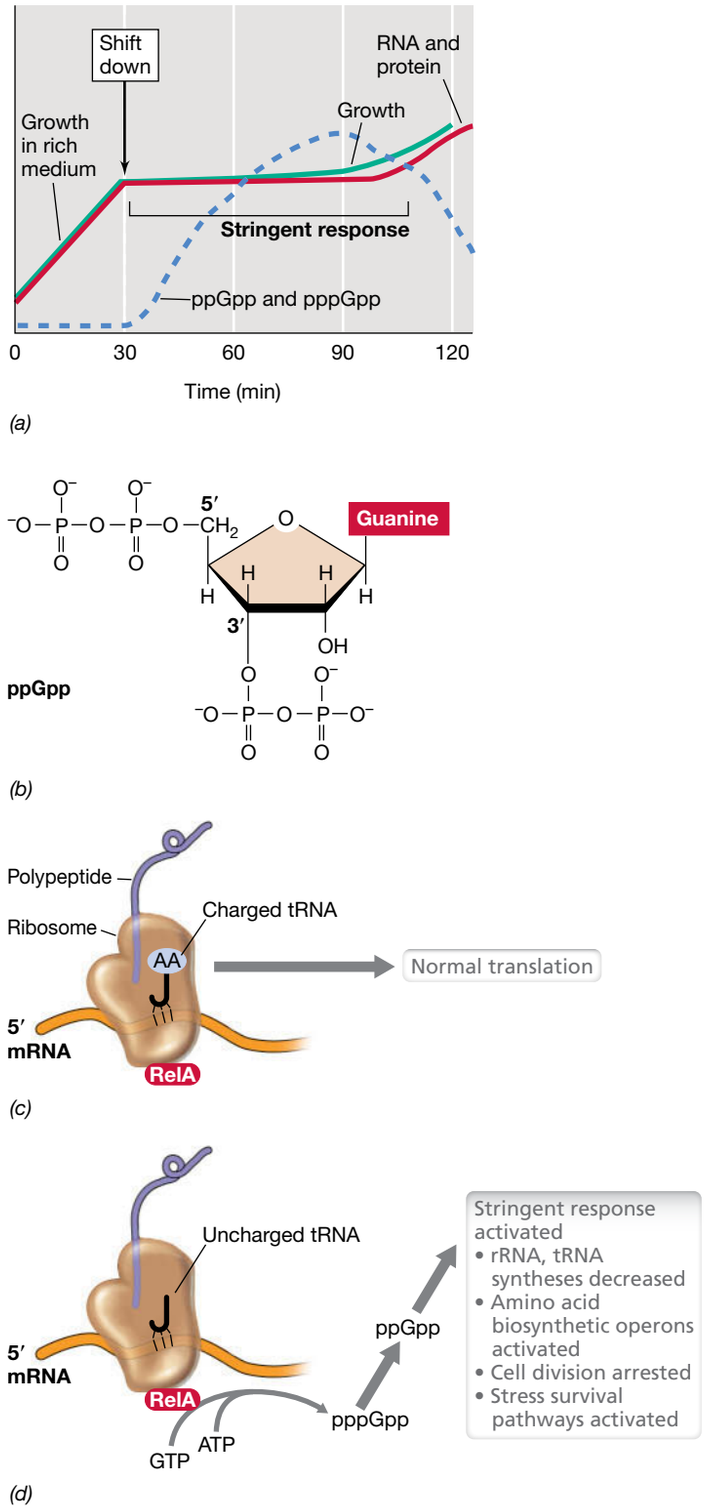


Figure 6.23 The stringent response in *Escherichia coli*. *(a)* Upon nutrient downshift, rRNA, tRNA, and protein syntheses temporarily cease. Sometime later, growth resumes at a decreased rate. *(b)* Structure of guanosine tetraphosphate (ppGpp), a trigger of the stringent response. *(c)* Normal translation, which requires charged tRNAs. *(d)* Synthesis of ppGpp. When cells are starved for amino acids, an uncharged tRNA can bind to the ribosome, which stops ribosome activity. This event triggers the RelA protein to synthesize a mixture of pppGpp and ppGpp.

synthesis are also curtailed, but the biosynthesis of new amino acids is activated. Following such a shift, new proteins must be made to synthesize the amino acids no longer available in the environment, and these proteins are made by existing ribosomes. After a while, rRNA synthesis (and hence, the production of new ribosomes) begins again but at a new rate commensurate with the cell's reduced growth rate (Figure 6.23*a*). This course of events is called the *stringent response* (or stringent control) and, like catabolite repression (Section 6.4), it is another example of global control.

The stringent response is triggered by a mixture of two regulatory nucleotides, *guanosine tetraphosphate* (ppGpp) and *guanosine pentaphosphate* (pppGpp); this mixture is often written as (p)ppGpp (Figure 6.23*b*). In *E. coli*, these nucleotides, which are also called *alarmones*, rapidly accumulate during stress or a shift down from amino acid excess to amino acid starvation. Alarmones are synthesized by a specific protein, called RelA, using ATP as a phosphate donor (Figure 6.23*c*, *d*). RelA adds two phosphate groups from ATP to GTP or GDP, thus producing pppGpp or ppGpp, respectively. RelA is associated with the 50S subunit of the ribosome and is activated by a signal from the ribosome during amino acid limitation. When the growth of the cell is limited by a shortage of amino acids, the pool of *uncharged* tRNAs increases relative to *charged* tRNAs. Eventually, an uncharged tRNA is inserted into the ribosome instead of a charged tRNA during protein synthesis. When this happens, the ribosome stalls, and this leads to (p)ppGpp synthesis by RelA (Figure 6.23*d*). The protein Gpp converts pppGpp to ppGpp so that ppGpp is the major overall product.

The alarmones ppGpp and pppGpp have global control effects. They strongly inhibit rRNA and tRNA synthesis by binding to RNA polymerase and preventing initiation of transcription of genes for these RNAs in gram-negative bacteria. In gram-positive bacteria, the same alarmones have been shown to interfere with initiating ribonucleotides for transcription. In both gram-negative and gram-positive bacteria, the alarmones activate both the stress response pathways and the biosynthetic operons for certain amino acids. In *E. coli*, the stringent response also inhibits the initiation of new rounds of DNA synthesis and cell division and slows down the synthesis of cell envelope components, such as membrane lipids.

In addition to RelA, a second protein called SpoT helps trigger the stringent response. SpoT can either make (p)ppGpp or degrade it. Under most conditions, SpoT is responsible for degrading (p)ppGpp; however, SpoT synthesizes (p)ppGpp in response to certain stresses or when nutrient deprivation is detected. Thus the stringent response results not only from the absence of precursors for protein synthesis, but also from the lack of energy for biosynthesis.

The Stringent Response and Microbial Ecology

Because the stringent response is a global mechanism that balances the metabolic state of the cell, the environment or habitat of the bacterium ultimately determines the response cascade. When cells of *E. coli* are voided in the feces and face a switch from the nutrient-rich intestine to an open water system, the reduction in nutrients triggers ppGpp synthesis to initiate the stringent response (**Figure 6.24*a***).

In contrast to *E. coli*, the aquatic bacterium *Caulobacter* naturally inhabits oligotrophic (nutrient-poor) freshwaters, and in this

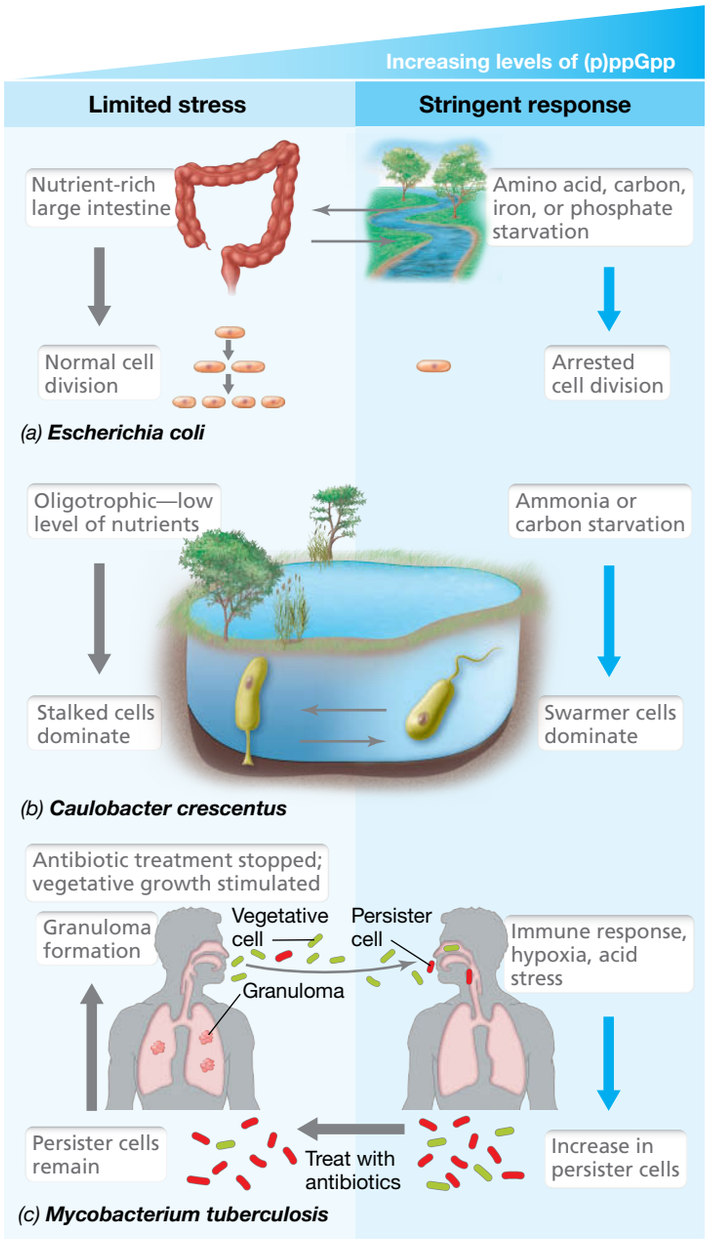


Figure 6.24 Environmental control of stringent response signaling in three separate bacteria, *Escherichia coli* and *Caulobacter crecentus* (both gram-negative), and *Mycobacterium tuberculosis* (gram-positive). *(a) Escherichia coli*. When *E. coli* cells move from the nutrient-rich intestine to an open water source, they experience nutrient limitation. As ppGpp production increases, cell division is inhibited. *(b) Caulobacter crescentus. C. crescentus* naturally inhabits freshwater systems that are limited in nutrients. If cells encounter severe nutrient limitation, the stringent response is induced and ppGpp production increases. This increase leads to the cell morphology changing from stalked cells to swarmer cells that can swim to find more nutrients. *(c) Mycobacterium tuberculosis*. When *M. tuberculosis* cells enter a host, they are exposed to the immune response. This leads to production of ppGpp, which converts some cells to relatively dormant persister cells. These persister cells are resistant to antibiotics and can dominate more sensitive vegetative cells, forming granulomas. Once persister cells are aerosolized and relieved of stress, they revert back to infective vegetative cells. Unlike many other bacterial pathogens that gain antibiotic resistance from mobile genetic elements, horizontal gene transfer is rare in *M. tuberculosis*. Antibiotic resistance in this pathogen is primarily due to chromosomal mutations affecting the target of the antibiotic.

bacterium, the stringent response is triggered by carbon and ammonia starvation, not amino acid limitation. *Caulobacter* can form two types of cells, stalked cells and swarmer cells (Figure 6.24*b*). To increase its probability of survival, the production of ppGpp in *Caulobacter* leads to an increase in swarmer cell formation. Unlike stalked cells, swarmer cells are motile and can therefore swim and perhaps reach a niche containing more nutrients (Section 7.7) (Figure 6.24*b*).

In a disease example, the stringent response has also been implicated in the persistence of the aerobic bacterial pathogen *Mycobacterium tuberculosis* (tuberculosis, Section 30.4) in dormant granulomas. Within the lung of the human host, the local environment is both hypoxic and limited for phosphate, and these conditions trigger the stringent response in cells of *M. tuberculosis*. Activation of the stringent response also leads to a subpopulation of *M. tuberculosis* cells that convert to dormant *persister cells* (Section 7.11); these cells are resistant to antibiotics and can survive in granulomas that form in the lung (Figure 6.24*c*). Once drug treatment of the infection has greatly reduced cell numbers and the environmental stresses have been relieved, the *M. tuberculosis* persister cells can then revert back to infective cells and trigger chronic tuberculosis, a not uncommon occurrence and one of the reasons drug therapy for tuberculosis is carried out for such a long period.

MINIQUIZ

- Which *Escherichia coli* genes are activated and which are repressed during the stringent response, and why?
- How are the alarmones ppGpp and pppGpp synthesized?
- What are some other conditions that trigger the stringent response in other bacteria?

6.10 Other Global Networks

Catabolite repression (Section 6.4) and quorum sensing (Section 6.8) are both examples of *global regulatory control*. There are several other global control systems in *Escherichia coli* (and probably in all *Bacteria* and *Archaea*), and some key ones are listed in Table 6.2. Global control systems regulate many genes comprising more than one regulon (Section 6.3). Global control networks may include activators, repressors, signal molecules, two-component regulatory systems, regulatory RNA (Section 6.11), and alternative sigma (σ) factors (Section 4.5).

Three well-studied examples of global responses in *Bacteria* include the *phosphate* (*Pho*) *regulon*, the **heat shock response**, and the *RpoS regulon*. The Pho regulon illustrates how cells respond to environmental phosphate concentrations and link this response to other metabolic and synthesis pathways, while the heat shock response is widespread in all three domains of life and in *Bacteria* is largely controlled by alternative σ factors. For general bacterial responses to stress, the alternative σ factor RpoS is the *master controller*, as it regulates over 400 different genes.

The Phosphate (Pho) Regulon

Phosphorus is not only essential for DNA, RNA, and membrane synthesis, but it is also critical for energy generation and cell signaling (Section 3.1). In nature, phosphorus is generally in the form of PO_4^{3-} (inorganic phosphate, abbreviated P_i) and is often the limiting nutrient in many environments. Thus regulatory mechanisms such as the Pho regulon have evolved in *Bacteria* to deal with P_i limitation. This regulon consists of a two-component regulatory system (Section 6.6) that regulates genes encoding extracellular enzymes for obtaining P_i from organic phosphates, P_i transporters, and enzymes for P_i storage using positive control mechanisms (Section 6.3).

In *Streptomyces*, the two-component Pho regulatory system consists of a membrane-bound histidine kinase sensor protein PhoR and a cytoplasmic transcriptional regulator PhoP (PhoB in *E. coli*). While the mechanism for actually *sensing* extracellular P_i levels is unknown, low environmental P_i levels are known to trigger PhoR kinase activity, yielding a phosphorylated PhoP (PhoP-P) (Figure 6.25). Once phosphorylated, PhoP-P then binds to conserved promoter regions called Pho boxes scattered across the genome. This binding signals the "general housekeeping" sigma subunit of RNA polymerase to bind, and transcription of genes needed for P_i uptake is *activated*. When P_i is in excess, PhoP is dephosphorylated, which leads to the removal of PhoP from the

TABLE 6.2 Examples of global control systems known in *Escherichia coli*[a]

System	Signal	Primary activity of regulatory protein	Number of genes regulated
Aerobic respiration	Presence of O_2	Repressor (ArcA)	> 50
Anaerobic respiration	Lack of O_2	Activator (FNR)	> 70
Catabolite repression	Cyclic AMP level	Activator (CRP)	> 300
Heat shock	Temperature	Alternative sigma factors (RpoH and RpoE)	36
Nitrogen utilization	NH_3 limitation	Activator (NRI)/alternative sigma factor (RpoN)	> 12
Oxidative stress	Oxidizing agents	Activator (OxyR)	> 30
SOS response	Damaged DNA	Repressor (LexA)	> 20
General stress response	Stress conditions	Alternative sigma factor (RpoS)	> 400

[a]For many of the global control systems, regulation is complex. A single regulatory protein can play more than one role. For instance, the regulatory protein for aerobic respiration is a repressor for many promoters but an activator for others, whereas the regulatory protein for anaerobic respiration is an activator protein for many promoters but a repressor for others. Regulation can also be indirect or require more than one regulatory protein. Many genes are regulated by more than one global system.

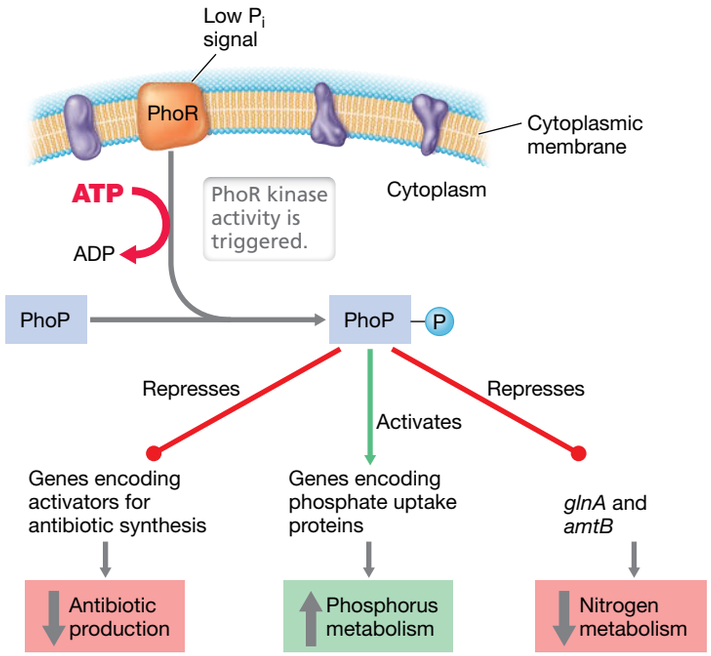


Figure 6.25 The phosphate (Pho) regulon of *Streptomyces*. The genus *Streptomyces* contains species of filamentous gram-positive *Bacteria*, many of which produce antibiotics (Section 16.12). If inorganic phosphate (P_i) concentrations are low, a signal is sent to the kinase PhoR to phosphorylate PhoP. PhoP-P recognizes and binds to promoter regions, acting as both an activator and a repressor. This regulation results in increasing phosphorus metabolism by activating phosphate uptake genes and decreasing antibiotic production and nitrogen metabolism by repressing genes regulating antibiotic production and glutamine synthetase (*glnA*) and ammonia transport (*amtB*), respectively.

Pho boxes and decreased transcription of genes encoding phosphate metabolism proteins.

While the Pho regulon is the mechanism many *Bacteria* use to respond to P_i starvation, why is this regulon considered an example of global control? Besides its role as an activator of P_i uptake and storage genes in *Streptomyces* and many other bacteria, PhoP-P also binds some promoters weakly without recruiting a sigma subunit. This binding blocks RNA polymerase and thus leads to repression of the linked gene. Somewhat surprisingly, PhoP-P appears to actually repress more genes than it activates. Genes regulated by P_i limitation and PhoP-P that are not associated with P_i uptake in *Streptomyces* include those whose products are necessary for nitrogen metabolism (glutamine synthetase, *glnA*; ammonium transport, *amtB*) and antibiotic synthesis (Figure 6.25).

The Pho regulon can also control some aspects of pathogenesis. Pathogenic bacteria are exposed to both P_i-limiting and P_i-rich environments within a host depending on the site of infection. While the mechanisms remain unclear, the Pho regulon has been implicated in regulating biofilm formation (Sections 5.1 and 7.9), antimicrobial resistance (Section 28.12), toxin production (Section 25.5), and resistance to acidity in pathogens such as *Vibrio cholerae, Pseudomonas* spp., and pathogenic *E. coli*. Thus the Pho regulon is a barometer of P_i starvation that leads to the regulation of a range of unrelated genes controlling a myriad of cellular functions.

Heat Shock Proteins

Most proteins are relatively stable, even to small increases in temperature. However, some proteins are less stable at elevated temperatures and tend to unfold (denature, Section 4.7). Improperly folded proteins are recognized by protease enzymes and are degraded. Consequently, heat stress triggers the synthesis of a set of proteins—the **heat shock proteins**—that help counteract the damage and assist the cell in recovering from stress. Heat shock proteins are induced not only by heat but also by several other stress factors, including the exposure to high levels of certain chemicals, such as ethanol, or exposure to high doses of ultraviolet (UV) radiation.

In most *Bacteria* and *Archaea*, there are three major classes of heat shock protein: Hsp70, Hsp60, and Hsp10. We have encountered these proteins before, although not by these names (Section 4.11 and Figure 4.40*a*). The Hsp70 protein of *E. coli* is DnaK, which prevents aggregation of newly synthesized proteins and stabilizes unfolded proteins. Major representatives of the Hsp60 and Hsp10 families in *E. coli* are the proteins GroEL and GroES, respectively. These are *molecular chaperones* that catalyze the correct refolding of misfolded proteins. Another class of heat shock proteins includes various proteases that degrade denatured or irreversibly aggregated proteins.

Heat Shock Response

In many bacteria, such as *E. coli*, the heat shock response is controlled by the alternative σ factor (Section 4.5) RpoH (σ^{32}) (Figure 6.26). RpoH controls expression of heat shock proteins and is normally degraded within a minute or two of its synthesis. However, when cells suffer a heat shock, degradation of RpoH is inhibited and its level therefore increases. As a consequence, transcription of those operons whose promoters are recognized by RpoH increases.

The rate of RpoH degradation depends on the level of free DnaK, which inactivates RpoH. In unstressed cells, the level of free DnaK is relatively high and the level of intact RpoH is correspondingly low. However, if heat begins to unfold proteins, DnaK binds preferentially to unfolded proteins and so is no longer free to degrade RpoH. Thus, the more denatured proteins there are, the lower the level of free DnaK and the higher the level of RpoH; the result is heat shock gene expression—the heat shock response. When the environmental stressor has passed, for example upon a temperature downshift, RpoH is once again inactivated by DnaK and the synthesis of heat shock proteins is reduced.

Because heat shock proteins perform vital functions in the cell, there is always a low level of these proteins present, even under optimal conditions. However, the rapid synthesis of heat shock proteins in stressed cells emphasizes their importance for surviving exposure to excessive heat, chemicals, or physical agents. Such stresses can generate large amounts of inactive proteins that need to be refolded (and in the process, reactivated) or degraded to release free amino acids for the synthesis of new proteins.

There is also a heat shock response in *Archaea*, even in species that grow best at very high temperatures. An analog of the bacterial Hsp70 is found in many *Archaea* and is structurally quite similar to that found in gram-positive species of *Bacteria*. Hsp70 is also

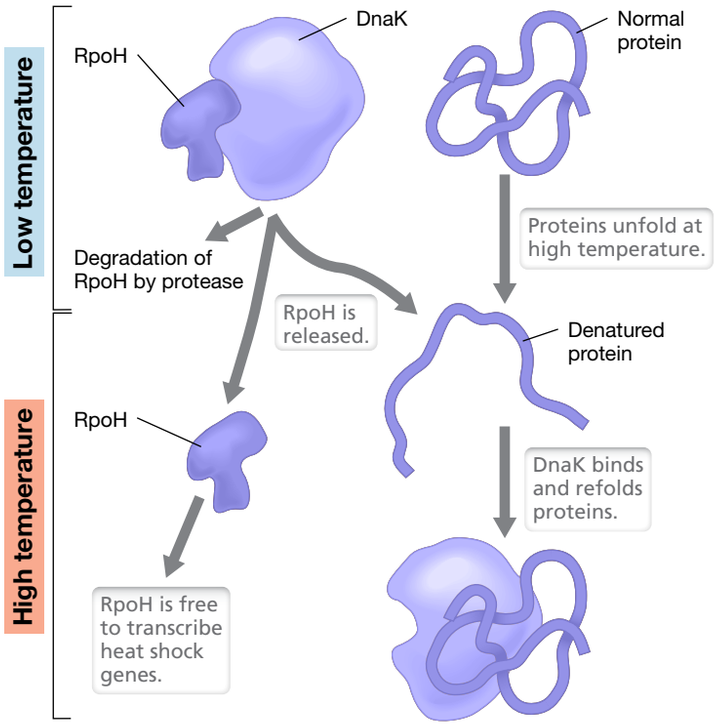


Figure 6.26 Control of heat shock in *Escherichia coli*. The RpoH alternative sigma factor is broken down rapidly by proteases at normal temperatures. This is stimulated by binding of the DnaK chaperone to RpoH. At high temperatures, some proteins are denatured, and DnaK recognizes, binds, and refolds these unfolded polypeptide chains. This removes RpoH from DnaK, which slows the degradation rate. When the level of RpoH rises, the heat shock genes are transcribed.

present in eukaryotes. In addition, other types of heat shock proteins are present in *Archaea* that are unrelated to stress proteins of *Bacteria*.

The General Stress Response: The RpoS Regulon

In nature microorganisms must survive under nutrient-limited conditions and exposure to environmental stressors such as extreme pH and oxidative stress. How do cells cope and adapt to such conditions? While some gram-positive cells undergo sporulation to withstand harsh conditions (↻ Section 2.10), many gram-negative *Bacteria* possess a general stress response in addition to the stringent response (Section 6.9) to activate cell survival pathways. These pathways are controlled by the alternative sigma factor RpoS (σ^S or σ^{38}, ↻ Table 4.3). Because RpoS is highly expressed during the transition from exponential to stationary phase, it is also known as the *stationary phase sigma factor*. The RpoS regulon comprises over 400 genes including those associated with nutrient limitation, resistance to DNA damage, biofilm formation, and responses to osmotic, oxidative, and acid stresses. Thus, RpoS not only senses environmental changes but also relays signals to other regulators.

Examples of *E. coli* genes that are recognized by RpoS include *dinB*, which encodes DNA polymerase IV of the SOS DNA repair system (↻ Section 11.4 and Figure 11.9) and catalase genes necessary for combating reactive oxygen species (↻ Section 5.14). Because of the expansive nature of the RpoS regulon, this alternative sigma factor is itself controlled by numerous transcriptional, translational, and post-translational regulatory mechanisms. For example, transcription of the *rpoS* gene increases in response to the presence of the alarmone ppGpp, thus directly linking the RpoS regulon with the stringent response (Section 6.9). Translation of the *rpoS* mRNA is also positively regulated by the presence of small RNAs expressed during stress conditions (Section 6.11). Conversely, the RpoS protein is susceptible to degradation during nonstressful conditions. Overall, the general stress response highlights the global control nature of RpoS and the coordinated processes that must occur for cell survival and adaptation.

MINIQUIZ

- What is the heat shock response?
- Why do cells have more than one type of σ factor?
- How do levels of RpoH control the heat shock response?

III • RNA-Based Regulation

Thus far we have focused on regulatory mechanisms in which proteins sense signals or bind to DNA. In some cases a single protein does both; in other cases, separate proteins carry out these two activities. Nonetheless, all of these mechanisms rely on regulatory *proteins*. However, in some cases *RNA* can regulate gene expression, both at the level of transcription and at the level of translation.

RNA molecules that are not translated to give proteins are collectively known as **noncoding RNA (ncRNA)**. This category includes the rRNA and tRNA molecules that participate in protein synthesis and the RNA present in the signal recognition particle that catalyzes some types of protein secretion (↻ Sections 4.8, 4.10, and 4.12). Noncoding RNA also includes small RNA molecules necessary for RNA processing, especially the splicing of mRNA in eukaryotes. *Small RNAs* (sRNAs) that range from 40–400 nucleotides long and regulate gene expression are widely distributed in both prokaryotic cells and eukaryotes. In *Escherichia coli*, for example, a number of sRNA molecules regulate various aspects of cell physiology in response to environmental or cellular signals by binding to other RNAs or in some cases to other small molecules; the end result is control of gene expression.

6.11 Regulatory RNAs

Small RNAs (sRNAs) exert their effects by base-pairing directly to other RNA molecules, usually mRNAs, which have regions of complementary sequence. This binding immediately modulates the rate of target mRNA translation because a ribosome cannot translate double-stranded RNA. Thus, sRNAs provide an additional mechanism to regulate the synthesis of a protein once its corresponding mRNA has already been transcribed.

Mechanisms of sRNA Activity

Small RNAs alter the translation of their mRNA target by four different mechanisms (**Figure 6.27**). Some sRNAs base-pair to their target mRNA, changing its secondary structure to either block a

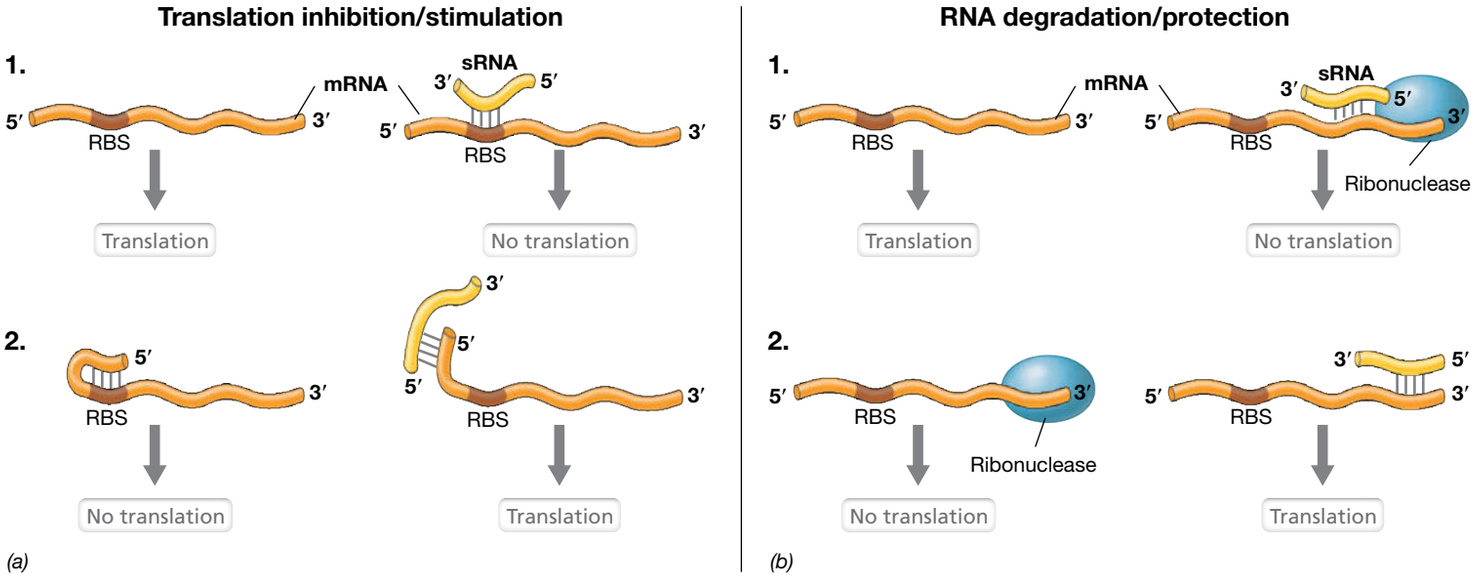


Figure 6.27 Small RNA mechanisms for modulating the translation of mRNA. *(a)* Binding of a ribosome to mRNA requires that the ribosome-binding site (RBS) of the mRNA be single-stranded. Binding of an sRNA to the RBS (shown in 1) can prevent translation, while the binding of an sRNA to an mRNA whose RBS has secondary structure (shown in 2) can stimulate translation. *(b)* Ribonuclease degrades RNA. Ribonuclease binding to partially double-stranded RNA results in RNA degradation (shown in 1), while sRNA binding at the ribonuclease binding site (shown in 2) can protect the mRNA from degradation. Compare the mechanisms for controlling translation by small RNAs shown in this figure with the mechanism of controlling translation by a riboswitch shown in Figure 6.30.

previously accessible ribosome-binding site (RBS) (Section 4.9) or to open up a previously blocked RBS, allowing access for the ribosome. These two events decrease or increase expression of the protein encoded by the target mRNA, respectively. The other two mechanisms of sRNA interaction affect mRNA stability; binding of the sRNA to its target can either increase or decrease degradation of the transcript by bacterial ribonucleases, thus modulating protein expression. Increased degradation of an mRNA prevents the synthesis of new protein molecules encoded by that mRNA. Alternatively, increasing the stability of mRNA will lead to higher corresponding protein levels in the cell (Figure 6.27).

Types of Small RNA

Small RNAs (sRNAs) are important modulators of various cellular processes including oxidative, iron, and glucose–phosphate stresses as well as quorum sensing, biofilm formation, and stationary phase events. Transcription of sRNAs is often enhanced under conditions in which their target genes need to be turned off. For example, the RyhB RNA of *Escherichia coli* is transcribed when iron is limiting for growth. RyhB sRNA binds to several distinct target mRNAs that encode proteins needed for iron metabolism or that use iron as cofactors. Binding of RyhB sRNA blocks the RBS of the mRNA and thus inhibits translation (Figure 6.27*a*). The base-paired RyhB/mRNA molecules are then degraded by ribonucleases, in particular, ribonuclease E, before translation of the mRNA can occur. This forms part of the mechanism by which *E. coli* and related bacteria respond to a shortage of iron. Other responses to iron limitation in *E. coli* include transcriptional controls by repressor and activator proteins (Sections 6.2 and 6.3) that decrease or increase, respectively, the capacity of cells to take up iron or to tap into intracellular iron reserves.

Similarly, SgrS is an sRNA in *E. coli* that is expressed during glucose–phosphate stress to avoid accumulation of glucose 6-phosphate. High levels of glucose 6-phosphate within the cell can block the glycolytic pathway (Section 3.8) and ultimately lead to cell death if not overcome. When SgrS is expressed, the sRNA molecule binds to the *ptsG* mRNA encoding a glucose transporter. This binding results in a double-stranded RNA complex that triggers degradation of *ptsG* mRNA by ribonuclease E (**Figure 6.28**). This degradation decreases the amount of *ptsG* mRNA that is

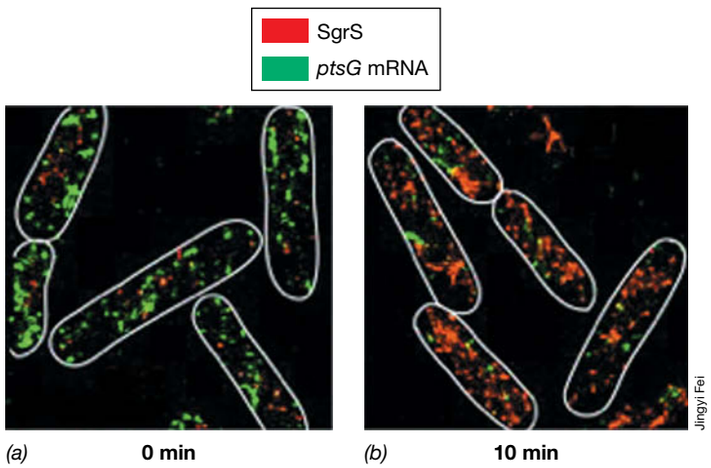


Figure 6.28 The interaction of SgrS sRNA and *ptsG* mRNA during glucose–phosphate stress. Three-dimensional super-resolution image of fluorescently labeled SgrS sRNA (red) and *ptsG* mRNA (green) in cells of *Escherichia coli*. *(a)* Before glucose–phosphate stress at time 0, *ptsG* mRNA is more abundant than SgrS sRNA. *(b)* After 10 min of glucose–phosphate stress, SgrS levels are higher due to degradation of *ptsG* mRNA.

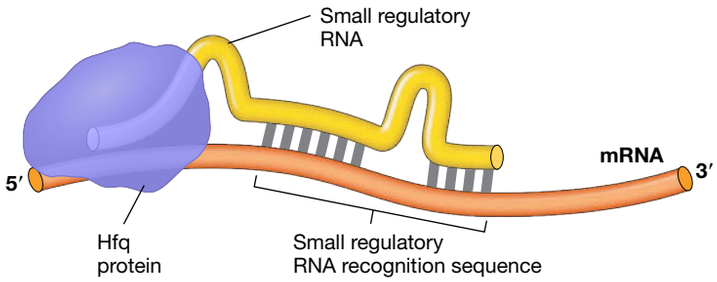


Figure 6.29 The RNA chaperone Hfq holds RNAs together. Binding of sRNA to mRNA often requires the Hfq protein. Small RNA molecules usually have several stem–loop structures. One consequence is that the complementary base sequence that recognizes the mRNA is noncontiguous.

translated, and thus lower levels of the glucose transporter protein are made. This in turn results in decreased levels of glucose 6-phosphate, which allows the glycolytic pathway to remain active.

Because many sRNAs, including RyhB and SgrS, are encoded in intergenic regions and can be spatially separated from their mRNA target, they are called *trans-sRNAs*. As such, these sRNAs usually have limited complementarity to their target molecule and may only base-pair with a 5- to 11-nucleotide stretch. The binding of trans-sRNAs to their targets often depends on a small protein called Hfq (**Figure 6.29**) that binds to both RNA molecules to facilitate their interaction. Hfq forms hexameric rings with RNA-binding sites on both surfaces. Hfq and functionally similar proteins are called *RNA chaperones*, as they help small RNA molecules, including many sRNAs, maintain their correct structure (Figure 6.29).

Small RNAs do not always work by affecting mRNA. For example, replication of the ColE1 plasmid in *Escherichia coli* is regulated by an sRNA that primes DNA synthesis on the plasmid and its antisense partner that blocks initiation of DNA synthesis. The level of the antisense RNA determines how often replication is initiated. Some sRNAs also bind to proteins and modulate their activity.

MINIQUIZ

- How do sRNAs alter the translation of target mRNAs?
- Why do trans-sRNAs often require a chaperone protein?
- How does SgrS help *Escherichia coli* prevent a potential metabolic disaster?

6.12 Riboswitches

RNA can carry out many roles once thought to be limited to proteins. In particular, RNA can specifically recognize and bind other molecules, including low-molecular-weight metabolites. It is important to emphasize that such binding does not require complementary base pairing (as does binding of the sRNAs described in the previous section) but results from the folding of the RNA into a specific three-dimensional structure that recognizes the target molecule, much as a protein enzyme recognizes its substrate in its active site. Catalytically active RNAs are called *ribozymes*. Other RNA molecules resemble repressors and activators in binding small metabolites and regulating gene expression; these are the **riboswitches**.

Mechanisms of Riboswitches

Earlier in this chapter we discussed the regulation of gene expression by negative control of transcription (Section 6.2). In this process, a specific metabolite interacts with a specific repressor protein to prevent transcription of genes encoding enzymes for the biosynthetic pathway of the metabolite. In contrast, in a riboswitch there is no regulatory protein. Instead, the metabolite binds directly to the mRNA. Because of this, riboswitches typically exert their control *after* the mRNA has already been synthesized; that is, most riboswitches control *translation* of the mRNA, rather than its *transcription*.

Riboswitch mRNAs contain regions upstream of their coding sequences that can fold into specific three-dimensional structures that bind small molecules. These recognition domains are the "switch" in the riboswitch and exist as two alternative secondary structures, one with the small molecule bound and the other without (**Figure 6.30**). Alternation between the two forms of the riboswitch thus depends on the presence or absence of the small molecule, which in turn controls expression of the mRNA. Riboswitches are known that control the synthesis of enzymes in biosynthetic pathways for various vitamins, a few amino acids, some nitrogen bases, and a precursor in peptidoglycan synthesis (**Table 6.3**).

The metabolite that is bound by the riboswitch is typically the product of a biosynthetic pathway whose constituent enzymes are encoded by the mRNAs that carry the corresponding riboswitches. For example, the thiamine riboswitch that binds the vitamin thiamine pyrophosphate lies upstream of the coding sequences for enzymes that participate in the thiamine biosynthetic pathway. When the pool of thiamine pyrophosphate is sufficient in the cell, this metabolite binds to its specific riboswitch mRNA. The new secondary structure of the riboswitch blocks the ribosome-binding

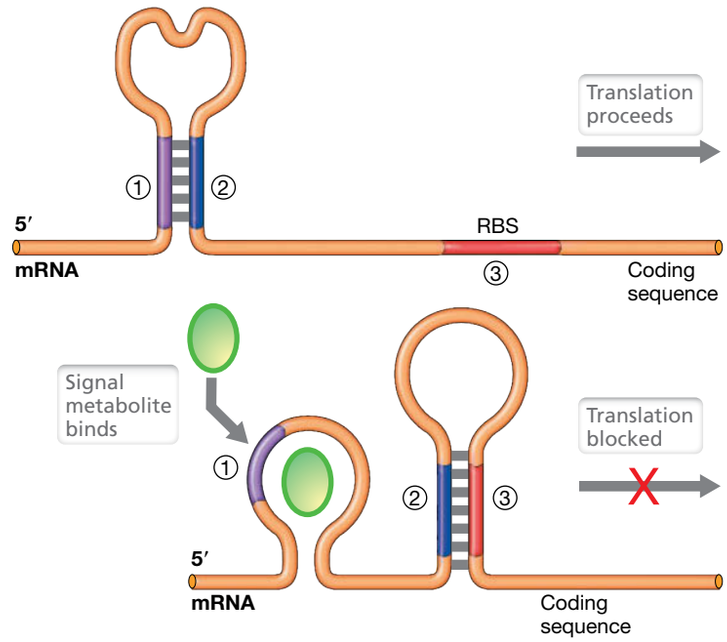


Figure 6.30 Regulation by a riboswitch. Binding of a specific metabolite alters the secondary structure of the riboswitch domain, which is located in the 5′ untranslated region of the mRNA, preventing translation. Numbers indicate regions within the riboswitch that can base-pair together. RBS, ribosome-binding site.

TABLE 6.3 Riboswitches in biosynthetic pathways of *Escherichia coli*

Type	*Example of biosynthetic pathway*
Vitamins	Cobalamin (B_{12}), tetrahydrofolate (folic acid), thiamine
Amino acids	Glutamine, glycine, lysine, methionine
Nitrogen bases of nucleic acids	Adenine, guanine (purine bases)
Others	Flavin mononucleotide (FMN), *S*-adenosylmethionine (SAM), glucosamine 6-phosphate (peptidoglycan precursor), cyclic di-GMP (biofilm signaling molecule)

site on the mRNA and prevents the mRNA from binding to the ribosome; this prevents translation (Figure 6.30). If the concentration of thiamine pyrophosphate drops sufficiently low, the molecule can dissociate from its riboswitch mRNA. This unfolds the mRNA and exposes the ribosome-binding site, allowing the mRNA to bind to the ribosome and be translated.

Despite being part of the mRNA, some riboswitches nevertheless do control transcription. The mechanism is similar to that seen in attenuation (Section 6.13) where a conformational change in the riboswitch causes premature termination of the synthesis of the mRNA that carries it.

Riboswitches and Evolution

How widespread are riboswitches and how did they evolve? Thus far riboswitches have been found only in some bacteria and a few plants and fungi. However, genomic analyses of *Archaea* indicate that at least some putative riboswitches are likely in this domain, too. Some scientists believe that riboswitches are remnants of the RNA world, a period eons ago before cells, DNA, and protein were present when it is hypothesized that catalytic RNAs were the only self-replicating "life forms." In such an environment, riboswitches may have been a primitive mechanism of metabolic control—a simple means by which RNA life forms could have controlled the synthesis of other RNAs. As proteins evolved, riboswitches might have been the first control mechanisms for their synthesis as well. If this is true, the riboswitches that remain today may be the last vestiges of this simple form of control because, as we have seen in this chapter, metabolic regulation is almost exclusively carried out by way of regulatory *proteins*.

MINIQUIZ

- What happens when a riboswitch binds the small metabolite that regulates it?
- What are the major differences between a repressor protein and a riboswitch in the control of gene expression?

6.13 Attenuation

Attenuation is a form of transcriptional control in *Bacteria* (and likely in *Archaea* as well) that functions by prematurely terminating mRNA synthesis. That is, in attenuation, control is exerted *after* the initiation of transcription but *before* its completion. Consequently, the number of completed transcripts from an operon is reduced, even though the number of initiated transcripts is not.

The basic principle of attenuation is that the first part of the mRNA to be made, called the *leader*, can fold into two alternative secondary structures. In this respect, the mechanism of attenuation resembles that of riboswitches (Figure 6.30). In attenuation, one mRNA secondary structure allows continued synthesis of the mRNA, whereas the other secondary structure causes premature termination. Folding of the mRNA depends either on events at the ribosome or on the activity of regulatory proteins, depending on the organism. The best examples of attenuation are the regulation of genes controlling the biosynthesis of certain amino acids in gram-negative *Bacteria*. The first to be described was in the tryptophan operon in *Escherichia coli*, and we focus on it here. Because the processes of transcription and translation are spatially separated in eukaryotes (Section 4.6), attenuation control is absent from *Eukarya*.

Attenuation in the Tryptophan Operon

The tryptophan operon contains structural genes for five proteins of the tryptophan biosynthetic pathway plus the usual promoter and regulatory sequences at the beginning of the operon (**Figure 6.31**). Like many operons, the tryptophan operon has more than one type of regulation. Transcription of the entire tryptophan operon is under negative control (Section 6.2). However, in addition to the promoter and operator regions needed for negative control, there is a sequence in the operon called the *leader sequence* that encodes a short polypeptide, the *leader peptide*. The leader

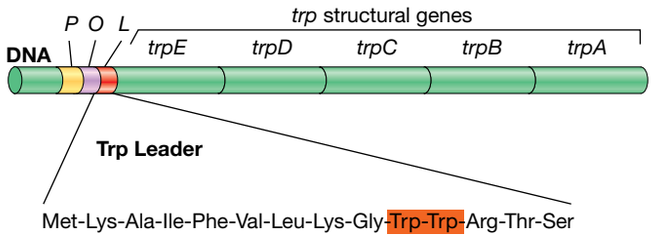


Figure 6.31 Attenuation and leader peptides in *Escherichia coli*. Structure of the tryptophan (*trp*) operon and of the tryptophan leader peptide and other leader peptides in *E. coli*. *(a)* Arrangement of the *trp* operon. Note that the leader (*L*) encodes a short peptide containing two tryptophan residues near its terminus (there is a stop codon following the Ser codon). The promoter is labeled *P*, and the operator is labeled *O*. The genes labeled *trpE* through *trpA* encode the enzymes needed for tryptophan synthesis. *(b)* Amino acid sequences of leader peptides of some other amino acid biosynthesis operons. Because isoleucine is made from threonine, it is an important constituent of the threonine leader peptide.

sequence contains tandem tryptophan codons near its terminus and functions as an attenuator (Figure 6.31).

The basis of control of the tryptophan attenuator is as follows. If tryptophan is plentiful in the cell, there will be plenty of charged tryptophan tRNAs and the leader peptide will be synthesized. Synthesis of the leader peptide results in termination of transcription of the remainder of the *trp* operon, which includes the structural genes for the biosynthetic enzymes. On the other hand, if tryptophan is scarce, the tryptophan-rich leader peptide will not be synthesized. If synthesis of the leader peptide is halted by a lack of tryptophan, the rest of the operon is transcribed.

Mechanism of Attenuation

How does translation of the leader peptide regulate transcription of the tryptophan genes downstream? Consider that in prokaryotic cells transcription and translation are simultaneous processes; as mRNA is released from the DNA, the ribosome binds to it and translation begins (Section 4.1). That is, while *transcription* of downstream DNA sequences is still proceeding, *translation* of transcribed sequences is already underway (**Figure 6.32**).

Transcription is attenuated because a portion of the newly formed mRNA folds into a unique stem–loop that inhibits RNA polymerase activity. The stem–loop structure forms in the mRNA because two stretches of nucleotides near each other are complementary and can thus base-pair. If tryptophan is plentiful, the ribosome translates the leader sequence until it comes to the leader stop codon. The remainder of the leader sequence then forms a stem–loop, a transcription pause site, which is followed by a uracil-rich sequence that actually triggers termination (Figure 6.32*a*).

If the concentration of tryptophan in the cell is limiting, transcription of genes encoding tryptophan biosynthetic enzymes is needed. Thus, during transcription of the leader, the ribosome pauses at a tryptophan codon because of a shortage of charged tryptophan tRNAs. The presence of the stalled ribosome at this position allows a stem–loop to form (sites 2 and 3 in Figure 6.32*b*) that differs from the terminator stem–loop. This alternative stem–loop is not a transcription termination signal but instead prevents the terminator stem–loop (sites 3 and 4 in Figure 6.32*a*) from forming. This allows RNA polymerase to move past the termination site and begin transcription of tryptophan structural genes. Thus, in attenuation control, the rate of transcription is ultimately influenced by the rate of translation.

MINIQUIZ

- Why does attenuation control not occur in eukaryotes?
- Explain how the formation of one stem–loop in the RNA can block the formation of another.

IV • Regulation of Enzymes and Other Proteins

We have just explored some of the key mechanisms for regulating the *amount* (or even the complete presence or absence) of an enzyme or other protein within a cell. Here we focus on the mechanisms that cells employ to control the *activity* of enzymes already present in the cell through processes such as feedback inhibition and post-translational regulation.

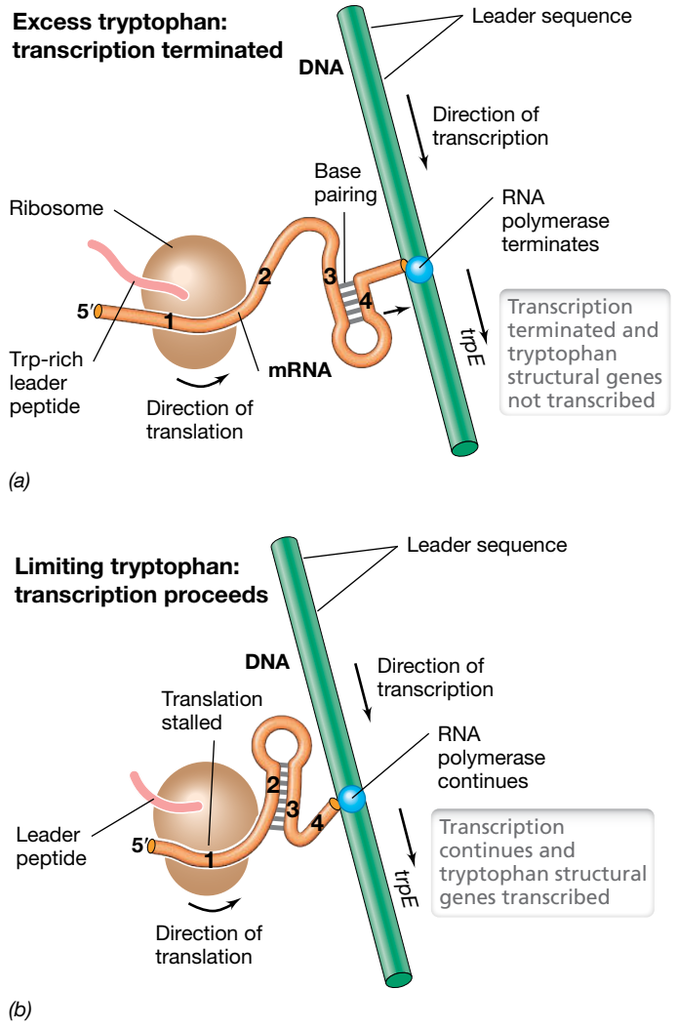
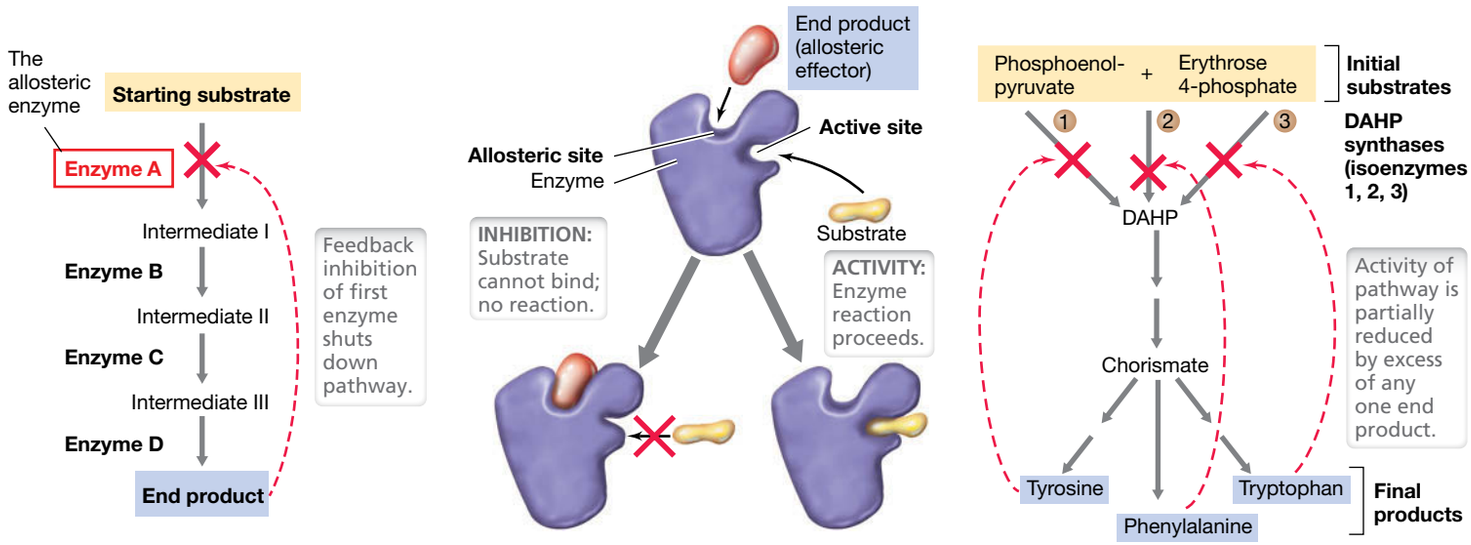


Figure 6.32 Mechanism of attenuation. Control of transcription of tryptophan (*trp*) operon structural genes by attenuation in *Escherichia coli*. The leader peptide is encoded by regions 1 and 2 of the mRNA. Two regions of the growing mRNA chain are able to form double-stranded loops, shown as 3:4 and 2:3. *(a)* When there is excess tryptophan, the ribosome translates the complete leader peptide, and so region 2 cannot pair with region 3. Regions 3 and 4 then pair to form a loop that terminates transcription. *(b)* If translation is stalled because of tryptophan starvation, a loop forms by pairing of region 2 with region 3, loop 3:4 does not form, and transcription proceeds past the leader sequence.

6.14 Feedback Inhibition

A major means of controlling enzymatic activity is by **feedback inhibition**. This mechanism temporarily shuts off the reactions in an entire biosynthetic pathway. The reactions are shut off because an excess of the end product of the pathway inhibits activity of an early (typically the *first*) enzyme of the pathway. Inhibiting an early step effectively shuts down the entire pathway because no intermediates are generated for subsequent enzymes in the pathway (**Figure 6.33*a***). Feedback inhibition is reversible,



(a) **Feedback inhibition** *(b)* **Allosteric inhibition** *(c)* **Isoenzyme inhibition**

Figure 6.33 Inhibition of enzyme activity. *(a)* In feedback inhibition, the activity of the first enzyme of the pathway is inhibited by the end product, thus shutting off the production of the three intermediates and the end product. *(b)* The mechanism of allosteric inhibition by the end product of a pathway. When the end product binds at the allosteric site, the conformation of the enzyme is so altered that the substrate can no longer bind to the active site. However, inhibition is reversible, and end product limitation will once again activate the enzyme. *(c)* Inhibition by isoenzymes. In *Escherichia coli*, the pathway leading to the synthesis of the aromatic amino acids contains three isoenzymes of DAHP synthase. Each of these enzymes is feedback-inhibited by one of the aromatic amino acids. However, note how an excess of all three amino acids is required to completely shut off the synthesis of DAHP. In addition to feedback inhibition at the DAHP site, each amino acid feedback inhibits its further metabolism at the chorismate step.

however, because once levels of the end product become limiting, the pathway again becomes functional.

How can the end product of a pathway inhibit the activity of an enzyme whose substrate is quite unrelated to it? This occurs because the inhibited enzyme has two binding sites, the *active site* (where substrate binds, ↪ Section 3.5), and the *allosteric site*, where the end product of the pathway binds. When the end product is in excess, it binds at the allosteric site, changing the conformation of the enzyme such that the substrate can no longer bind at the active site (Figure 6.33*b*). When the concentration of the end product in the cell begins to fall, however, the end product no longer binds to the allosteric site, so the enzyme returns to its catalytic form and once again becomes active.

Isoenzymes

Some biosynthetic pathways controlled by feedback inhibition employ *isoenzymes* ("iso" means "same"). Isoenzymes are different proteins that catalyze the same reaction but are subject to different regulatory controls. Examples are enzymes required for the synthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine in *Escherichia coli* (Figure 6.33*c*).

The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase plays a central role in aromatic amino acid biosynthesis, whose pathway contains a branch point late in the pathway (Figure 6.33*c*). In *E. coli*, three DAHP synthase isoenzymes catalyze the first reaction in this pathway, each regulated independently by a different one of the end product amino acids. However, unlike the example of feedback inhibition where an end product completely inhibits enzyme activity (Figure 6.33*a*, *b*), enzyme activity in the DAHP-controlled pathway is diminished incrementally; enzyme activity falls to zero only when *all three* end products are present in excess. This prevents the excess of one or two of the amino acids from shutting down the entire pathway and thereby starving the cell for the third amino acid (Figure 6.33*c*).

MINIQUIZ

- What is feedback inhibition?
- What is the difference between an allosteric site and an active site?
- What is an isoenzyme?

6.15 Post-Translational Regulation

We have already discussed *phosphorylation* and *methylation*, two very common mechanisms for regulating a protein post-translationally, when we considered two-component regulatory systems and chemotaxis, respectively (Sections 6.6 and 6.7). Biosynthetic enzymes can also be regulated by the attachment of other small molecules, such as the nucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP), and uridine monophosphate (UMP). Throughout this chapter we have also encountered avenues in which the cell regulates the activity of proteins by protein–protein interactions. In contrast to these, some enzymes are regulated by *covalent modification*, typically the attachment or removal of a small molecule to or from the enzyme that subsequently affects its activity, and we consider this regulatory mechanism here.

Regulation of PII Signal Transduction Proteins

PII proteins are a widespread family of signal-transducing proteins (Section 6.6) found in *Bacteria, Archaea*, and the plastids of plants, and they play an important role in nitrogen metabolism by regulating a diverse range of transcription factors, enzymes, and membrane transport proteins. The activity of PII proteins on their regulatory cascade depends on whether they have been covalently modified or not. These modifications range from *uridylylation* (addition of a UMP group), *adenylylation* (addition of AMP), or even *phosphorylation* (in some cyanobacteria). Because these covalent modifications affect protein activity and occur *after* the PII proteins have been fully synthesized, the regulation is considered post-translational. Here we consider PII and its uridylylation by GlnD, a bifunctional enzyme that possesses both uridyltransferase activity and uridyl-removal activity in response to the presence of glutamine (**Figure 6.34**).

One of the main ways the cell determines if ammonia (NH_3) assimilation is necessary is through glutamine sensing by the GlnD protein. If the cellular glutamine pool is low, it signals that NH_3 assimilation is necessary. GlnD transduces this signal through the binding of glutamine itself. When GlnD is not bound to glutamine, the enzyme possesses uridyltransferase activity and adds a UMP group to PII, yielding PII-UMP through post-translational modification.

One protein target of PII-UMP within the nitrogen metabolism pathway is glutamine synthetase adenyltransferase (Figure 6.34*a*). Once uridylylated, PII-UMP stimulates glutamine synthetase adenyltransferase to remove adenyl groups from glutamine synthetase (GS). GS is a key enzyme in ammonia (NH_3) assimilation (↔ Section 3.14), which must be tightly regulated to conserve energy if cellular levels of nitrogen are high, as sensed by the glutamine pool. As GS becomes less adenylylated, its activity *increases* and NH_3 assimilation increases (Figure 6.34*c*). Once glutamine levels are sufficient, the cell no longer needs to assimilate NH_3. This results in GlnD binding to available glutamine, which stimulates the protein to remove uridyl groups from PII-UMP (Figure 6.34*b*). PII in its unmodified form stimulates glutamine synthetase adenyltransferase to adenylate GS, forming GS-AMP. Fully adenylylated GS is *less active* and thus less NH_3 assimilation occurs.

Why does all this elaborate regulation surround the enzyme GS? The activity of GS requires ATP, and nitrogen assimilation is a major biosynthetic process in the cell. However, when NH_3 is present at high levels in the cell, it can be assimilated into amino acids by enzymes that do not consume ATP; under these conditions, GS remains inactive. When NH_3 levels are low, however, GS becomes catalytically active. By having GS active only when NH_3 is limiting, the cell conserves ATP that would be unnecessarily consumed if GS were active when NH_3 is present in excess.

Inactivation of Sigma Factors

In Section 6.10 we described how the σ factor RpoH is inactivated by DnaK under normal temperature conditions in the heat shock response (Figure 6.26). Proteins known as *anti-sigma* factors can also bind to sigma factors, inactivating them in a form of post-translational regulation.

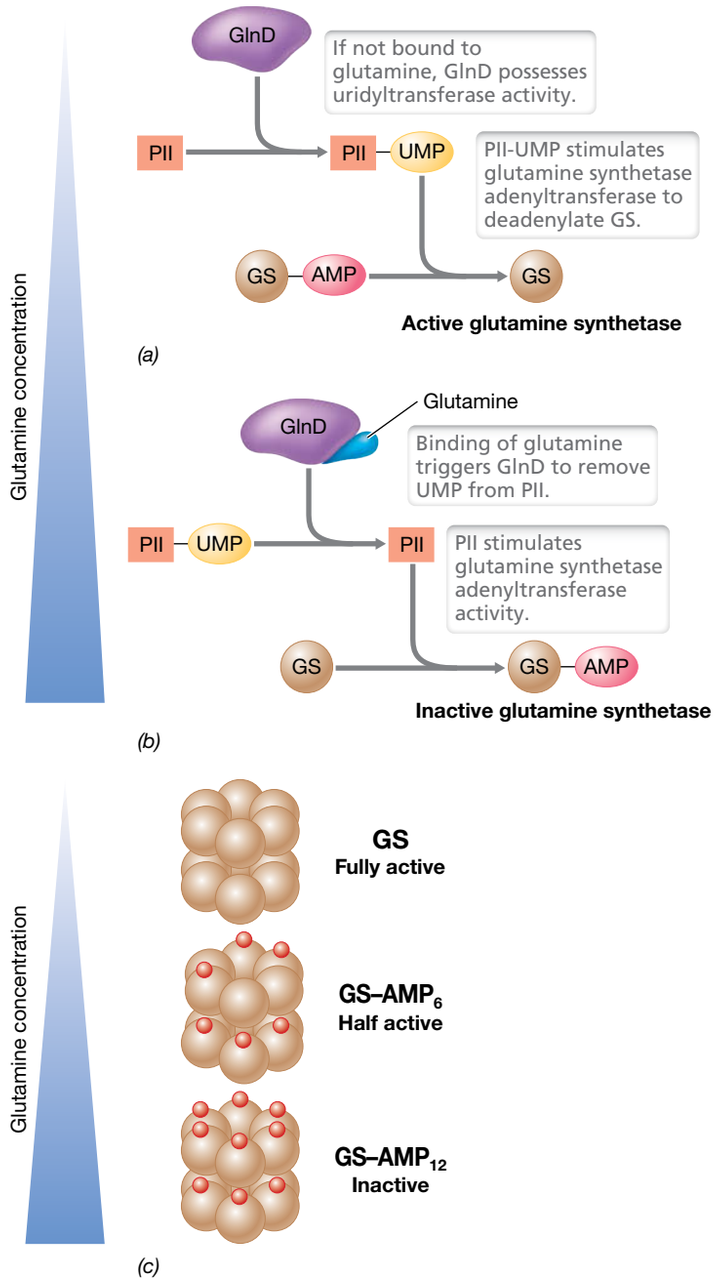


Figure 6.34 Post-translational regulation of PII and glutamine synthetase. *(a)* Uridylylation of PII and activation of glutamine synthetase (GS). In response to low cellular pools of glutamine, the uridyltransferase activity of GlnD is stimulated. This results in PII becoming PII-UMP, which triggers glutamine synthetase adenyltransferase to activate GS through AMP removal. *(b)* Removal of UMP from PII and inactivation of GS. In response to high cellular pools of glutamine, the UMP removal activity of GlnD is activated. This results in PII-UMP becoming PII, which triggers glutamine synthetase adenyltransferase to inactivate GS through adenylylation. *(c)* Control of GS activity. Adenylylated GS subunits are catalytically inactive, so the overall GS activity increases progressively as glutamine levels decrease and more subunits are de-adenylylated.

RpoE (σ^{24} or σ^{E}, ↔ Table 4.3) is a sigma factor conserved in many *Bacteria* that responds to extracytoplasmic stress by recognizing promoters for genes that encode products necessary for proper folding, expression, and turnover of proteins of the outer membrane (↔ Section 2.5). One of these promoters is for *dnaK*.

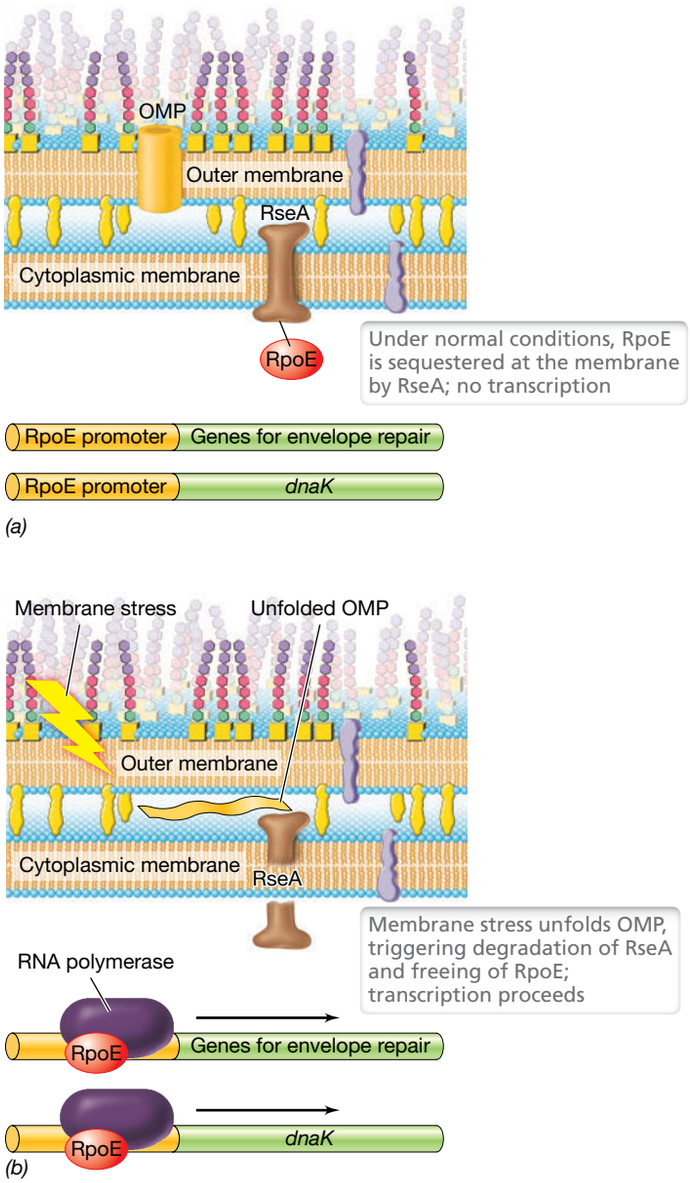


Figure 6.35 Anti-sigma–sigma factor interactions. *(a)* Sequestration of the RpoE sigma factor by the anti-sigma factor RseA. When the outer membrane is not stressed, cytoplasmic membrane–bound RseA binds to RpoE. This binding prevents the sigma factor from binding to promoters of genes for envelope repair and *dnaK*. *(b)* Inactivation of RseA and release of the RpoE sigma factor. When the outer membrane is exposed to stress, outer membrane proteins (OMPs) are denatured. These unfolded OMPs trigger proteolytic degradation of RseA. Without RseA, the RpoE sigma factor can bind to its promoter regions and transcribe genes for envelope stress and *dnaK* to help repair the membrane and refold proteins.

Under normal nonstressful conditions, the membrane-bound anti-sigma factor RseA sequesters RpoE from binding to the promoters it targets. However, if a cell encounters a condition stressful to the membrane such as heat or osmotic stress, outer membrane proteins are denatured, and this *activates* a protease specific for RseA. Once RseA has been degraded, RpoE is free to activate transcription of genes necessary for envelope repair such as refolding outer membrane proteins and synthesis of phospholipids and lipopolysaccharides (**Figure 6.35**). Release of RpoE also includes increasing the level of DnaK produced so that the chaperone (Section 4.11) can assist with folding and turnover of outer membrane proteins.

Once the membrane has been repaired, RseA is no longer targeted for degradation and reassociates with the cytoplasmic membrane. In this form, RseA can resume its anti-sigma factor activity by binding and sequestering RpoE from promoter regions. This leads to a *decreased* expression of both genes encoding products needed to respond to membrane stress and *dnaK*. Another well-studied example of regulation by anti-sigma–sigma interactions occurs during the formation of endospores in *Bacillus* when the anti-sigma factor SpoIIAB binds to σ^F, thereby preventing its association with RNA polymerase; we pick up on this control process in Section 7.6 and Figure 7.15.

Regardless of the mechanism, in the final analysis it should be clear that regulating the synthesis and activities of a cell's RNAs and proteins is (1) very important to its biology, (2) possible in many different ways, and (3) a major genetic investment. But the costs to the cell are worth it and are grounded in billions of years of evolutionary refinement. At every turn in a highly competitive world, a microbe's growth and very survival may well depend on its ability to conserve its resources and maximize its progeny.

MINIQUIZ

- What types of covalent modifications commonly alter the activity of proteins?
- How does GlnD signal to the cell that NH_3 assimilation is necessary?
- Explain the role of an anti-sigma factor.

Chapter Review

I • DNA-Binding Proteins and Transcriptional Regulation

6.1 Proteins can bind to DNA when specific domains of the proteins bind to specific regions of the DNA molecule. In most cases the interactions are sequence-specific. Proteins that bind to DNA are often regulatory proteins that affect gene expression.

Q Explain how a protein ensures that it binds specifically to only a certain region of DNA and not to another.

6.2 The amount of a specific enzyme in the cell can be controlled by regulatory proteins that bind to DNA and increase (induce) or decrease (repress) the amount of mRNA that encodes the enzyme. In negative control of transcription, the regulatory protein is called a repressor and it functions by inhibiting mRNA synthesis.

Q How is β-galactosidase induced in cells?

6.3 Positive regulators of transcription are called activator proteins. They bind to activator-binding sites on the DNA and stimulate transcription. Inducers modify the activity of activating proteins. In positive control of enzyme induction, the inducer promotes the binding of the activator protein and thus stimulates transcription.

Q How does an activator protein help RNA polymerase begin transcription?

6.4 Global control systems regulate the expression of many genes simultaneously. Catabolite repression is a global control system that helps cells make the most efficient use of available carbon sources. The *lac* operon is under the control of catabolite repression as well as its own specific negative regulatory system.

Q Describe the mechanism by which cAMP receptor protein (CRP), the regulatory protein for catabolite repression, functions. Use the lactose operon as an example.

6.5 *Archaea* resemble *Bacteria* in using DNA-binding activator and repressor proteins to regulate gene expression at the level of transcription.

Q What are the two mechanisms used by archaeal repressor proteins to repress transcription?

II • Sensing and Signal Transduction

6.6 Signal transduction systems transmit environmental signals to the cell. In *Bacteria* and *Archaea*, signal transduction is typically carried out by a two-component regulatory system that includes a membrane-integrated sensor kinase and a cytoplasmic response regulator. The activity of the response regulator depends on its state of phosphorylation.

Q What are the two components that give their name to the signal transduction system in prokaryotic cells? What is the function of each of the components?

6.7 Chemotactic behavior allows cells to respond to attractants and repellents in their environment. The regulation of chemotaxis affects the activity of proteins rather than their synthesis. Adaptation by methylation allows the system to reset itself to the continued presence of a signal.

Q Adaptation allows the mechanism controlling flagellar rotation to be reset. How is this achieved?

6.8 Quorum sensing allows cells to monitor their environment for cells of their own kind. Quorum sensing depends on the sharing of specific small molecules known as autoinducers. Once a sufficient concentration of the autoinducer is present, specific gene expression is triggered.

Q How can quorum sensing be considered a regulatory mechanism for conserving cell resources?

6.9 The stringent response is employed to survive nutrient limitation and stresses by decreasing expression of genes for macromolecule biosynthesis and activating stress survival pathways.

Q Explain the sequence of molecular events that leads to the synthesis of (p)ppGpp in *Escherichia coli* during the stringent response.

6.10 Cells can control sets of genes by employing transcriptional regulators and alternative sigma factors. These recognize only certain promoters and thus allow transcription of a select category of genes that is most appropriate for the environmental conditions. Cells respond to both excessive temperature and nutritional limitations by expressing sets of genes whose products help the cell overcome stress.

Q Describe the proteins produced when cells of *Escherichia coli* experience a heat shock. Of what value are they to the cell?

III • RNA-Based Regulation

6.11 Cells can control genes in several ways by employing regulatory RNA molecules. One way is to take advantage of base pairing and use sRNA to promote or prevent translation of mRNAs.

Q What are the mechanisms by which regulation by sRNA occurs?

6.12 Riboswitches are sequences at the 5′ ends of certain mRNAs that recognize small molecules and respond by changing their three-dimensional structure to affect translation or transcriptional termination of the mRNA. Riboswitches are mostly employed to control biosynthetic pathways for vitamins, amino acids, purines, and a few other metabolites.

Q What is the mechanism by which a riboswitch regulates translation?

6.13 Attenuation is a mechanism whereby transcription is controlled after initiation of mRNA synthesis. Attenuation mechanisms depend upon alternative stem–loop structures in the mRNA that result in either read-through or stalling of the ribosome.

Q Describe in detail how a tryptophan attenuator is controlled.

IV • Regulation of Enzymes and Other Proteins

6.14 In feedback inhibition, an excess of the final product of a biosynthetic pathway inhibits an allosteric enzyme at the beginning of the pathway. Enzyme activity can also be modulated by isoenzymes.

Q Describe how feedback inhibition is reversible.

6.15 Protein activity can be regulated after translation. Reversible covalent modification or interactions with other proteins can modulate protein activity.

Q How can post-translational modifications regulate the activity of an enzyme?

Application Questions

1. What would happen to regulation from a promoter under negative control if the region where the regulatory protein binds was deleted? What if the promoter was under positive control? Promoters from *Escherichia coli* under positive control are not close matches to the promoter consensus sequence for *E. coli*. Why?
2. Most of the regulatory systems described in this chapter employ regulatory proteins. However, regulatory RNA is also important. Describe how one could achieve negative control of the *lac* operon using either of two different types of regulatory RNA.
3. Many amino acid biosynthetic operons under attenuation control are also under negative control. Considering that the environment of a bacterium can be highly dynamic, what advantage could be conferred by having attenuation as a second layer of control?
4. How would you design a regulatory system to make *Escherichia coli* use succinic acid in preference to glucose? How could you modify it so that *E. coli* prefers to use succinic acid in the light but glucose in the dark?

Chapter Glossary

Activator protein a regulatory protein that binds to specific sites on DNA and stimulates transcription; involved in positive control

Allosteric protein a protein containing an active site for binding substrate and an allosteric site for binding an effector molecule such as the end product of a biochemical pathway

Attenuation a mechanism for controlling gene expression that terminates transcription after initiation but before a full-length messenger RNA is produced

Autoinducer a small signal molecule that takes part in quorum sensing

Catabolite repression the suppression of _alternative catabolic pathways by a preferred source of carbon and energy

Cyclic AMP a regulatory nucleotide that participates in catabolite repression

Domains regions of a protein with specific structure and function

Feedback inhibition a process in which an excess of the end product of a multistep pathway inhibits activity of the first enzyme in the pathway

Gene expression transcription of a gene followed by translation of the resulting mRNA into protein

Heat shock proteins proteins induced by high temperature (or certain other stresses) that protect against high temperature, especially by refolding partially denatured proteins or by degrading them

Heat shock response response to high temperature that includes the synthesis of heat shock proteins together with other changes in gene expression

Induction production of an enzyme in response to a signal (often the presence of the substrate for the enzyme)

Negative control a mechanism for regulating gene expression in which a repressor protein prevents transcription of genes

Noncoding RNA (ncRNA) RNA that is not translated into protein; examples include ribosomal RNA, transfer RNA, and small regulatory RNAs

Operon two or more genes transcribed into a single RNA and under the control of a single regulatory site

Positive control a mechanism for regulating gene expression in which an activator protein functions to promote transcription of genes

Quorum sensing a regulatory system that monitors the population level and controls gene expression based on cell density

Regulatory nucleotide a nucleotide that functions as a signal rather than being incorporated into RNA or DNA

Regulon a series of operons controlled as a unit

Repression prevention of the synthesis of an enzyme in response to a signal

Repressor protein a regulatory protein that binds to specific sites on DNA and blocks transcription; involved in negative control

Response regulator protein one of the members of a two-component regulatory system; a protein that is phosphorylated by a sensor kinase and then acts as a regulator, often by binding to DNA

Riboswitch an RNA domain, usually in a messenger RNA molecule, that can bind a specific small molecule, altering its own secondary structure; this, in turn, controls translation of the mRNA

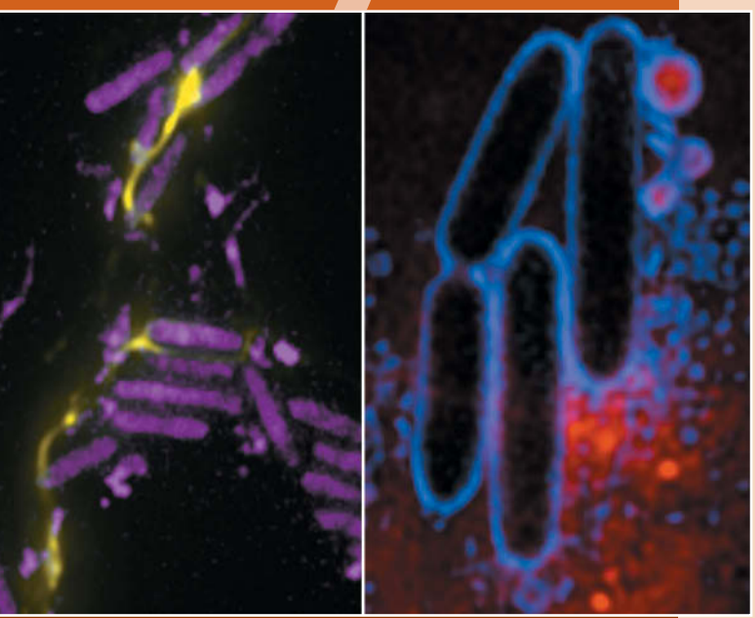
Sensor kinase protein one of the members of a two-component regulatory system; a protein that phosphorylates itself in response to an external signal and then transfers the phosphoryl group to a response regulator protein

Signal transduction see *two-component regulatory system*

Stringent response a regulatory mechanism that detects nutrient or environmental stresses and antibiotics

Two-component regulatory system a regulatory system consisting of two proteins: a sensor kinase and a response regulator

7 Molecular Biology of Microbial Growth



microbiologynow

Explosive Cell Death Promotes Biofilm Formation

To withstand environmental challenges and take best advantage of nutrient cycling, microbes often form slimy, three-dimensional structural communities called biofilms. While the major requirement for a biofilm lifestyle is a surface for attachment, specific phenotypic changes must occur that lead to attached growth instead of a planktonic (free-floating) existence. These changes, as well as the release of sticky substances called extracellular polymeric substances (EPS), require cell-to-cell communication.

EPS is composed of polysaccharides, extracellular DNA, and cytoplasmic proteins and is essential for protecting and holding attached cells together. Often the switch to a biofilm lifestyle can lead to beneficial outcomes for humans, such as in enhanced wastewater treatment. However, biofilm development by pathogens can lead to chronic disease and enhanced antibiotic resistance. This is especially true of the best-studied biofilm-forming bacterium—the opportunistic pathogen *Pseudomonas aeruginosa*.

Because of the robust ability of *P. aeruginosa* to form biofilms resistant to antibiotic treatment, microbiologists have focused on the biofilm developmental cycle. In doing so, they have discovered a previously unknown phenomenon—explosive death in a subpopulation of cells promotes biofilm formation. This explosive death is caused by a lysis enzyme used by an intrinsic virus to disrupt the bacterial cell wall. The DNA of this virus resides as a prophage in the genome of *P. aeruginosa* cells. As the left photo shows, production of the lysis enzyme causes the bacterial cells (purple) to break open, releasing their DNA (yellow). This DNA is critical to forming the EPS that holds the biofilm together. Cell bursting not only leads to the release of DNA (right photo, red), but also to shattered cell membrane fragments that curl together to form vesicles surrounding proteins and DNA that contribute to EPS formation (right photo, small blue circles with red centers).

While exposure to a DNA-damaging agent or an antibiotic normally leads to "activation" of the virus, random expression of the viral lysis gene occurs in a discrete subpopulation of cells. This annihilation of a few cells ultimately benefits the rest of the population. These findings also provide a potential answer to the following conundrum: Why does antibiotic treatment often enhance (rather than diminish) biofilm formation?

Source: Turnbull, L., et al. 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun. 7:* 1120.

In previous chapters we discussed how prokaryotic cells replicate their chromosome(s) (Chapter 4), grow and divide (Chapter 5), and regulate gene expression (Chapter 6). Here we focus on the key molecular processes that occur in the microbial growth cycle. We also consider cellular differentiation in some model bacteria, revisit the process of biofilm growth, and close by exploring how some common antibiotics affect microbial growth and what weapons bacteria deploy to counter such attacks.

I • Bacterial Cell Division

Most cells divide by binary fission (Section 5.1 and Figure 5.1), and this process occurs in a defined series of steps such that each daughter cell obtains a copy of the genome. Because the process of cell division in prokaryotic cells requires both temporal and spatial control elements, it is controlled by a cell cycle (Figure 7.1).

Temporally, two copies of the cell's genome must exist prior to cell division. Spatially, the two copies must be equally segregated and the septum must form at the correct location in the cell for a successful cell cycle to occur (Figure 7.1). During the division cycle, the cell must also produce new peptidoglycan and cytoskeleton elements to prevent bursting from osmotic forces. To successfully orchestrate all of these events, various regulatory cascades are put into play. In this first part of the chapter we focus on the molecular mechanisms employed by two well-studied gram-negative bacteria, *Escherichia coli* and *Caulobacter crescentus*, and introduce advanced microscopic techniques that have revealed the molecular pathways associated with cell division and cell morphology.

7.1 Visualizing Molecular Growth

While the standard light microscope nicely reveals cell size and shape, more powerful techniques are required to visualize macromolecules such as chromosomes, proteins, and membranes. To observe the tiniest details within a cell, **super-resolution microscopy**, a powerful new form of light microscopy that employs a suite of fluorescent molecules, was developed. Super-resolution techniques are capable of resolving structures as small as 10–50 nm in living cells, allowing for dynamic cellular behaviors to be observed in real time.

Fluorescent Tagging

To visualize the localization of specific proteins and to monitor gene expression, **reporter genes** have long been used. Reporter genes encode proteins that are easy to detect or assay and are fused to genes of interest. For visualizing molecular events, reporter genes that encode fluorescent products such as the **green fluorescent protein (GFP)** are routinely used (Figure 7.2). Through the use of fluorescence microscopy (Section 1.6) and multiple fluorescent reporter proteins, the activity and localization of different macromolecules can be determined simultaneously. Figure 7.2*a* demonstrates use of GFP and another fluorescent protein to determine the cellular locations of σ^F and σ^E, two spore-specific sigma factors (Section 4.5) that play key roles in endospore development in *Bacillus* (Section 7.6).

Fluorescent tagging can also be used to resolve different nucleic acids in a cell, such as a chromosome and a plasmid

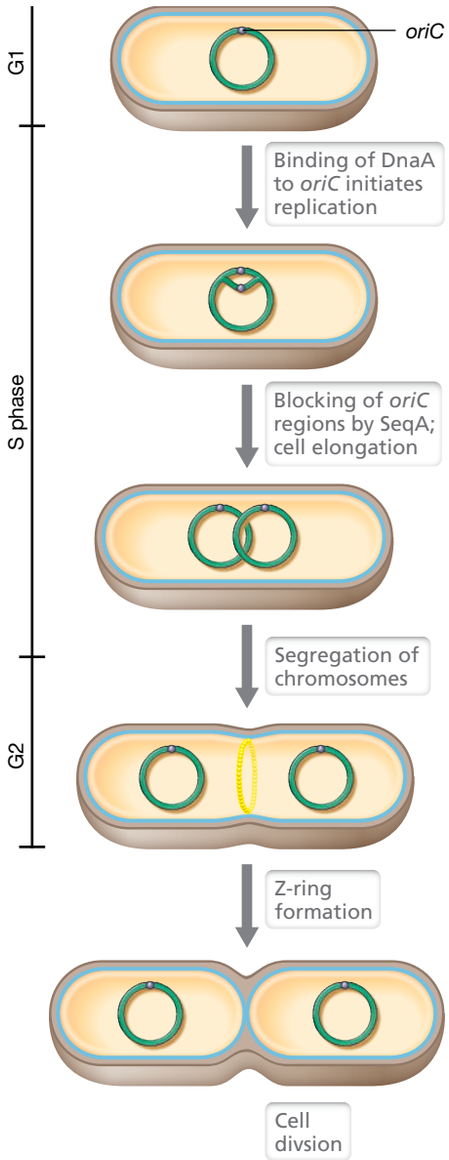
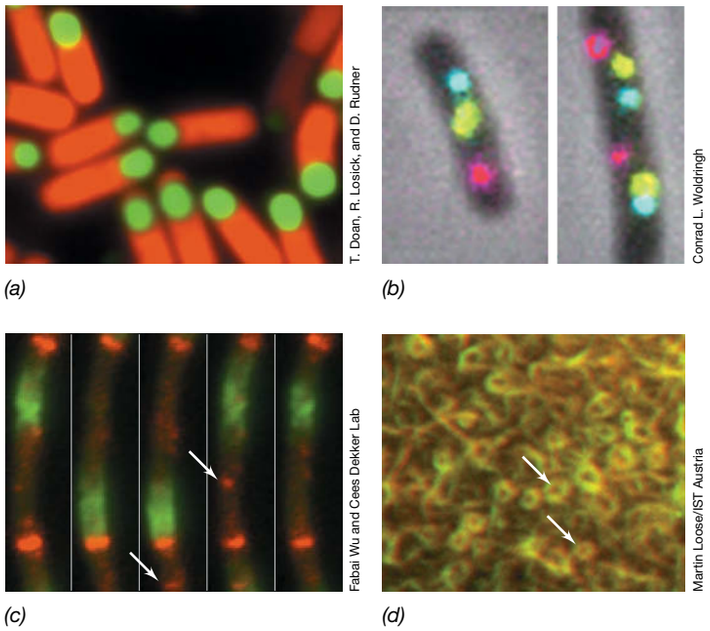


Figure 7.1 Overview of the bacterial cell cycle. Phases analogous to the growth 1 (G1), synthesis (S), and growth 2 (G2) phases of the eukaryotic cell cycle are represented.

(see Figure 7.7). In fact, even different loci within an individual nucleic acid molecule can be observed in a living cell. While DNA or RNA loci are not linked directly to a reporter gene like GFP, nucleic acid–binding proteins specific for target loci can be fused to a reporter gene. If necessary, the corresponding DNA or RNA binding sites can be introduced into the nucleic acid region of interest using recombinant techniques (Chapter 12). Figure 7.2*b* illustrates how separate "arms" of the *Escherichia coli* chromosome can be visualized in a living cell by introducing specific DNA sequences into the chromosome and tagging the corresponding DNA-binding proteins with different fluorescent reporter genes.

Advances in protein labeling and microscopy techniques have resulted in new fluorescent variants that fold quickly and allow for visualization of both spatial and temporal protein dynamics in



(a) (b) (c) (d)

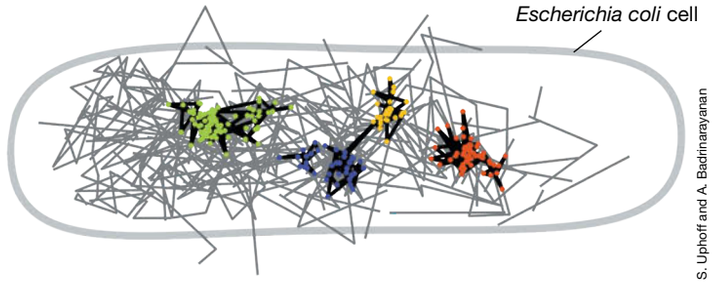
Figure 7.2 Fluorescence micrographs illustrating molecular growth characteristics. *(a)* During endospore formation in *Bacillus*, alternative sigma factors are localized to specific regions of the cell (Section 7.6). σ^F linked to GFP indicates activity of the protein in the developing endospore (at one end of each cell). σ^E linked to a reporter protein that fluoresces red indicates expression and activity of the protein throughout the mother cell prior to endospore formation. Regions correspond to the model depicted in Figure 7.15*b*. *(b)* Location and orientation of the *Escherichia coli* chromosome during cell cycle progression (Section 7.2). Regions are visible due to tagged DNA-binding proteins targeting the *oriC* (green), left arm (red), and right arm (cyan). Magnification bar represents 2 μm. Modified from Woldringh, C.L., Hansen, F.G., Vischer, N.O.E., and Atlung, T. 2015. *Front. Microbiol. 6:* 448. *(c)* Time-lapse series showing FtsZ (red) and MinD (green) in an elongated *E. coli* cell. Cell division was inhibited by antibiotic treatment and arrows indicate locations where unstable FtsZ proteins polymerize and depolymerize. Modified from Wu, F., Van Rijn, E., Van Schie, B.G.C., Keymer, J.E. and Dekker, C. 2015. *Front. Microbiol. 6:* 607. *(d)* Co-assembly of the cell-division proteins FtsZ (yellow) and FtsA (brown) on a membrane (Section 7.3). Swirls of FtsZ are visible (arrows).

live cells. Figure 7.2*c* illustrates the spatiotemporal dynamics of MinD and FtsZ (proteins of the divisome, see Section 7.3) in a growing cell over a time-lapse series. To visualize membrane-associated proteins, lipid bilayers can be created and applied to microscope slides before introducing fluorescently tagged proteins. This technique has been used to visualize interactions between the cell-division proteins FtsZ and FtsA and the rotating rings of FtsZ (Figure 7.2*d* and Section 7.3).

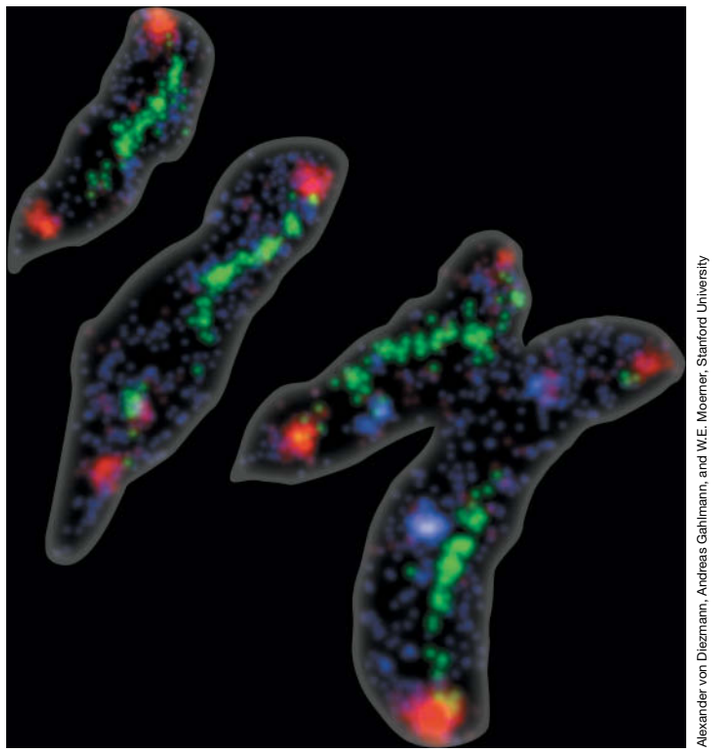
Super-Resolution Techniques

Although fluorescence microscopy enables observation of tagged internal cellular structures, resolution is insufficient to observe the molecular events occurring during the cell cycle. By contrast, super-resolution techniques can reveal and quantify elements as small as *single molecules* in living cells. Super-resolution techniques employ photoactivatable probes that switch from bright to dark emission states depending on the wavelength of light that strikes them; this allows for only one fluorescent molecule (or target) to be excited at a time. By recording the position of individual molecules over time, a super-resolution image is generated (**Figure 7.3*a***).

Super-resolution techniques can reveal the details of cellular superstructures, for example by precisely mapping the diffusion and movement of individual proteins. Figure 7.3*a* illustrates the use of *photoactivated localization microscopy*, a form of super-resolution microscopy, to map the movement of individual MukB molecules in a living *E. coli* cell. MukBEF is a protein complex that binds to the nucleoid and localizes to distinct regions in the cell to assist with unknotting daughter chromosomes after replication (Section 7.2). Not only can super-resolution microscopy be used to observe structural changes to cellular complexes over time, the micrographs can also be modified to construct



(a)



(b)

Figure 7.3 Super-resolution imaging of molecular growth characteristics. *(a)* Photoactivated localization micrograph (PALM) mapping the movement of the nucleoid-binding protein MukB in an individual *Escherichia coli* cell. The four different colors represent clusters of nucleoid-bound MukB molecules, while tracks corresponding to their movement are represented by black lines. Gray lines represent tracks of free MukB (Section 7.2). *(b)* Three-dimensional quantitative multicolor subdiffraction imaging of *Caulobacter* cellular structures. PopZ is tagged with a red fluorescent reporter, while crescentin is tagged with a green fluorescent reporter (Section 7.4).

three-dimensional (3D) images. Figure 7.3*b* illustrates the use of a super-resolution method called 3D subdiffraction imaging to observe the arrangement of different protein complexes in the budding bacterium *Caulobacter crescentus*.

By providing spatiotemporal information about individual proteins, super-resolution techniques have been instrumental in proving that like their eukaryotic counterparts, prokaryotic cells contain a highly dynamic subcellular organization and one whose activities during the cellular growth process can be tracked at the molecular level. We now focus on some of these key molecular events.

MINIQUIZ

- What is the utility of a reporter gene?
- What are the advantages of using super-resolution microscopy versus standard fluorescence microscopy?

7.2 Chromosome Replication and Segregation

A prerequisite for bacterial growth is replication of the genome. Chromosome replication must be tightly regulated to coincide with cell division. After genome replication and prior to cell division, molecular mechanisms must also ensure that the daughter chromosomes are segregated (Figure 7.1).

Regulation of Chromosome Replication Initiation

How does the cell ensure that the genome is completely replicated before cell division while also preventing multiple rounds of replication? Several different proteins play a role in initiating and inhibiting chromosome replication in *Escherichia coli*. Here we focus on a key protein in this regard, DnaA.

As discussed in Section 4.3, binding of DnaA to specific DNA sequences within the *oriC* region of the chromosome leads to unwinding of the DNA and loading of the replisome for chromosome replication in *Escherichia coli* (Figure 7.1). DnaA is most active when it is linked to a molecule of ATP, forming DnaA–ATP. To tightly control chromosome replication, multiple regulatory mechanisms come into play to inactivate DnaA–ATP once replication has initiated. These mechanisms include competition for *oriC* binding, repression of *dnaA* expression, titration of DnaA–ATP away from *oriC*, and inactivation of DnaA–ATP.

Before DNA replication initiates, both strands of the chromosome contain methyl groups on the adenine residue of –GATC– sequences within the chromosome. However, immediately after replication has initiated, only the parental strand remains methylated. This results in *hemimethylated DNA* and facilitates a competition for origin binding between DnaA–ATP and a protein called SeqA (**Figure 7.4**). Because hemimethylated *oriC* sequences are strongly bound by SeqA, DnaA–ATP is blocked from binding and reinitiating chromosome replication (Figures 7.1 and 7.4). Approximately 10 min after replication has initiated, GATC sequences within the newly synthesized daughter strand are methylated by a DNA adenine methylase.

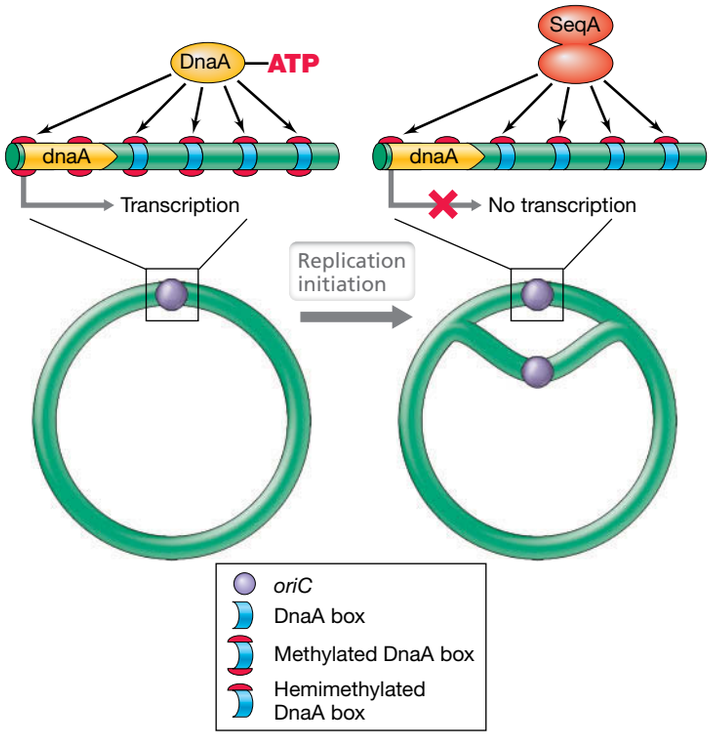


Figure 7.4 Binding of the *Escherichia coli oriC* by DnaA and SeqA proteins. DnaA–ATP only binds to fully methylated DnaA boxes, while SeqA binds to hemimethylated DNA. The binding of SeqA upstream of the *dnaA* gene leads to repression of transcription.

As a result both of the chromosomal proximity of the *dnaA* gene to *oriC* and of the promoter region (↔ Section 4.5) of *dnaA* possessing a GATC sequence, binding of SeqA also plays a role in repressing the expression of *dnaA*. Once replication has initiated, the promoter region for *dnaA* is quickly hemimethylated, and the binding of SeqA prevents RNA polymerase from binding and transcribing *dnaA* (Figure 7.4). Subsequently, expression of *dnaA* is also *autoregulated* by its corresponding protein binding to DnaA boxes within its promoter region.

The final mechanism of preventing DnaA–ATP from binding *oriC* is to decrease the ratio of DnaA–ATP to DnaA–ADP. How does the cell increase the level of DnaA–ADP when ATP dominates over ADP in a growing cell? This is controlled by the ATPase HdaA, which associates with DNA in the proximity of the replisome and specifically targets DnaA–ATP. This enzyme promotes the hydrolysis of ATP associated with DnaA in a replication-dependent manner and thus functions as a final method of regulating the initiation of chromosome replication. As a result of these combined mechanisms, the cellular level of DnaA–ATP oscillates during the cell cycle, reaching the maximum active amount when initiation of chromosome replication is needed and waning thereafter.

Genome Replication in Fast-Growing Cells

As we learned in Chapter 4, the circular nature of the chromosome of *E. coli* (and that of most other *Bacteria* and *Archaea*) creates an opportunity for speeding up DNA replication. This is because replication of circular genomes is *bidirectional* from the origin of

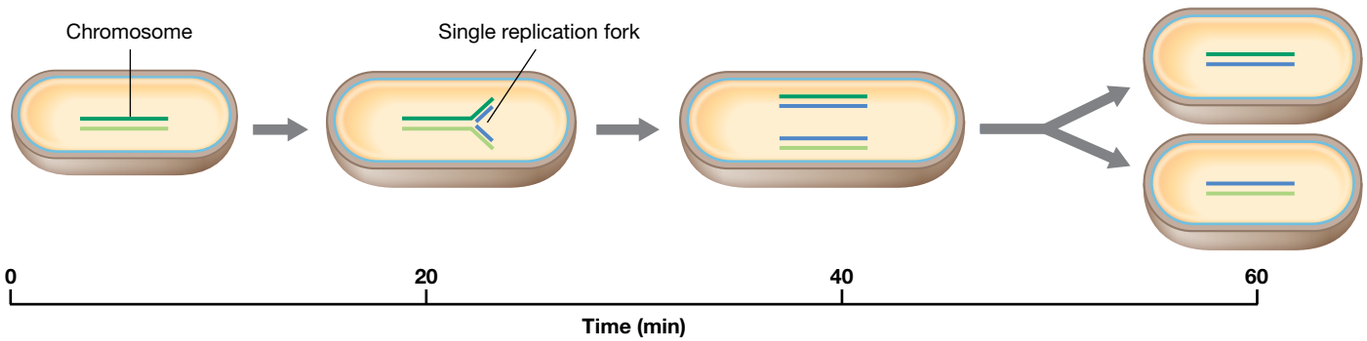
replication. During bidirectional replication, synthesis occurs in both leading and lagging directions on each template strand, and this allows DNA to replicate as rapidly as possible (Figure 4.16). Studies of chromosome replication in *E. coli* have shown that about 40 min is required for genome replication and that this value is independent of the generation time, which in *E. coli* can be as little as 20 min. If an *E. coli* cell is growing at twice the rate that its chromosome is replicating, how does the cell resolve this conundrum?

In cells growing at doubling times shorter than 40 min, *multiple DNA replication forks* are present in each cell. That is, a new round of DNA replication begins before the last round has been completed (Figure 7.5), and therefore, some genes are present in more than one copy. This can occur after the DNA in the *oriC* region of the newly synthesized DNA has been methylated, which releases SeqA from the DNA and allows DnaA–ATP to be recruited to reinitiate another round of replication (Figure 7.4). This ensures that at generation times shorter than the time required for replication (a process that occurs at a constant and maximal speed), each daughter cell receives a complete genome at the time of septum formation.

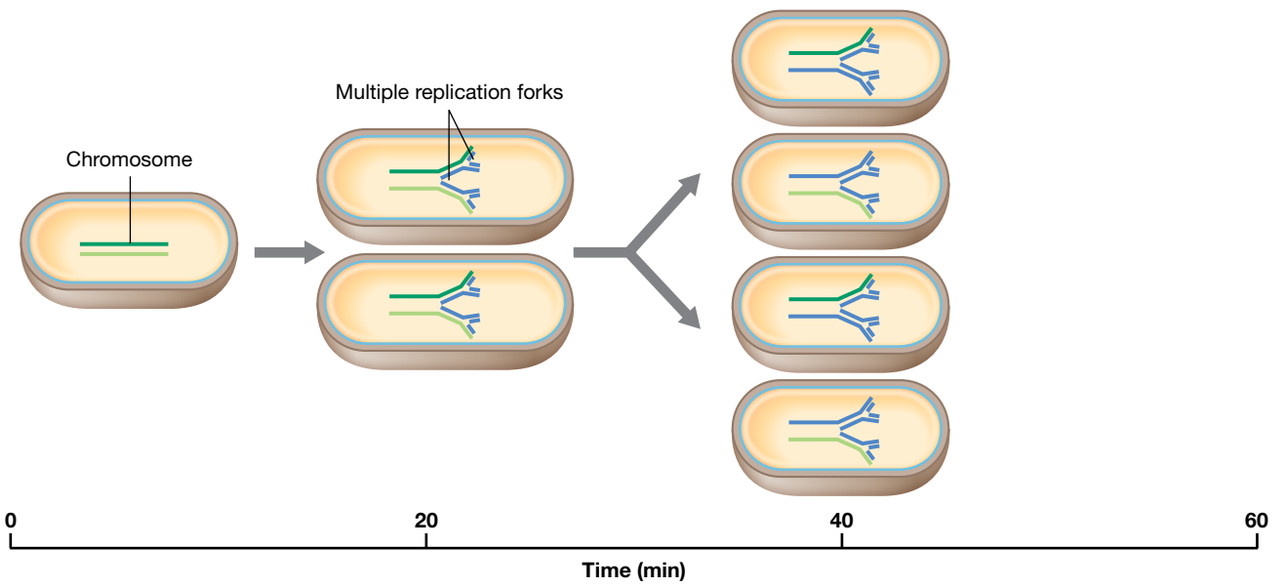
Chromosome Segregation

During cell division, segregation of the chromosomes to the cell poles is needed not only to ensure that each daughter cell gets a copy of the genome, but also to allow for septum formation (Figure 7.1). If the two copies of the nucleoid remained in the center of the cell, nucleoid occlusion (a process that prevents the cell from dividing across the nucleoids) would impede proper cell division. In eukaryotes, mitotic spindles (Section 2.14) separate replicated chromosomes. In many bacteria including the budding bacterium *Caulobacter*, a similar mechanism known as the *Par* (partitioning) *system* is used to distribute chromosomes and plasmids equally to progeny cells during growth (Figure 7.6). This system is composed of the ParA ATPase, the ParB chromosome-binding protein, and the PopZ complex, as well as a centromere-like *parS* sequence located near *oriC*.

Unlike eukaryotic mitotic spindles, the Par system does not segregate fully replicated chromosomes. Instead, PopZ proteins localize to the old pole of the cell and facilitate anchoring of the chromosome to this location by interacting with ParB bound to the *parS* sequence (Figure 7.6). Once chromosome replication



(a) **Generation time, 1 h; replication time, 40 min.**



(b) **Generation time, 20 min; replication time, 40 min.**

Figure 7.5 Genome replication in cells of *Escherichia coli* growing at 60 min or 20 min generation times. In cells doubling every 20 min, multiple replication forks are needed to ensure that each daughter cell gets a complete copy of the genome, which takes 40 min to replicate. In cells doubling every 60 min, multiple replication forks are unnecessary.

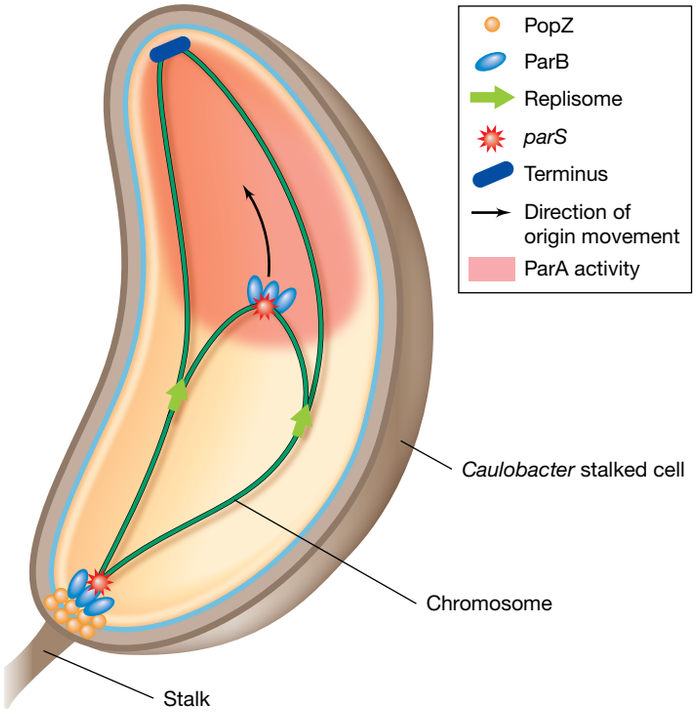


Figure 7.6 Chromosome partitioning in *Caulobacter*. After replication initiates, PopZ localizes to the old pole of the cell. Next, ParB binds to the *parS* sequence on the parental chromosome and the complex associates with PopZ at the pole. As replication continues, the *parS* sequence on the daughter chromosome is bound by ParB and moved to the new cell pole by the activity of diffuse ParA molecules.

initiates, the newly synthesized *parS* sequence binds to another molecule of ParB, which is then pulled to the new cell pole by the ATPase activity of ParA. Not only does PopZ help anchor *parS* of the parent chromosome to the old pole of the cell, it also helps recruit ParA to transfer the newly synthesized *parS* sequence bound to ParB to the new cell pole (Figure 7.6).

While *E. coli* lacks Par, the daughter chromosomes must still be segregated prior to cell division. After replication the resulting circular chromosomes remain interlinked or tangled, much like the links in a chain. This linkage is broken by the *structural maintenance of chromosome* complex, which is composed of a topoisomerase (IV) (Section 4.1) and MukBEF proteins. Super-resolution images show that the MukBEF proteins move to discrete locations within the nucleoid (Figure 7.3*a*) and recruit a topoisomerase to separate replicated sister chromosomes (a process called *decatenation*) prior to segregation. While the actual segregation process is still not completely understood, the chromosome "arms" appear to remain distinctly separated from one another during replication and the chromosomal loci are pushed to the cell poles in their order of replication (Figure 7.2*b*). This separation of daughter chromosomes in *E. coli* appears to be independent of specific proteins and proceeds instead by the physical action of replication and DNA accumulation.

How are extrachromosomal elements segregated between daughter cells? Although plasmids are not considered essential for cell survival under all conditions, they are replicated using the same cellular machinery as the chromosome (Figure 7.7). Various

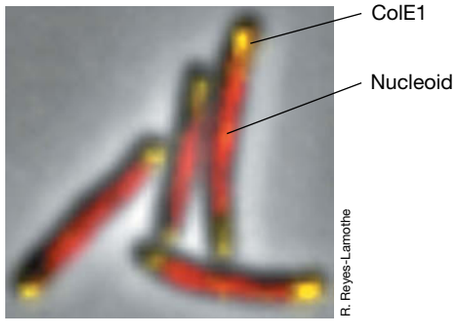


Figure 7.7 Cellular location of ColE-1 plasmid in *Escherichia coli* during replication. The plasmid (yellow) localizes to the cell poles, while the nucleoid remains in the center of the cell during DNA replication. The separate DNA molecules are visible due to fluorescently labeled DNA-binding proteins.

control mechanisms exist to ensure that a relatively constant copy number is disseminated to progeny cells. Replication of large ColE1-type plasmids occurs at the poles instead of in the center of the cell where the nucleoid exists (Figure 7.7). This location helps ensure that efficient transfer to daughter cells occurs during cell division and that stable inheritance is achieved over generations. Other mechanisms for segregating plasmids include partitioning systems similar to the Par system in *Caulobacter* (Figure 7.6).

MINIQUIZ

- How does SeqA prevent DnaA–ATP from binding to the *oriC* regions immediately after chromosome replication initiates?
- Explain how the minimum generation time for the bacterium *Escherichia coli* can be less than the time needed to replicate its chromosome.

7.3 Cell Division and Fts Proteins

Cell division is both spatially and temporally controlled to ensure that each daughter cell has a copy of the genome before the septum seals off the two cells (Figure 5.1). Here we discuss regulation of septum formation and a series of key proteins that identify the site of cell division and control the overall process.

The Divisome

Several essential proteins play roles in cell division in *Bacteria*. Collectively, these proteins are called *Fts proteins* and a key one, **FtsZ**, plays a crucial role in binary fission. FtsZ is related to tubulin, the important cell-division protein in eukaryotes (Section 2.16), and is also found in virtually all *Archaea*. Other Fts proteins are found only in *Bacteria* and not in *Archaea*, so our discussion here will be restricted to the *Bacteria*. The gram-negative *Escherichia coli* and the gram-positive *Bacillus subtilis* have been the model bacterial species for the study of cell-division events.

Fts proteins interact in the cell to form a division apparatus called the *divisome*. In rod-shaped cells, formation of the divisome begins with the attachment of molecules of FtsZ in a ring precisely around the center of the cell; this ring becomes the cell-division plane. In a cell of *E. coli*, about 10,000 FtsZ molecules polymerize

to form the ring; once formed, the FtsZ ring attracts other divisome proteins, including *FtsA* and *ZipA* (**Figure 7.8**). ZipA is an anchor that connects the FtsZ ring to the cytoplasmic membrane and stabilizes it. FtsA, a protein related to actin, an important cytoskeletal protein in eukaryotes (Section 2.16), both recruits FtsZ and other divisome proteins and helps connect the ring to the cytoplasmic membrane (Figure 7.2*d*). The divisome forms about three-quarters of the way into the cell-division cycle. However, long before the divisome forms, the cell is already elongating and DNA replication has begun (see Figure 7.9).

The divisome also contains Fts proteins needed for peptidoglycan synthesis, such as FtsI (Figure 7.8). FtsI is one of several *penicillin-binding proteins* present in the cell. Penicillin-binding proteins are so named because their activities are inhibited by the antibiotic penicillin (Section 7.5). The divisome orchestrates synthesis of new cytoplasmic membrane and cell wall material, called the *division septum*, at the center of a rod-shaped cell until the cell reaches twice its original length. Following elongation, the cell divides, yielding two daughter cells (Figure 7.1).

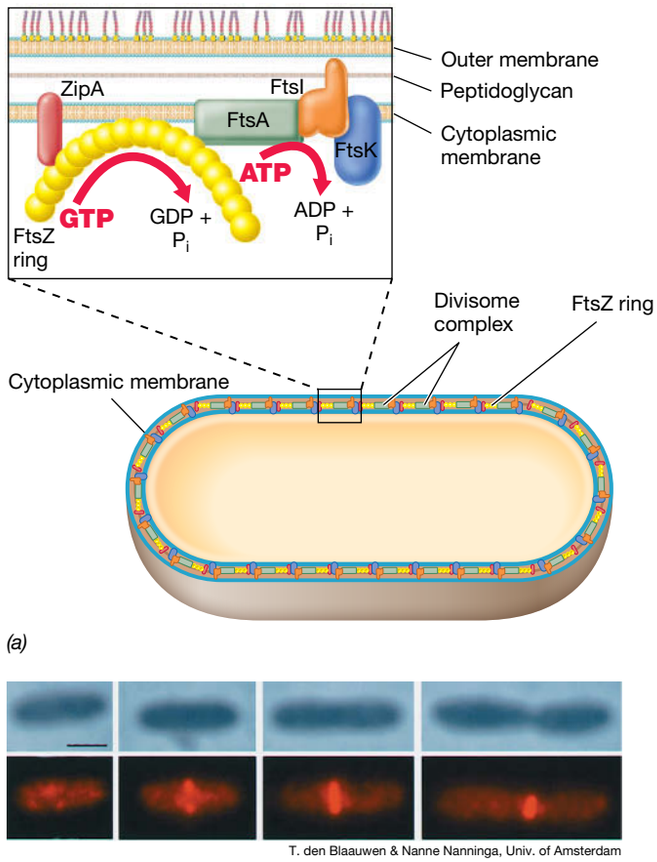


Figure 7.8 The FtsZ ring and cell division. *(a)* Cutaway view of a rod-shaped cell showing the ring of FtsZ molecules around the division plane. Blowup shows the arrangement of individual divisome proteins. ZipA is an FtsZ anchor, FtsI is a peptidoglycan biosynthesis protein, FtsK assists in chromosome separation, and FtsA is an ATPase. *(b)* Appearance and breakdown of the FtsZ ring during the cell cycle of *Escherichia coli*. Microscopy: upper row, phase-contrast; bottom row, cells stained with a specific reagent against FtsZ. Cell division events: first column, FtsZ ring not yet formed; second column, FtsZ ring appears as nucleoids start to segregate; third column, full FtsZ ring forms as cell elongates; fourth column, breakdown of the FtsZ ring and cell division. Marker bar in upper left photo, 1 μm.

Min Proteins and Cell Division

DNA replicates before the FtsZ ring forms (**Figure 7.9**) because the ring forms in the space between the duplicated nucleoids; before the nucleoids segregate, they effectively block formation of the FtsZ ring in a process known as nucleoid occlusion (Section 7.2). The proteins MinC, MinD, and MinE interact to help guide FtsZ to the cell midpoint. MinD forms a spiral structure on the inner surface of the cytoplasmic membrane and helps to localize MinC to the cytoplasmic membrane. The MinD spiral oscillates back and forth along the long axis of the growing cell and functions to *inhibit* cell division by preventing the FtsZ ring from forming (Figure 7.9). Simultaneously, however, MinE also oscillates from pole to pole, and as it does, it functions to sweep MinC and MinD aside. Hence, because MinC and MinD dwell longer at the poles than anywhere else during their oscillation cycle, the center of the cell will have, on average, the lowest concentration of these proteins. As a result, the cell center becomes the most permissive site for FtsZ binding and so the FtsZ ring forms there. In this unusual series of events, the Min proteins ensure that the divisome forms only at the *cell center* and not at the cell poles (Figure 7.9).

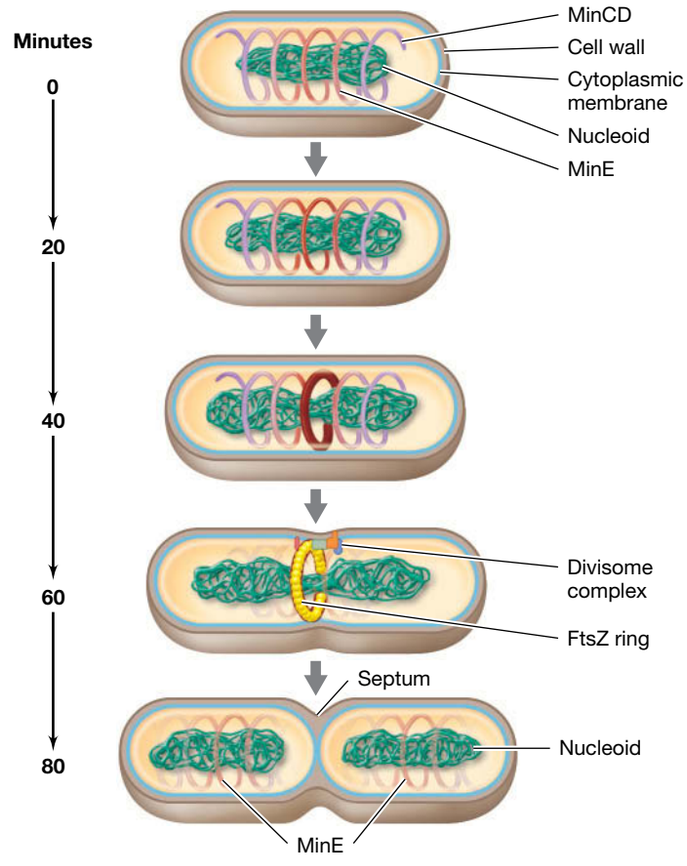


Figure 7.9 DNA replication and cell-division events. The protein MinE directs formation of the FtsZ ring and divisome complex at the cell-division plane. Shown is a schematic for cells of *Escherichia coli* growing with a doubling time of 80 min. MinC and MinD are most abundant at the cell poles during FtsZ ring formation.

As cell elongation continues and septum formation begins, the two copies of the chromosome are pulled apart, each to its own daughter cell (Figure 7.9). The Fts protein *FtsK* and several other proteins assist in this process. As the cell constricts, the FtsZ ring begins to depolymerize, triggering the inward growth of wall materials to form the septum and seal off one daughter cell from the other. The enzymatic activity of FtsZ also hydrolyzes guanosine triphosphate (GTP, an energy-rich compound) to yield the energy necessary to fuel the polymerization and depolymerization of the FtsZ ring (Figures 7.8 and 7.9).

There are significant practical reasons for understanding the details of bacterial cell division because such knowledge could lead to the development of new drugs that target specific steps in the growth of pathogenic bacteria. Like penicillin (a drug that targets bacterial cell wall synthesis), drugs that interfere with the function of specific Fts or other bacterial cell-division proteins could have broad applications in clinical medicine.

MINIQUIZ

- What is the divisome?
- How does FtsZ find the cell midpoint of a rod-shaped cell?

7.4 MreB and Cell Morphology

Just as specific proteins direct cell *division* in bacteria, other specific proteins form the cell *cytoskeleton*. The cytoskeleton is the scaffolding that directs cell *shape*, yielding the spheres, spirals, crescents, and other morphologies typical of bacterial cells (Section 2.1). Interestingly, these shape-determining proteins show significant homology to key cytoskeletal proteins in eukaryotic cells (Section 2.16). Like eukaryotes, bacteria also possess a dynamic and multifaceted cell cytoskeleton.

Cell Shape and MreB

The major shape-determining factor in *Bacteria* is a protein called *MreB*. MreB forms a simple cytoskeleton in *Bacteria* and in a few species of *Archaea*. MreB forms patchlike filaments around the inside of the cell, just below the cytoplasmic membrane (**Figure 7.10**). The MreB cytoskeleton presumably defines cell shape by recruiting other proteins that function in cell wall growth to group into a specific pattern. Inactivation of the gene encoding MreB in rod-shaped bacteria causes the cells to become coccus-shaped. Moreover, most naturally occurring coccoid bacteria lack the *mreB* gene and thus do not make MreB. This suggests that the "default" morphology for a bacterium is a sphere. Variations in the arrangement of MreB filaments in cells of nonspherical bacteria are likely responsible for the different common morphologies of prokaryotic cells.

How does MreB define a cell's shape? The filament structures formed by MreB (Figure 7.10*a*) are not static, but instead passively move from one side to the other within the cytoplasm of a growing cell. MreB filaments localize the synthesis of peptidoglycan (Section 7.5) at points where the rod-shaped filaments contact the cytoplasmic membrane (Figure 7.10*a*). This allows new cell wall to form at several points along the cell rather than from a single location at the FtsZ site outward, as in spherical bacteria (see Figure 7.12). By moving progressively in tracks perpendicular to the cell cylinder and initiating cell wall synthesis at multiple sites along the cell wall, MreB helps direct new wall synthesis in such a way that a rod-shaped cell elongates only along its long axis with cell wall synthesis dispersed at intervals (Figure 7.10).

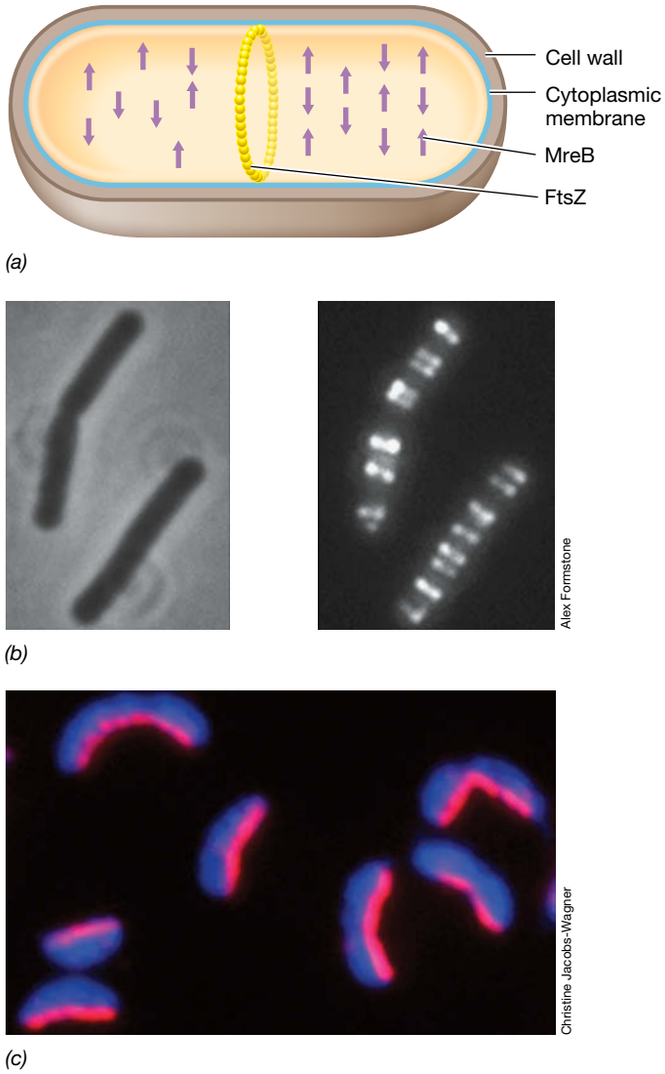


Figure 7.10 MreB and crescentin as determinants of cell morphology. *(a)* The cytoskeletal protein MreB is an actin analog that moves in tracks perpendicular to the long axis of a rod-shaped cell, making contact with the cytoplasmic membrane in several locations (indicated by arrows). *(b)* Photomicrographs of the same cells of *Bacillus subtilis*. Left, phase-contrast; right, fluorescence. The cells contain a substance that makes the MreB protein fluoresce, shown here as bright white. *(c)* Cells of *Caulobacter crescentus*, a naturally curved (vibrio-shaped) cell. Cells are stained to show the shape-determining protein crescentin (red), which lies along the concave surface of the cell, and with DAPI, which stains DNA and thus the entire cell (blue).

Crescentin

In the vibrio-shaped bacterium *Caulobacter*, a shape-determining protein called *crescentin* is present in addition to MreB. Copies of crescentin protein organize into filaments about 10 nm wide that localize onto the concave face of the curved cell (Figure 7.3*b*).

The arrangement and localization of crescentin filaments are thought to impart the characteristic curved morphology to the *Caulobacter* cell (Figure 7.10*c*). *Caulobacter* is an aquatic bacterium that undergoes a life cycle in which swimming cells, called *swarmers*, eventually form a stalk and attach to surfaces. Attached cells then undergo cell division to form new swarmer cells that are released to colonize new habitats (see Section 7.7 and Figure 7.16). The steps in this life cycle are highly orchestrated at the genetic level, and *Caulobacter* has been used as a model system for the study of gene expression in cellular differentiation. Although crescentin seems to be unique to *Caulobacter*, proteins similar to crescentin have been found in other curved cells, such as the bacterium that causes peptic ulcers, *Helicobacter pylori*. This suggests that these proteins may be necessary for the formation of curved cells.

Evolution of Cell Division and Cell Shape

How do the determinants of cell shape and cell division in *Bacteria* compare with those in eukaryotes? Despite not being helical in structure, MreB functions similarly to the eukaryotic protein actin. Interestingly, FtsZ is both structurally and functionally related to the eukaryotic protein tubulin. Actin forms structures called *microfilaments* that function as scaffolding in the eukaryotic cell cytoskeleton and in cell division, whereas tubulin forms *microtubules* that are important in mitosis and other processes. In addition, the shape-determining protein crescentin in *Caulobacter* is related to the keratin proteins that make up *intermediate filaments* in eukaryotic cells. Intermediate filaments form part of the eukaryotic cytoskeleton, and genes encoding similar proteins have been found in some other *Bacteria*. It thus appears that several proteins that control cell division and the cell cytoskeleton in eukaryotic cells (Section 2.16) have evolutionary roots in the *Bacteria*. However, with the exception of FtsZ, genes encoding homologs of these proteins appear to be absent from most *Archaea*.

While our focus has been on the filament proteins MreB and crescentin in bacteria that grow by synthesizing new peptidoglycan at the center of the cell (or dispersively), a diversity of morphologies exists in the bacterial world. This is especially evident in species of *Alphaproteobacteria* (**Figure 7.11**), gram-negative bacteria that show not only significant morphological diversity but also significant metabolic diversity. Hence, *Alphaproteobacteria* fill a host of diverse ecological niches in nature, from soil and water to symbiotic plant and animal associations.

Besides growing as rod-shaped cells and producing new cell material as we have seen in Figures 7.8–7.10, the *Alphaproteobacteria* group also contains species that can grow by synthesizing peptidoglycan at the poles (polar elongation), while others grow in a process known as budding. Budding results in diverse cell morphologies (Figure 7.11) and is a consequence of synthesizing peptidoglycan at various regions within the cell. However, the precise positioning of the peptidoglycan-synthesizing machinery in budding cells has yet to be discovered. Figure 7.11 also illustrates a principle we will see repeated many times later in this book: The phylogenetic position of a bacterium cannot be predicted from its morphology, and vice versa.

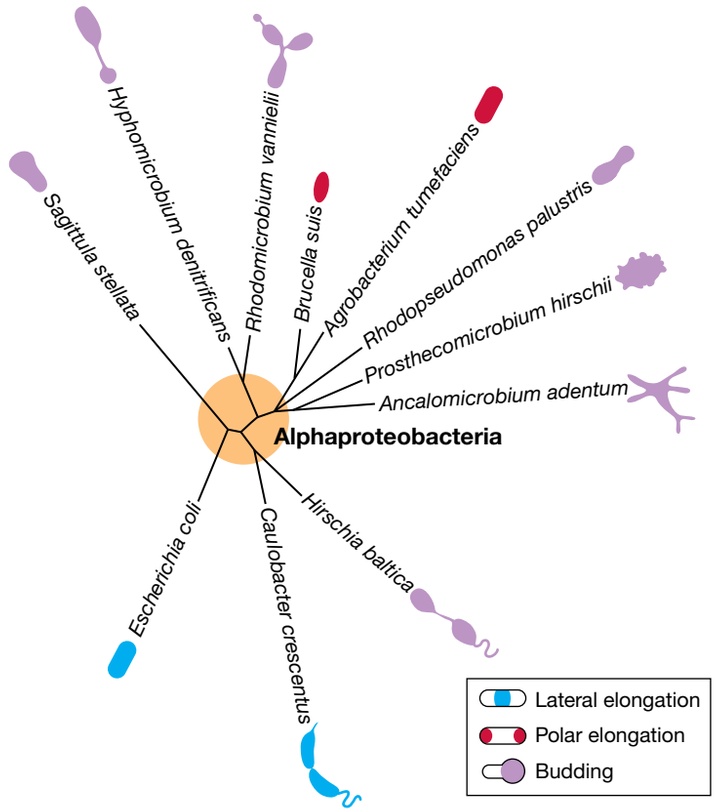


Figure 7.11 Phylogeny and morphology of select diverse bacteria. The tree was constructed from comparative sequences of *rpoC*, a gene encoding one of the subunits of RNA polymerase. Colored shading represents areas of new peptidoglycan synthesis as indicated. Note the extensive morphological diversity within the *Alphaproteobacteria*. Modified from Randich, A.M., and Brun, Y.V. 2015. *Front. Microbiol. 6:* 580.

MINIQUIZ

- How does MreB control the shape of a rod-shaped bacterium?
- What protein is thought to control the shape of cells of *Caulobacter*?
- What relationships exist between cytoskeletal proteins in *Bacteria* and those in eukaryotes?

7.5 Peptidoglycan Biosynthesis

In cells of *Bacteria* that contain peptidoglycan—and virtually all species do—preexisting peptidoglycan has to be temporarily severed to allow newly synthesized peptidoglycan to be inserted during the growth process. In cocci, new cell wall material grows out in opposite directions from the FtsZ ring (**Figure 7.12**). By contrast, as we have just seen in rod-shaped cells, new cell wall grows at several locations along the length of the cell (Figure 7.10*a*), and it is localized in those cells that divide by budding. However, regardless of morphology, a growing bacterial cell must both synthesize new peptidoglycan and export it outside the cytoplasmic membrane. We consider this problem here.

Insertion of New Peptidoglycan

Peptidoglycan can be thought of as a stress-bearing fabric, much like a thin sheet of rubber. Synthesis of new peptidoglycan during

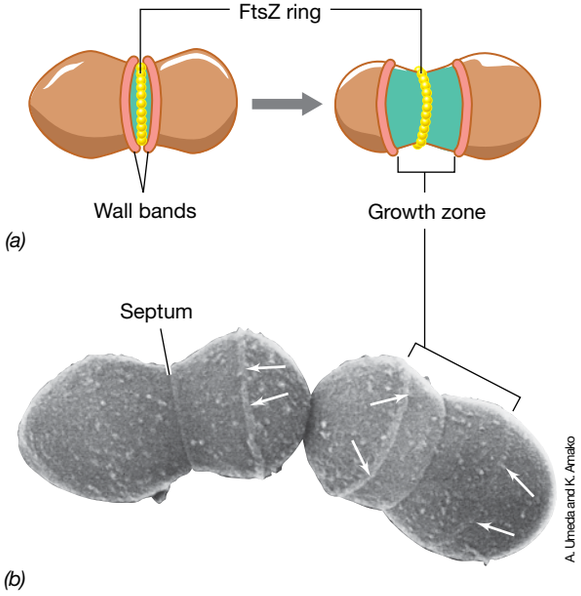


Figure 7.12 Cell wall synthesis in gram-positive *Bacteria*. *(a)* Localization of cell wall synthesis during cell division. In cocci, cell wall synthesis (shown in green) is localized at only one point (compare with Figure 7.10*a*). *(b)* Scanning electron micrograph of cells of *Streptococcus hemolyticus* showing wall bands (arrows). A single cell is about 1 μm in diameter.

growth requires the controlled cutting of preexisting peptidoglycan along with the simultaneous insertion of peptidoglycan precursors. A lipid carrier molecule called *bactoprenol* plays a major role in the latter process. Bactoprenol is a hydrophobic C_{55} alcohol that is bonded to an *N*-acetylglucosamine/*N*-acetylmuramic acid/pentapeptide peptidoglycan precursor (**Figure 7.13**). Bactoprenol

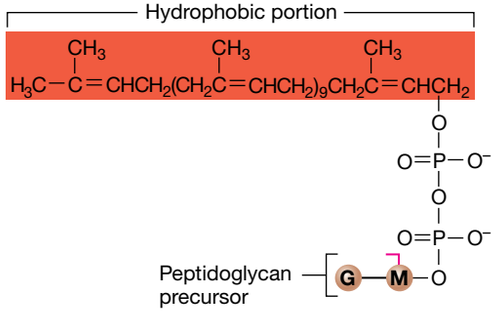


Figure 7.13 Bactoprenol (undecaprenol diphosphate). This highly hydrophobic molecule carries cell wall peptidoglycan precursors through the cytoplasmic membrane.

transports peptidoglycan precursors across the cytoplasmic membrane by rendering them sufficiently hydrophobic to pass through the membrane.

Once outside the cell, the bactoprenol complex interacts with enzymes called *transglycosylases* that insert peptidoglycan precursors into a growing point in the cell wall and catalyze glycosidic bond formation (**Figure 7.14**). Prior to this, small gaps in the existing peptidoglycan are made by enzymes called *autolysins*, enzymes that function to hydrolyze the bonds that connect *N*-acetylglucosamine and *N*-acetylmuramic acid in the peptidoglycan backbone. New cell wall material is then added across the gaps (Figure 7.14*a*). The junction between new and old peptidoglycan forms a ridge on the cell surface of gram-positive bacteria that can be observed as a *wall band* (Figure 7.12*b*). Peptidoglycan synthesis must be a highly coordinated process and peptidoglycan precursors must be readily available during autolysin activity. This is because tetrapeptide units must be spliced into existing peptidoglycan immediately

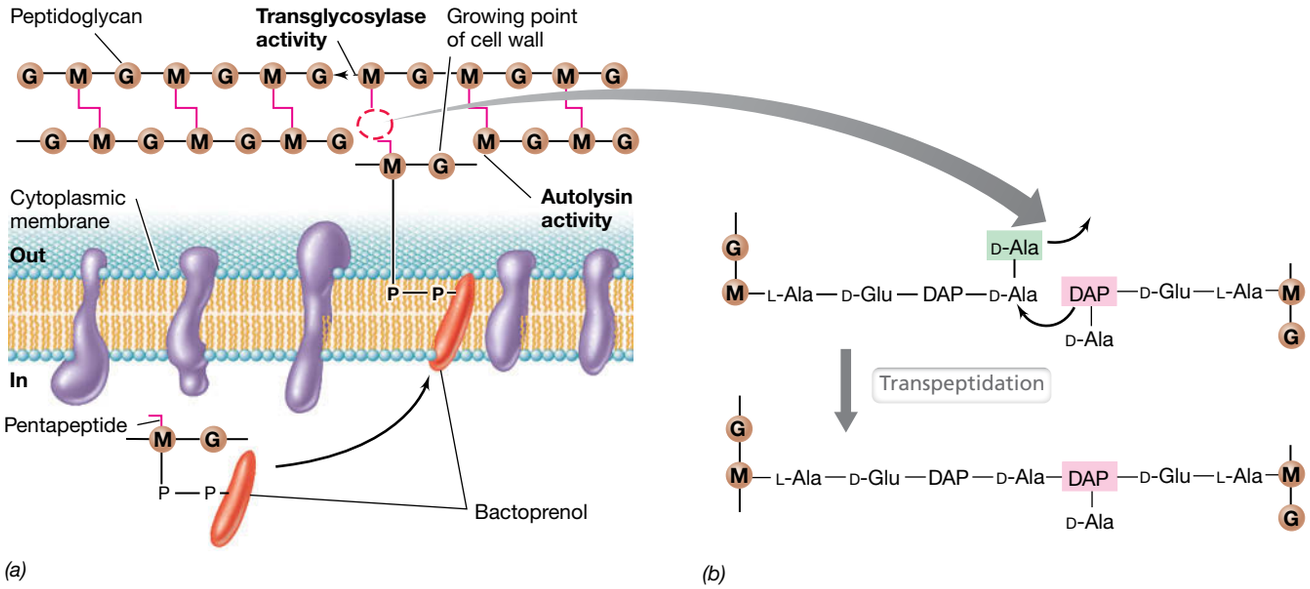


Figure 7.14 Peptidoglycan synthesis. *(a)* Transport of peptidoglycan precursors across the cytoplasmic membrane to the growing point of the cell wall. Autolysin breaks glycolytic bonds in preexisting peptidoglycan, while transglycosylase synthesizes them, linking old peptidoglycan with new. *(b)* The transpeptidation reaction that leads to the final cross-linking of two peptidoglycan chains. Penicillin inhibits this reaction.

after autolysin activity in order to prevent a breach in the peptidoglycan fabric at the splice point; a breach could cause spontaneous cell lysis, called *autolysis*.

Transpeptidation

The final step in peptidoglycan synthesis is transpeptidation. Transpeptidation forms the peptide cross-links between muramic acid residues in adjacent glycan chains (Section 2.4 and Figures 2.10 and 2.11). In gram-negative bacteria such as *Escherichia coli*, cross-links form between diaminopimelic acid (DAP) on one peptide and D-alanine on the adjacent peptide. Although there are two D-alanine residues at the end of the peptidoglycan precursor, only one remains in the final molecule, as the other is removed during transpeptidation (Figure 7.14*b*). This reaction is exergonic (energy-releasing, Section 3.4) and supplies the energy necessary to drive transpeptidation forward. In *E. coli*, the protein FtsI (Figure 7.8*a*) functions as a transpeptidase.

Transpeptidation is medically noteworthy because it is the reaction inhibited by the antibiotic penicillin. Several penicillin-binding proteins have been identified in bacteria, including FtsI (Figure 7.8*a*). When penicillin is bound to penicillin-binding proteins, the proteins are inactivated. If transpeptidation is blocked in an otherwise growing cell, the continued activity of autolysins (Figure 7.14) so weakens the peptidoglycan that the cell eventually bursts. The absence of peptidoglycan in eukaryotes (such as humans) is the basis of the clinical efficacy of penicillin—the antibiotic destroys only growing bacteria and thus targets pathogenic bacteria that often grow rapidly in an active infection.

MINIQUIZ

- What is the function of bactoprenol?
- What is transpeptidation and why is it important to both the cell and to clinical medicine?

II • Regulation of Development in Model *Bacteria*

Now that we have an understanding of the basic molecular processes that occur during bacterial growth, we move on to explore how certain bacteria differentiate to form specialized cells and how bacteria can transition from growing in suspensions (planktonic growth) to form multicellular biofilms.

Development and differentiation are largely characteristics one associates with multicellular organisms. Because most *Bacteria* and *Archaea* grow as single cells, few show differentiation. But a few important examples are known and are classical cases of differential gene expression yielding two genetically identical descendants whose functions differ. Here we discuss three well-studied examples of development and differentiation: the formation of endospores in the gram-positive soil bacterium *Bacillus*; the formation of two cell types—motile and stationary—in the gram-negative aquatic bacterium *Caulobacter*; and the formation of heterocysts in the nitrogen-fixing cyanobacterium *Anabaena*. We conclude by considering the formation of biofilms in the gram-negative and pathogenic bacteria *Pseudomonas aeruginosa* and *Vibrio cholerae*.

7.6 Regulation of Endospore Formation

Many microorganisms respond to adverse conditions by converting growing (vegetative) cells into spores (Section 2.10). Once favorable conditions return, the spore germinates and the organism returns to the vegetative state. Among *Bacteria*, the genus *Bacillus* is well known for the formation of *endospores*, that is, spores formed inside a mother cell. Prior to endospore formation, the cell divides asymmetrically. The smaller cell develops into the endospore, which is surrounded by the larger mother cell. Once development is complete, the mother cell bursts, releasing the endospore.

Endospore Formation: Sporulation Factors

Endospore formation in *Bacillus subtilis* is triggered by unfavorable conditions, such as starvation, desiccation, or growth-inhibitory temperatures. A cell of *B. subtilis* monitors its environment through a group of five sensor kinases. These enzymes function via a phosphotransfer relay system whose mechanism resembles that of a two-component regulatory system (Section 6.6) but is considerably more complex (**Figure 7.15**). The net result of multiple adverse conditions is the successive phosphorylation of several proteins called *sporulation factors*, culminating with sporulation factor Spo0A. When Spo0A is highly phosphorylated, sporulation proceeds. Spo0A controls the expression of several sporulation-specific genes. The product of one of these, SpoIIE, removes the phosphate from SpoIIAA, and this triggers the latter to remove the anti-sigma factor SpoIIAB; this liberates the sigma factor σ^F, a key step in the sporulation process.

Endospore Formation: Alternative Sigma Factors

Once a cell of *B. subtilis* commits to sporulation, endospore development is controlled by four different σ factors, two of which, σ^F and σ^G, activate genes needed inside the developing endospore (called the *forespore*) and two of which, σ^E and σ^K, activate genes needed in the mother cell surrounding the forespore (Figure 7.15*b*). The sporulation signal, transmitted via Spo0A (see earlier), activates σ^F in the forespore (σ^F is already present in the forespore but is inactive because it is bound by an anti-sigma factor, Figure 7.15*a*). Once free, σ^F is active and can bind to RNA polymerase and promote transcription of genes whose products are needed for the next stage of sporulation (Figures 7.2*a* and 7.15*b*). These include the gene encoding the sigma factor σ^G and the genes for proteins that cross into the mother cell and activate σ^E.

Active σ^E is required for transcription of yet more genes inside the mother cell, including the gene for σ^K. The sigma factors σ^G (in the forespore) and σ^K (in the mother cell) are required for transcription of genes needed even later in the sporulation process (Figure 7.15). Eventually, the many spore coats and other unique structures typical of the endospore (Section 2.10 and Table 2.2) are formed, and the mature endospore is released.

Nutrients for Endospore Formation

Nutrient limitation is the major trigger of sporulation in *Bacillus* (Section 2.10). But if this is the case, how do cells obtain

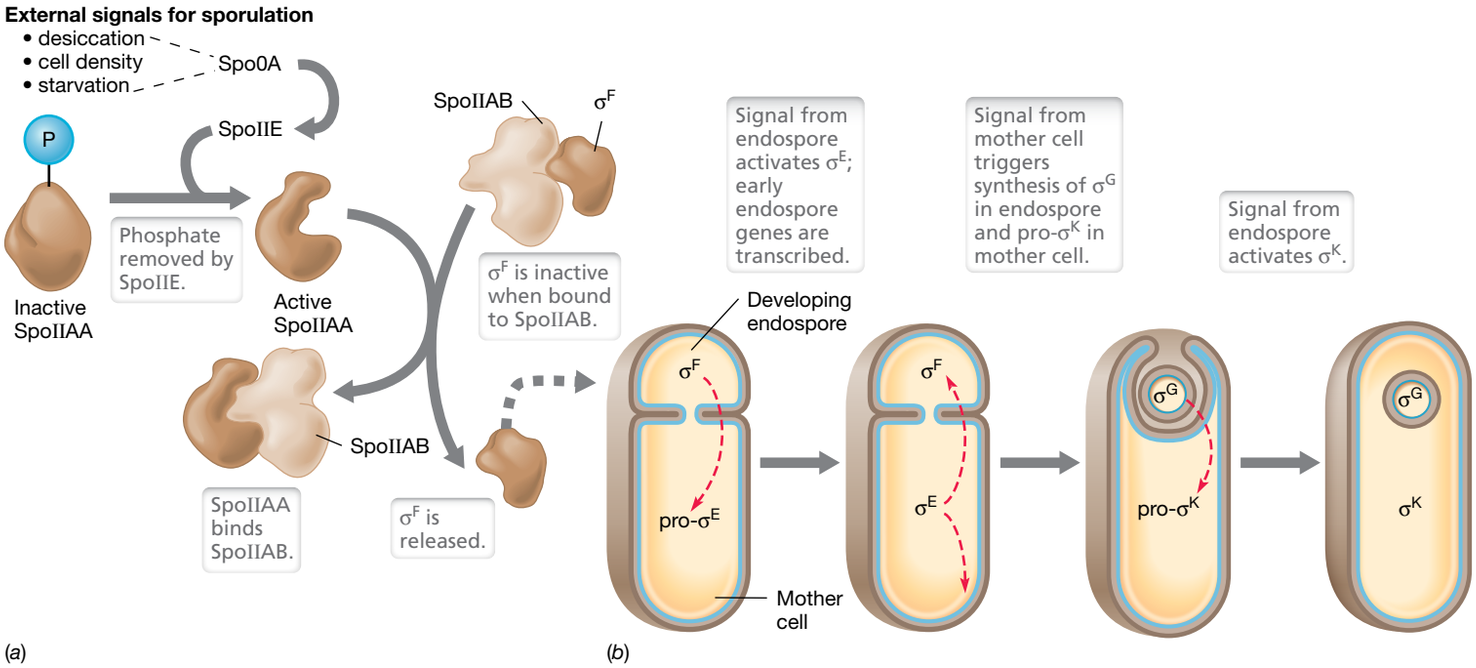


Figure 7.15 Control of endospore formation in *Bacillus*. After an external signal is received, a cascade of sigma (σ) factors controls differentiation. *(a)* Active SpoIIAA binds the anti-σ factor SpoIIAB, thus liberating the first σ factor, σ^F. *(b)* σ^F initiates a cascade of sigma factors, some of which already exist and need to be activated, others of which are not yet present and whose genes must be expressed. These σ factors then promote transcription of genes needed for endospore development.

sufficient nutrients to complete the formation of endospores? One fascinating aspect of the regulation of endospore formation is another regulatory event in which sporulating cells cannibalize cells of their own species. Those *Bacillus* cells in which Spo0A has already become activated secrete a toxic protein that lyses nearby *Bacillus* cells whose Spo0A protein has not yet become activated. This lytic protein is produced along with a second protein that functions to delay sporulation of neighboring cells. Cells committed to sporulation also make an antitoxin protein to protect themselves against the effects of their own toxic protein. When lysed, their sacrificed sister cells are used as a source of nutrients for developing endospores. Shortages of certain key nutrients, in particular phosphate, increase transcription of the gene that encodes the toxic protein.

In sporulation we thus see not only a strategy by which cellular differentiation allows the species to form cells that can withstand adverse conditions, but a strategy in which survival of a few (as opposed to all) cells of the species in a population is a priority and is facilitated by the sacrifice of other cells of the same species.

MINIQUIZ

- How are different sets of genes expressed in the developing endospore and the mother cell?
- What is an anti-sigma factor and how can its effect be overcome?

7.7 *Caulobacter* Differentiation

The gram-negative bacterium *Caulobacter* provides a second example of a cell that divides into two genetically identical daughter cells that are both structurally and functionally distinct and express different sets of genes.

Caulobacter is a genus of *Alphaproteobacteria* (Figure 7.11) that undergoes a simple life cycle and is a common bacterium in oligotrophic (nutrient-poor) aquatic environments (Section 15.20). In the *Caulobacter* life cycle, free-swimming (swarmer) cells alternate with cells that lack flagella and instead are attached to surfaces by a stalk with a holdfast at its end. The role of the swarmer cells is strictly dispersal, as swarmers cannot divide to form new swarmer cells nor can they replicate their DNA. Conversely, the role of the stalked cell is strictly reproduction. In order to divide, swarmer cells must first differentiate into stalked cells, and to swim, stalked cells must first produce swarmers; this is the nature of the *Caulobacter* life cycle (**Figure 7.16**) (Section 6.9 and Figure 6.24*b*).

Regulatory Features

The *Caulobacter* cell cycle is controlled by three major regulatory proteins whose concentrations oscillate in succession. Two of these are the transcriptional regulators GcrA and CtrA. The third is DnaA, a protein that functions both in its normal role in initiating DNA replication (Section 7.2) and also as a transcriptional regulator. Each of these regulators is active at a specific stage in the cell cycle, and each controls many other genes that are needed at that particular stage in the cycle.

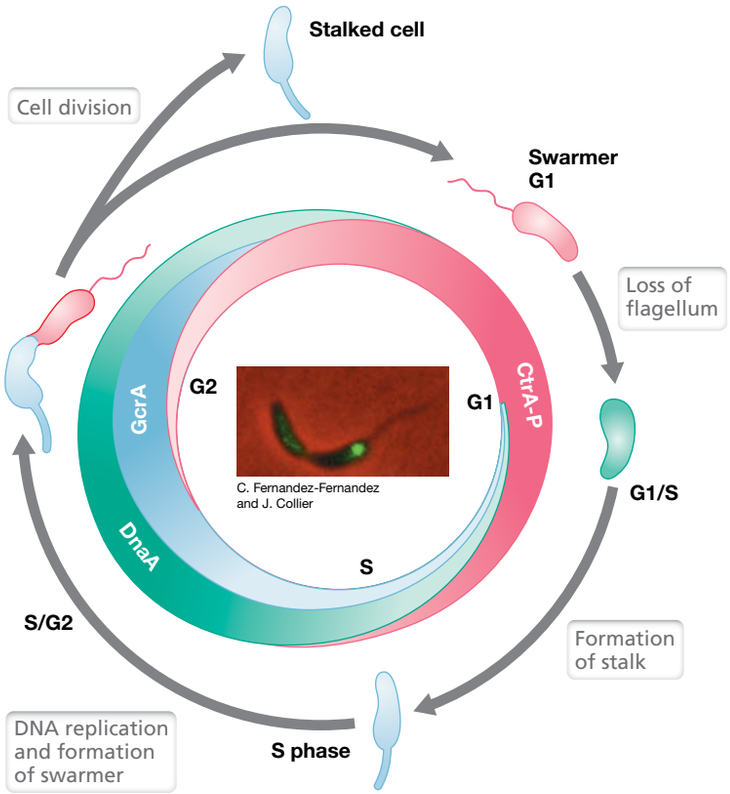


Figure 7.16 Cell cycle regulation in *Caulobacter*. Three global regulators, CtrA, DnaA, and GcrA, oscillate in levels through the cycle as shown. G1 and G2 are the two growth phases and S is the DNA synthesis phase. In G1 swarmer cells, CtrA represses initiation of DNA replication and expression of GcrA. At the G1/S transition, CtrA is degraded and DnaA levels rise. DnaA binds to the origin of replication and initiates replication (see inset photo). GcrA also rises and activates genes for cell division and DNA synthesis. At the S/G2 transition, CtrA levels begin to rise again and shut down GcrA expression. GcrA levels slowly decline in the stalked cell but are rapidly degraded in the swarmer. CtrA is degraded in the stalked cell. Inset: Fused to the green fluorescent protein as a reporter (Section 7.1), a subunit of DNA polymerase is localized in the end of the stalked *Caulobacter* cell where DNA replication occurs. Each cell of the dividing *Caulobacter* pair is about 2 μm long.

CtrA is activated by phosphorylation in emerging swarmer cells in response to external signals. Once phosphorylated, CtrA-P activates genes that encode synthesis of the flagellum and other functions specific to swarmer cells. Conversely, CtrA-P represses the synthesis of GcrA and also inhibits the initiation of DNA replication in swarmer cells by binding to and blocking the origin of replication (Figure 7.16).

As the cell cycle proceeds, CtrA is degraded by a specific protease, and as a consequence, levels of DnaA begin to rise. The absence of CtrA-P allows access to the chromosomal origin of replication, and, as in all *Bacteria*, DnaA binds to the origin and triggers the initiation of DNA replication (Section 7.2). In addition, *Caulobacter* DnaA activates several other genes needed for chromosomal replication. The level of DnaA then falls due to protease degradation, and the level of GcrA rises. The GcrA regulator promotes the elongation phase of chromosome replication, cell division, and the growth of the stalk on the immobile daughter cell. Eventually, GcrA levels fall and CtrA reappears (in the daughter cell destined to swim away) (Figure 7.16) and the cell cycle is repeated.

Caulobacter and the Eukaryotic Cell Cycle

Both external stimuli and internal factors such as nutrient and metabolite levels coordinate the events of the *Caulobacter* cell cycle (Section 6.9). Since its genome has been sequenced and good genetic transfer systems are available, the *Caulobacter* cell cycle has been used as a model for studying cell developmental processes in other organisms as well. This focus is primarily due to the strict cell cycle followed by *Caulobacter*, which resembles the cell cycle of eukaryotic cells in many respects. In fact, the resemblance is so striking that terminology used to describe the eukaryotic cell cycle has been adapted to the *Caulobacter* system.

In eukaryotic cells, phase G1 of cell division is where growth and normal metabolic events occur, and in phase G2 the cell prepares for subsequent mitotic events, which occur in the M phase. Between G1 and G2 is the S phase, where DNA replication occurs. In the *Caulobacter* life cycle there is no mitosis, of course, but analogs of the G1, G2, and S phases are apparent (Figure 7.16), making this bacterium an excellent model for studying cell-division events in higher organisms.

MINIQUIZ

- Why are the levels of DnaA protein controlled during the *Caulobacter* cell cycle?
- When do the regulators CtrA and GcrA carry out their main roles during the *Caulobacter* life cycle?

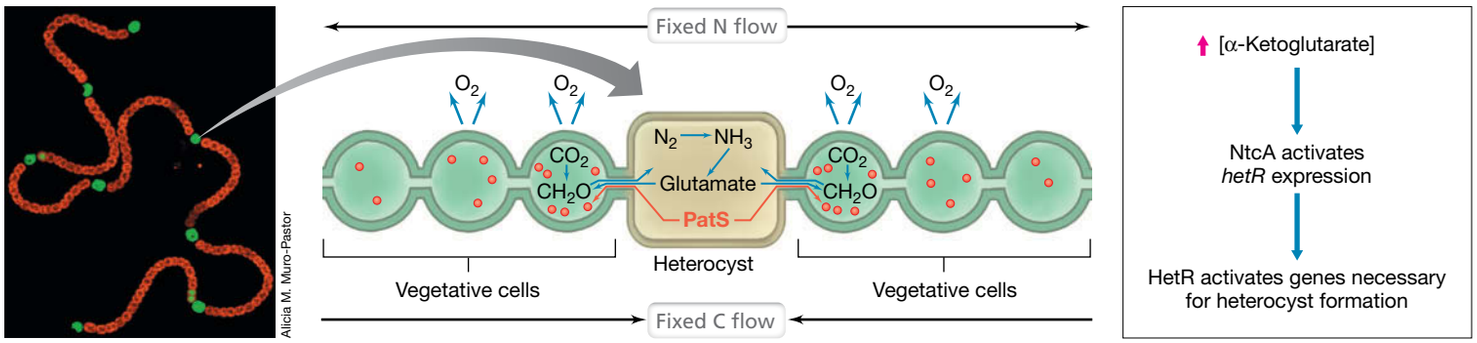
7.8 Heterocyst Formation in *Anabaena*

Cyanobacteria are oxygenic phototrophs that yield oxygen from their photosynthetic metabolism (Sections 14.4 and 15.3). They are also able to perform nitrogen fixation—the reduction of N_2 to NH_3 as a nitrogen source (Section 14.6). Nitrogen fixation is a highly energy-demanding process catalyzed by *nitrogenase*, an enzyme extremely sensitive to oxygen. How then is it possible for both nitrogen fixation and oxygenic photosynthesis to occur simultaneously in the same bacterium? To solve this problem, some filamentous cyanobacteria such as *Anabaena* and *Nostoc* undergo a developmental process to form specialized cells called **heterocysts** that are dedicated to nitrogen fixation.

Heterocyst Formation

Because heterocysts lack photosystem II—the pigment–protein complex that produces O_2 during oxygenic photosynthesis—they are anoxic cells. This anaerobic lifestyle provides a hospitable environment for nitrogenase and thus nitrogen fixation. Heterocysts arise from the differentiation of phototrophic vegetative cells that produce O_2, and typically develop in a regular pattern along a filament (**Figure 7.17*a***). As we will see, this *patterning* separates two incompatible metabolic processes while still allowing for necessary nutrient exchanges and growth.

Heterocyst formation requires several morphological and metabolic changes that are regulated by a network of systems that sense both external conditions and intracellular signaling molecules. These changes include the formation of a thickened cell wall to prevent O_2 diffusion into the cell, inactivation of photosystem II,



(a) **A filament of *Anabaena*** *(b)* **Heterocyst—vegetative cell interactions** *(c)* **Triggering heterocyst formation**

Figure 7.17 Regulation of heterocyst formation. *(a)* Fluorescence microscopy showing *Anabaena* filaments expressing the green fluorescent protein linked to heterocyst-specific genes; vegetative cells are red from chlorophyll *a* fluorescence. *(b)* Molecule dispersion in heterocysts. Fixed carbon from photosynthesis in the vegetative cells is transferred to the heterocyst, while fixed nitrogen produced in the heterocyst is shared with the vegetative cells. The protein PatS, which is synthesized by heterocysts, is also dispersed to neighboring vegetative cells where it inhibits expression of genes necessary for heterocyst formation. *(c)* Cascade of events in the activation of genes necessary for heterocyst formation. The cascade is initiated by an increase in α-ketoglutarate concentration.

expression of nitrogenase, and "patterning" of heterocyst differentiation along the filament (Figure 7.17*a*). Because nutrients can be exchanged between heterocysts and adjacent vegetative cells (Figure 7.17*b*), other regulatory steps are initiated to prevent nearby vegetative cells from undergoing the developmental conversion to heterocysts.

Regulation of Heterocyst Formation

The cascade of events leading to heterocyst formation is triggered by a limitation of fixed nitrogen (nitrate, ammonia, etc.); the limitation is sensed in the vegetative cell as an elevation in levels of α-ketoglutarate, the acceptor molecule for formation of the amino acid glutamate (↪ Section 3.14). When the cell is starved for fixed nitrogen, α-ketoglutarate accumulates and activates the transcriptional global regulator NtcA. NtcA then activates transcription of the gene *hetR*, which encodes HetR, the major transcriptional regulator controlling heterocyst formation. HetR activates a cascade of genes necessary for differentiation of the heterocyst, expression of cytochrome *c* oxidases to remove traces of O_2, as well as expression of the *nif* operon for the synthesis and regulation of nitrogenase (Figure 7.17*c*).

Only specific cells along the filament form heterocysts, and the consistent pattern observed (Figure 7.17*a*) is under strict control. Intercellular connections between cells in an *Anabaena* filament allow vegetative cells to provide fixed carbon to the heterocyst as an electron donor (for N_2 reduction by nitrogenase) in exchange for some of the NH_3 produced. However, the cell connections also allow for intercellular communication by regulatory molecules. In this regard, differentiating cells produce a small peptide called PatS that diffuses away from the developing heterocyst to form a gradient along the vegetative cells in the filament (Figure 7.17*b*). PatS is thought to inhibit differentiation in vegetative cells by preventing HetR from activating genes necessary for heterocyst formation. A second regulator called PatA, a response regulator analogous to the chemotaxis response regulator CheY (↪ Figure 6.19), also participates in heterocyst pattern development. PatA promotes the activity of HetR, decreases the activity of PatS, and may also participate in cell division.

While other regulatory links in heterocyst formation remain active areas of study, the differentiation of vegetative cells to heterocysts in heterocystous cyanobacteria is a unique example of multicellular patterning and intercellular communication in prokaryotic cells.

MINIQUIZ

- Vegetative cells produce oxygen, while heterocysts do not. Why?
- What is the major transcriptional regulator that controls heterocyst formation?

7.9 Biofilm Formation

In Chapter 5 we briefly discussed the basic characteristic of bacterial biofilms—attached polysaccharide matrices containing embedded bacterial cells—along with their environmental and medical significance. In this section we focus on the molecular mechanisms that control biofilm development. Biofilm formation is a diverse process that is controlled in different ways. We focus here on two well-studied models of biofilm formation in gram-negative bacteria, those of *Pseudomonas aeruginosa* and *Vibrio cholerae*.

Steps to Biofilm Formation

Biofilm formation can be considered a type of developmental cycle that has four basic stages: (1) attachment, (2) colonization, (3) development, and (4) dispersal. Random collision of cells with a surface accounts for the initial cell attachment. Cell attachment is facilitated by structures such as flagella and pili or by proteins on the cell surface. Attachment of a cell to a surface (**Figure 7.18**) is a signal for the expression of biofilm-specific genes. The latter include genes encoding proteins that produce intercellular signaling molecules and extracellular polysaccharides that initiate matrix formation. Once committed to biofilm formation, a previously suspended (planktonic) cell typically loses its flagella and becomes nonmotile. However, biofilms are not static entities

UNIT 2

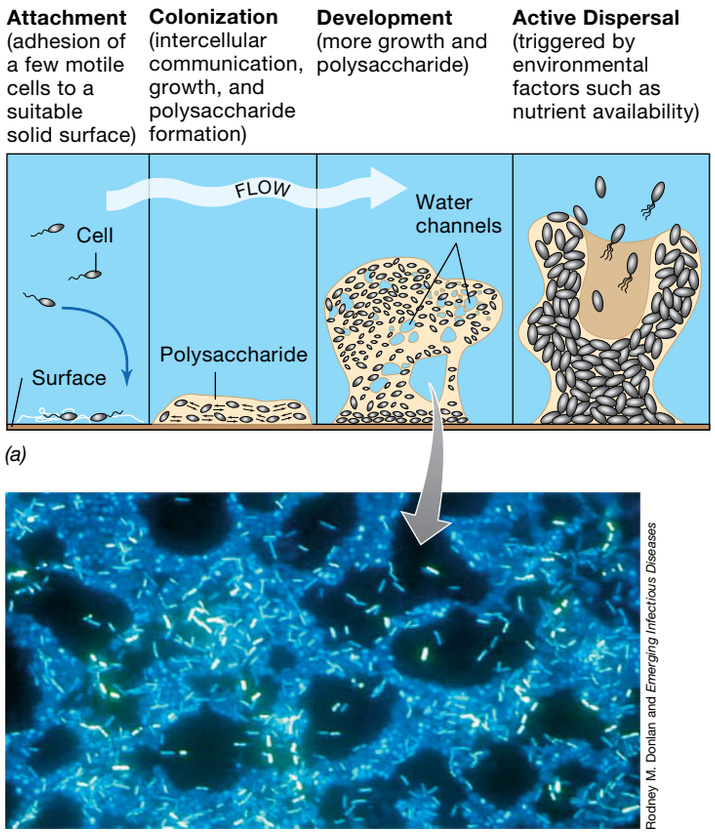


Figure 7.18 Biofilm formation. *(a)* Biofilms begin with the attachment of a few cells that then grow and communicate with other cells. The matrix is formed and becomes more extensive as the biofilm grows, eventually releasing cells. *(b)* Photomicrograph of a DAPI-stained biofilm that developed on a stainless steel pipe. Note the water channels.

and cells can be released from the biofilm through an active process of dispersal.

Several signals guide bacteria in transitioning from planktonic growth to life in a semisolid matrix. The actual switch from planktonic to biofilm growth in many bacteria is triggered by the cellular accumulation of the regulatory nucleotide *cyclic di-guanosine monophosphate* (c-di-GMP) (**Figure 7.19**). Although various nucleotides play important regulatory roles in all domains of life (Section 6.4), c-di-GMP is widely distributed only in *Bacteria*. The synthesis or degradation of c-di-GMP depends on both environmental and cellular cues, and its synthesis triggers a variety of physiological events. For example, c-di-GMP binds to proteins that reduce the activity of the flagellar motor, regulates cell surface proteins required for attachment, and mediates the biosynthesis of extracellular matrix polysaccharides of the biofilm.

Pseudomonas aeruginosa and Biofilms

Pseudomonas aeruginosa can form a tenacious biofilm (**Figure 7.20**) containing specific polysaccharides that subsequently increase its pathogenicity and prevent the penetration of antibiotics. *P. aeruginosa* is a classic opportunistic pathogen and from its primary reservoir in soil can infect the blood, lungs, urinary tract, ears, skin, and other

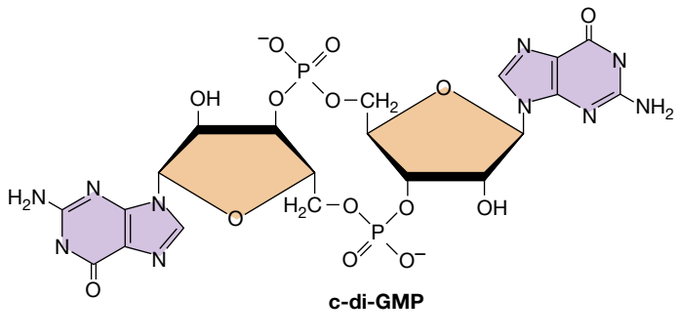


Figure 7.19 Molecular structure of the second messenger cyclic di-guanosine monophosphate. This is used as an intracellular signaling molecule by many bacteria to control specific physiological processes.

tissues of humans. The major symptoms of the genetic disease cystic fibrosis are caused by thick biofilms of *P. aeruginosa* that develop in the lungs, and the bacterium is a significant nosocomial (hospital-acquired) pathogen.

Besides the intracellular activities triggered by c-di-GMP, intercellular communication by quorum sensing (Section 6.8) is critical for the development and maintenance of *P. aeruginosa* biofilms. As *acyl homoserine lactones* (AHLs) accumulate, they signal to adjacent *P. aeruginosa* cells that the population of this species is enlarging. The production of AHL also triggers expression of a subset of the

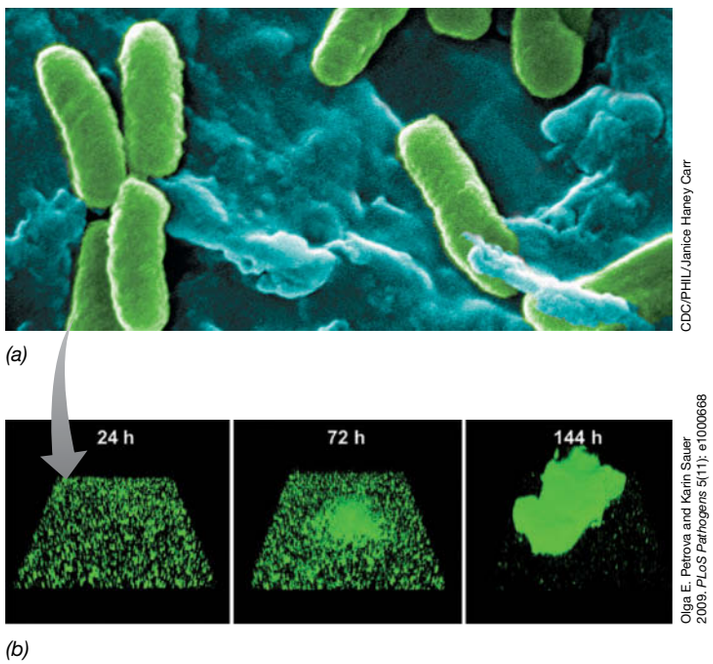


Figure 7.20 Biofilm formation in *Pseudomonas*. *(a)* Biofilms begin with the attachment of a few cells that then grow and communicate with other cells. The matrix is formed and becomes more extensive as the biofilm grows, eventually releasing cells. *(b)* Confocal scanning laser microscopy showing the progression of *P. aeruginosa* biofilm formation over a 144-h period. Cells are stained with the LIVE/DEAD viability stain, which stains live cells green. Each rectangular pattern of cells is about 0.2 mm wide. The mature biofilm is about 0.1 mm wide and 60 μm high. Data adapted from Petrova, O.E., and K. Sauer. 2009. A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. *PLoS Pathogens 5*(11): e1000668.

genes necessary for biofilm formation including those that increase extracellular polysaccharide and c-di-GMP synthesis.

Elevated c-di-GMP levels initiate the production of extracellular polysaccharide, including Pel, which functions as both a primary scaffold for the microbial community and a mechanism for resisting antibiotics. c-di-GMP also leads to decreased flagellar function. Over time in nutrient-rich conditions, *P. aeruginosa* cells can develop mushroom-shaped microcolonies that can be more than 0.1 mm high and contain millions of cells. The final architecture of the biofilm is determined by multiple factors in addition to signaling molecules including nutritional factors and local flow environment.

In the biofilm-forming *Pseudomonas* species *P. fluorescens*, increases in c-di-GMP also promote biofilm formation. However, the biofilm machinery regulated by c-di-GMP in *P. fluorescens* is very different from that of *P. aeruginosa*. In *P. fluorescens*, changes in c-di-GMP levels affect secretion and cell surface localization of a large adhesion protein called LapA that helps stick the cell to the surface. For example, in response to low extracellular phosphate, *P. fluorescens* cells maintain a low c-di-GMP level that prevents localization of LapA to the outer membrane, thereby disabling the attachment mechanism required to initiate biofilm formation. If phosphate levels continue to fall within the biofilm, the associated reduction in c-di-GMP levels also results in the activation of a protease that cleaves LapA; this releases already attached cells and promotes their dispersal to explore for available nutrients in habitats nearby.

Vibrio cholerae and Biofilms

Vibrio cholerae, the causative agent of cholera (Section 32.3), also uses both inter- and intracellular signaling to control biofilm formation. While signaling by c-di-GMP activates the expression of genes for biofilm formation, quorum sensing acts in an opposite manner from that in *P. aeruginosa*. As *V. cholerae* cell density increases, the level of quorum signaling molecules also increases. The accumulation of these molecules triggers a regulatory cascade that ultimately results in the *repression* of biofilm formation genes and the *activation* of flagellar and virulence genes. Thus, in contrast to *P. aeruginosa*, biofilm formation in *V. cholerae* is triggered by low cell densities and repressed by high cell densities.

The ecological significance of this phenomenon is that biofilm formation is more likely to occur when *V. cholerae* is found in its natural marine environment where nutrients are typically scarce, thus leading to smaller populations than those found inside the close quarters of an intestinal cell where nutrients are more plentiful. Biofilm formation allows the *V. cholerae* cell to attach to marine surfaces such as plankton, crustaceans, and sediments for greater access to nutrients and protection from perturbations. The final step in biofilm formation, dispersal, ultimately aids in the transmission of *V. cholerae* to new host cells.

Because the biofilm lifestyle of many pathogenic bacteria often leads to either persister cells (Section 7.11) or antibiotic-resistant cells (or both), and because biofilms themselves function as barriers to the penetration of antimicrobial drugs, there is great medical interest in understanding the molecular biology of biofilm development. We continue our discussion of the ecology of biofilms and strategies to control their formation in Chapter 20.

MINIQUIZ

- What are the four basic stages of biofilm formation?
- Besides autoinducer synthesis, what intracellular molecule promotes biofilm formation in many bacteria?

III • Antibiotics and Microbial Growth

Throughout this chapter we have discussed how cells coordinate their molecular biology to optimize steps in microbial growth. But what about mechanisms that short-circuit these synchronized processes? Here we focus on how antibiotics target growth processes and the bacterial responses that can lead to resistance and persistence. In Chapter 28 we will focus on the clinical significance of antibiotics and consider their spectrum of activity and spread of resistance.

7.10 Antibiotic Targets and Antibiotic Resistance

Antibiotics are antimicrobial agents naturally produced by microorganisms, primarily certain bacteria and fungi. These agents are characterized by their ability to either kill or inhibit the growth of bacteria, and all target molecular processes essential to growth and survival. In this section we consider how antibiotics work and some key resistance mechanisms that bacteria have evolved to counter their effects.

Antibiotics That Target Major Molecular Processes

Because all steps within the central dogma (which describes the flow of genetic information; Chapter 4) are essential for growth, many antibiotics specifically target enzymes that catalyze DNA replication, RNA synthesis, and translation (**Figure 7.21*a***). Quinolones such as ciprofloxacin target DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive bacteria. Thus quinolones lead to cell death by interfering with DNA unwinding and replication (Section 4.1). Likewise, when transcription is inhibited in a growing cell, mRNA cannot be made and new protein synthesis is interrupted. The antibiotics rifampin and actinomycin prevent RNA synthesis by either blocking the RNA polymerase active site (rifampin) or blocking RNA elongation by binding to the major groove in DNA (Section 4.5).

Many antibiotics inhibit bacterial growth by targeting some aspect of protein synthesis (Figure 7.21*a*). Recall that ribosomes in *Bacteria* are 70S structures while eukaryotic ribosomes are 80S. The antibiotic puromycin contains a region that mimics the 3′ end of a tRNA, and this structural mimicry results in specific binding of the antibiotic to the A site in the 70S ribosome (Section 4.10); this induces chain termination and inhibits protein synthesis. Aminoglycoside antibiotics such as streptomycin specifically target the 16S rRNA of the 30S ribosome and result in the ribosome misreading mRNAs, thus leading to error-filled proteins that accumulate in the cell and ultimately inhibit growth.

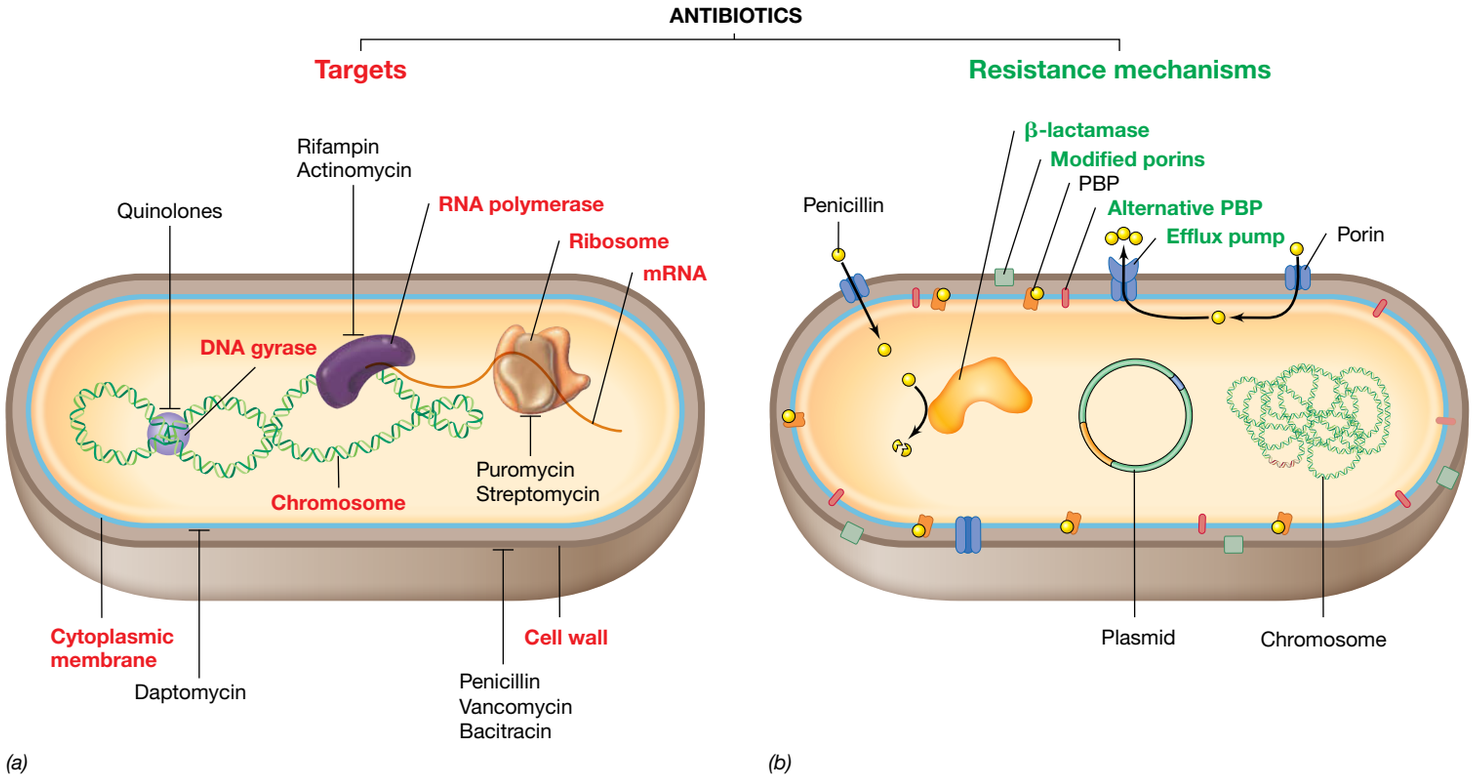


Figure 7.21 Antibiotics and antibiotic resistance. *(a)* Growth targets of select antibiotics. Antibiotic targets are in red bold. *(b)* Mechanisms of antibiotic resistance. Penicillin (β-lactam) is used to illustrate the mechanisms of modified target (modified porin), drug inactivation (β-lactamase), efflux pump, and metabolic bypass (alternative penicillin-binding protein). Genes for efflux pumps and β-lactamase enzymes can be plasmid or chromosomally encoded, while the alternative penicillin-binding protein is encoded only on the chromosome. PBP: Penicillin-binding protein. Mechanisms of antibiotic resistance are in green bold.

Antibiotics That Target the Cell Membrane and Wall

Other common antibiotic targets include the cell membrane, the cell wall, and specific metabolic processes (Figure 7.21*a*). Daptomycin is a lipopeptide produced by *Streptomyces* that specifically binds to phosphatidylglycerol residues of the bacterial cytoplasmic membrane (Section 2.3); this leads to pore formation and depolarization of the membrane, ultimately resulting in cell death. Some antibiotics target the gram-negative cell outer membrane. For example, polymyxins are cyclic peptides whose long hydrophobic tails specifically target the LPS layer and ultimately disrupt membrane structure, causing leakage and cell death.

Several antibiotics target the synthesis of peptidoglycan in bacteria such as the β-lactams penicillin, cephalosporin, and their derivatives. These antibiotics inhibit growth by interfering with proteins that catalyze transpeptidation (penicillin-binding proteins, Section 7.5); transpeptidation is the formation of cross-links between muramic acid residues that contribute to the structural strength of peptidoglycan (Section 2.4). Similarly, the antibiotic vancomycin inhibits peptidoglycan synthesis in gram-positive bacteria by binding to the pentapeptide of peptidoglycan precursors and preventing the formation of peptide interbridges by transpeptidases. The topical antibiotic bacitracin prevents peptidoglycan synthesis by binding to the peptidoglycan precursor transport system (bactoprenol, Section 7.5) and preventing new peptidoglycan precursors from reaching the site of peptidoglycan synthesis. As autolysins continue to introduce small gaps in the existing peptidoglycan (Figure 7.14*a*), a shortage of precursors to patch the gaps weakens the cell wall and leads to cell lysis.

Many other antibiotics and related antimicrobials are known, some of which target other aspects of a bacterium's biology, such as particular metabolic reactions. But the bulk of clinically useful antibiotics strike one of the targets shown in Figure 7.21*a*. We finish this section with a look at how bacteria have countered the activity of antibiotics by evolving major mechanisms of antibiotic resistance.

Antibiotic Resistance: Spontaneous Mutations and Antibiotic Modification

If bacteria are to survive the onslaught of antibiotics produced by other microbes (or from their own antibiotics if they are an antibiotic-producing organism) they require resistance mechanisms of one sort or another. Resistance mechanisms are genetically encoded and fall into four classes: (1) modification of the drug target, (2) enzymatic inactivation, (3) removal from the cell via efflux pumps, and (4) metabolic bypasses (Figure 7.21*b*).

Random chromosomal mutations can lead to antibiotic resistance. For example, spontaneous mutants of *Escherichia coli* or other bacteria resistant to the antibiotic rifampin—which inhibits

the activity of RNA polymerase—can be obtained by simply exposing a large cell population to the antibiotic. Under such conditions, spontaneous mutants that produce an altered RNA polymerase unaffected by rifampin are strongly selected for.

Resistance genes can also exist on a variety of mobile genetic elements (Chapter 4), and such genes can be readily transmitted between bacteria of the same or different species by horizontal gene flow (Chapter 11). Unlike a spontaneous mutation that affects the target of the antibiotic, many of these mobile resistance genes, especially ones transmitted by R plasmids, encode enzymes that inactivate the antibiotic by altering its structure, either through chemical modification or actual cleavage. As examples, β-lactamase binds to β-lactam-type antibiotics and cleaves a key ring structure in the molecule, and an acetylating enzyme adds acetyl groups to free hydroxy groups of chloramphenicol; once cleaved or chemically modified, the drugs can no longer bind to their target and microbial growth can continue.

Antibiotic Resistance: Efflux Pumps and Metabolic Bypasses

Another effective resistance strategy is to pump out antibiotics that have entered the cell. *Efflux pumps* are ubiquitous in gram-negative bacteria and work by transporting various molecules, including antibiotics, out of the cell (Figure 7.21*b*). Efflux lowers the intracellular concentration of an antibiotic and thus allows the cell to survive at higher external concentrations. Many efflux pumps act promiscuously and by transporting different classes of antibiotics outside of the cell, they contribute to the problem of multidrug resistance. The AcrAB-TolC efflux system of *E.coli* is one of the best-characterized efflux pumps and can pump out several antibiotics including rifampicin, chloramphenicol, and fluoroquinolones.

Growth as a biofilm (Section 7.9) leads to increased antibiotic resistance, a characteristic that makes infections caused by biofilm-forming bacteria difficult to treat. Although the exopolysaccharide matrix of a biofilm decreases the permeability of antibiotics, efflux pumps also play a major role. For example, genes encoding the AcrAB-TolC efflux pump in *E. coli* are upregulated when cells enter a biofilm growth mode. *Pseudomonas aeruginosa*, a classic biofilm former, encodes several multidrug efflux pumps that are also more active when cells grow in an attached state.

A final form of antibiotic resistance can occur when the target of the antibiotic is no longer essential to the cell's metabolism or survival. A classic example of this is *methicillin-resistant Staphylococcus aureus* (*MRSA*), the causative agent of a variety of serious, even life-threatening, infections. Methicillin is a β-lactam antibiotic that, like other penicillins, targets the activity of penicillin-binding proteins. However, unlike many penicillin derivatives, methicillin is resistant to β-lactamase cleavage. Although many strains of *S. aureus* are killed by methicillin, MRSA strains contain a 20- to 60-kilobase DNA chromosomal (or genomic) island (↔ Section 9.7) called the *Staphylococcus chromosomal cassette for methicillin resistance* (*SCCmec*) that encodes an *alternative* penicillin-binding protein called MecA that is not recognized by methicillin or other β-lactams (Figure 7.21*b*).

Interestingly, MRSA strains synthesize MecA *only* in the presence of methicillin or other β-lactams. This regulation is due to the presence of the repressor protein MecI and the β-lactam sensor MecR1. In the absence of a β-lactam-type antibiotic, MecI binds to the operator of *mecA* and represses transcription. If the cell is treated with methicillin or other β-lactams, it triggers the MecR1 protease to degrade the MecI repressor. This induces expression of *mecA* when methicillin is present and thus the production of the alternative penicillin-binding protein allows MRSA strains to synthesize peptidoglycan in the presence of the antibiotic. The chromosomal island also possesses a transposable element (↔ Section 4.2) that encodes resistance to the antibiotics erythromycin and spectinomycin. The island is thus likely subject to horizontal transfer to confer multidrug resistance.

MINIQUIZ

- Describe two targets of antibiotics and discuss why the drugs are effective.
- Some antibiotics target peptidoglycan synthesis. What is a molecular growth target of an antibiotic that inhibits peptidoglycan synthesis?
- Why are efflux pumps capable of conferring multidrug resistance?

7.11 Persistence and Dormancy

Besides being sensitive or resistant to antibiotics, a third growth response is possible—**persistence**. Persistence occurs when a population of antibiotic-sensitive bacteria produces rare cells that *transiently* become tolerant to multiple antibiotics (**Figure 7.22*a***). These *persisters*, as they are called, are genetically identical to their antibiotic-susceptible siblings and thus persistence is not inheritable or conferred by genetic transfer. Instead, persisters, which form at a rate of 10^{-6} to 10^{-4} in exponentially growing cultures, derive from a state of *dormancy* in which the cells are viable but do not grow.

Because antibiotics target active processes, the dormant state prevents the antibiotic from killing the cell. When antibiotic treatment is stopped, the dormant cells emerge from dormancy and grow. Persistence is believed to be the cause of recurring infections of *Mycobacterium tuberculosis* in those with chronic tuberculosis (↔ Section 30.4) and of *Pseudomonas aeruginosa* in those suffering from the genetic disease cystic fibrosis.

How does a subpopulation of cells go dormant while genetically identical daughter cells do not? For a cell to become a persister and be able to later reverse the process, coordinated molecular events that affect cell growth must take place. The keys to this unusual phenomenon are chromosomally encoded toxin–antitoxin modules, the stringent response (↔ Section 6.9), and phenotypic heterogeneity.

Toxin–Antitoxin Modules

Toxin–antitoxin (TA) modules are genetic loci that encode two components: a toxin whose production inhibits cell growth and an antitoxin that counteracts the activity of the toxin. TA modules are found in almost all *Bacteria* and in many *Archaea*. The identification of over 30 TA systems in *Escherichia coli* and

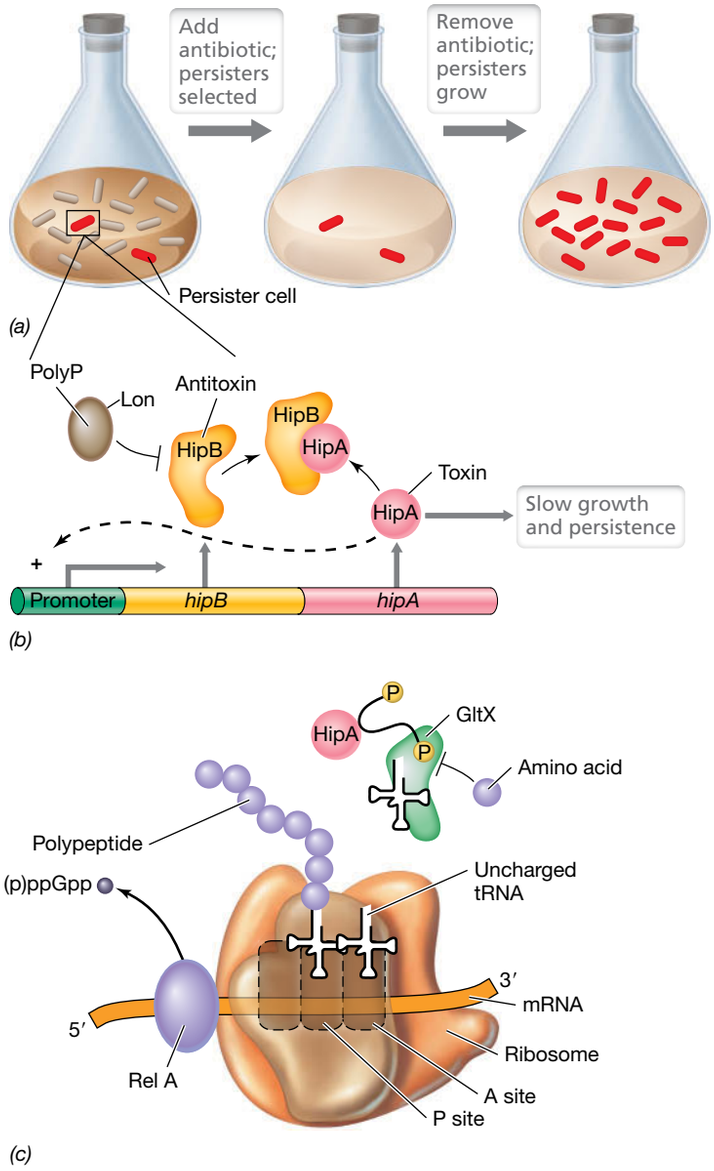


Figure 7.22 Persistence and the HipAB toxin–antitoxin module. *(a)* Antibiotic selection of persister cells and recovery from dormancy. *(b)* HipAB toxin–antitoxin module. During normal conditions, HipB sequesters the toxin HipA. The presence of Lon-PolyP leads to inactivation of the HipB antitoxin and free HipA toxin. HipA leads to slow growth and persistence and positively regulates the *hipAB* operon. *(c)* Mechanism of HipA toxin. HipA phosphorylates GltX, preventing the tRNA synthetase from charging amino acids. This leads to an uncharged tRNA entering the A site of the ribosome and the stimulation of RelA to produce (p)ppGpp.

60 TA systems in *M. tuberculosis* suggests that the TA modules play a role not only in normal physiology but also in pathogenicity. It is thought that toxin activity promotes cellular adaptation to ever-changing environments by slowing down cell growth to help ensure survival during stressful conditions.

The *hipAB* genes encode a TA module that has been shown to trigger persistence in *E. coli* (Figure 7.22*b*). In this module, HipA is a toxin that inhibits translation and HipB is an antitoxin that is susceptible to a protease called Lon. Under normal cellular conditions, the HipA toxin and HipB antitoxin form a stable complex that prevents HipA from exerting its toxicity. However, if Lon is activated by its signal molecule polyphosphate (PolyP), the HipB antitoxin is degraded. Without the HipB antitoxin present to neutralize the HipA toxin, translation is inhibited and cell growth is arrested (Figure 7.22*b*).

How does the HipA toxin inhibit translation? HipA acts as a kinase (phosphorylating protein) targeting glutamyl-tRNA synthetase (GltX). If HipA is free to phosphorylate GltX, GltX can no longer charge its respective tRNAs with glutamine. This leads to uncharged tRNAs entering the A site of the ribosome (Section 4.10) and ultimately to stalling of the ribosome and activation of RelA (Figure 7.22*c*). Stalling, in turn, inhibits translation and thus protein synthesis. Free HipA also binds upstream of the *hipAB* operon, further activating transcription and thus increasing the concentration of cellular HipA (Figure 7.22*b*).

Steps to Dormancy

As we have seen, free HipA induces ribosome stalling. This stalling not only inhibits translation, but it also leads to the most important criteria for the development of persistence—production of the alarmone (p)ppGpp (guanosine tetraphosphate and guanosine pentaphosphate) by RelA and induction of the *stringent response pathway* (Figure 7.22*c*).

Recall that the stringent response leads to decreased rRNA and tRNA synthesis, and thus protein synthesis is inhibited (Section 6.9). The pathway also leads to decreased DNA replication and cell division. Thus, those cells in a population that have triggered the stringent response no longer actively grow and enter a state of dormancy. Although this cascade of events only occurs in a small subpopulation of cells, antibiotic treatment strongly selects and enriches for multidrug-tolerant persister cells. Once the antibiotic exposure has ended, the persisters can exit the stringent response pathway and resume producing antitoxin to neutralize the effect of the toxin. When this happens, protein synthesis returns to normal levels, allowing cells to grow.

Why does this cascade of events only occur in a *subpopulation* of cells without any sort of genetic direction? HipA-induced persistence occurs in cells that randomly produce higher amounts of the signal molecule PolyP, a phenomenon called *phenotypic heterogeneity*. While it is often assumed that cells within a clonal population are homogeneous, cell-to-cell differences in gene expression and in metabolite and protein content do occur. These differences can be the result of RNA polymerase producing a subset of mRNAs through random interactions with DNA or of unequal distribution of macromolecules between daughter cells during cell division. These cell-to-cell variations can have many outcomes, but a major one for clinical medicine is the persistence of difficult-to-eradicate pathogenic bacteria whose dormant state can falsely signal a cure, only to be followed by reinfection by the same pathogen at a later date.

MINIQUIZ

- Is persistence a heritable trait?
- What prevents the toxin component of TA modules from killing the cell under normal conditions?
- What occurs in the cell that frees HipA toxin?

Chapter Review

I • Bacterial Cell Division

7.1 Macromolecules such as proteins and nucleic acids can be visualized in living cells using fluorescent tagging and microscopy techniques. Super-resolution microscopy, the most advanced form of microscopy, can even resolve two individual molecules, generate 3D images, and track the movement of molecules throughout the cell.

Q What type of probes must be used for super-resolution microscopy and why?

7.2 Before cell division occurs in prokaryotic cells, two complete copies of the genome must be present. While multiple replication forks can exist to decrease generation times, the process must be regulated. The nucleoid must also segregate to the poles of the cell to allow the septum to form.

Q What is the role of PopZ in segregating the *Caulobacter* daughter chromosomes?

7.3 Cell division and chromosome replication are coordinately regulated, and the Fts proteins are keys to these processes. With the help of MinE, FtsZ defines the cell-division plane and helps assemble the divisome, the protein complex that orchestrates cell division.

Q What is the role of the penicillin-binding protein FtsI in cell division?

7.4 MreB helps define cell shape, and in rod-shaped cells, MreB forms patchlike filaments that direct cell wall synthesis along the long axis of the cell. The protein crescentin plays an analogous role in *Caulobacter*, leading to formation of a curved cell. The eukaryotic proteins actin and tubulin involved in shape and cell division have prokaryotic counterparts.

Q How does a rod-shaped bacterial cell differ morphologically if its MreB function is disrupted?

7.5 During bacterial growth, new cell peptidoglycan is synthesized by the insertion of peptidoglycan precursors into the preexisting peptidoglycan fabric. Bactoprenol facilitates transport of these units through the cytoplasmic membrane. Transpeptidation completes the process of cell wall synthesis by cross-linking adjacent ribbons of peptidoglycan at muramic acid residues.

Q What step is necessary prior to inserting a new peptidoglycan subunit into a growing cell wall?

II • Regulation of Development in Model *Bacteria*

7.6 Sporulation in *Bacillus* during adverse conditions is triggered via a complex phosphotransfer relay system that monitors multiple aspects of the environment. The sporulation factor Spo0A then sets in motion a cascade of regulatory responses under the control of several alternative sigma factors.

Q What is a common trigger for sporulation in *Bacillus*?

7.7 Differentiation in *Caulobacter* consists of the alternation between motile cells and those that are attached to surfaces. Three major regulatory proteins—CtrA, GcrA, and DnaA—act in succession to control the three phases of the cell cycle. Each in turn controls many other genes needed at specific times in the cell cycle.

Q How is the *Caulobacter* life cycle similar to the eukaryotic cell cycle?

7.8 Heterocyst formation requires expression of the major regulatory protein HetR in the protoheterocysts. However, the protein must be inactivated in vegetative cells by diffusion of the PatS peptide along the filament.

Q What is meant by "patterning" during heterocyst formation?

7.9 During biofilm formation, cells make contact with a surface using cell structures such as flagella and pili. Once cells are attached to a surface, both intra- and intercellular signaling molecules lead to the production of an extracellular matrix and colonization. Biofilms are not static entities, and the final step in their development is dispersal and release of cells.

Q What are the regulatory roles of c-di-GMP in biofilm formation?

III • Antibiotics and Microbial Growth

7.10 Antibiotics are antimicrobial agents produced by microorganisms that target processes critical for growth such as DNA replication, transcription, protein synthesis, and cell wall synthesis. Specific mechanisms of antibiotic resistance include modification of the drug target, enzymatic inactivation, removal via efflux pumps, and metabolic bypass. These mechanisms are either chromosomally or plasmid encoded.

Q Why do antibiotics that target bacterial ribosomes not make humans sick?

7.11 Persistence is a dormant growth state in which cells are not susceptible to antibiotics. This lack of sensitivity is transient and thus not considered antibiotic resistance. Persistence is the result of toxin–antitoxin modules and the induction of the stringent response. Cells in the persistent state can resume growth once the antibiotic exposure has been stopped.

Q What are the steps to inducing the stringent response during the development of persistence?

Application Questions

1. If DnaA was not regulated in *Escherichia coli* and multiple rounds of replication were completed before cell division, what would be the consequence to the daughter cell and why? Would the resulting cell still be considered haploid?
2. Explain how cells exhibiting different phenotypes under the same conditions can possess the same genotype. Give examples in your explanation.
3. Describe how you would genetically design a superbug resistant to β-lactams, methicillin, streptomycin, daptomycin, and trimethoprim.

Chapter Glossary

Antibiotic an antimicrobial agent naturally produced by microorganisms

FtsZ a protein that forms a ring at the future site of septum formation in a prokaryotic cell

Green fluorescent protein (GFP) a protein that fluoresces green and is widely used in genetic analysis

Heterocysts specialized cells dedicated to nitrogen fixation

Persistence a dormant growth state in which a few members of a genetically identical population are transiently tolerant to multiple antibiotics

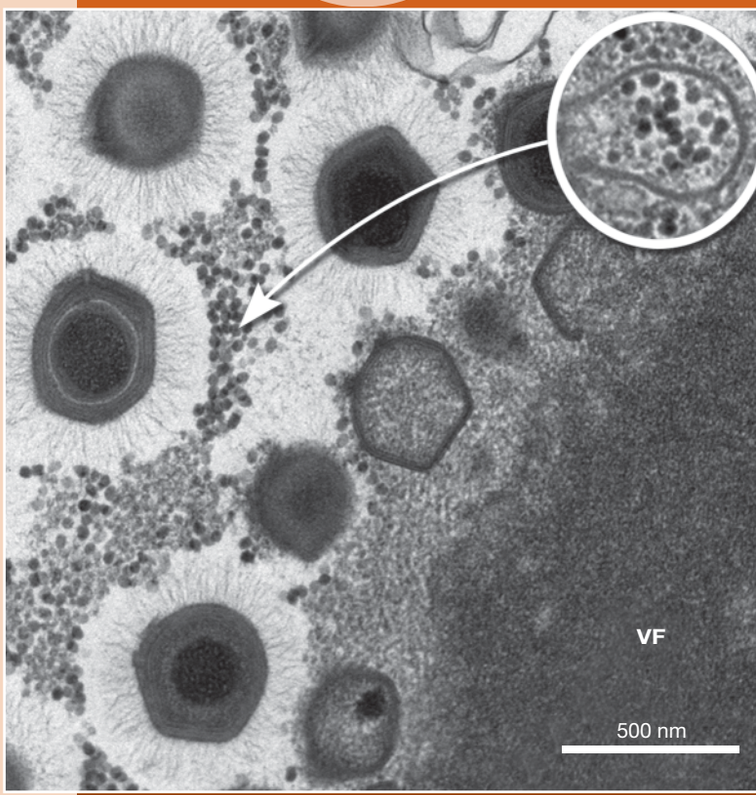
Reporter gene a gene whose product is easy to assay or detect

Super-resolution microscopy a form of light microscopy that employs special fluorescent molecules to resolve structures as small as 10–50 nm

Toxin–antitoxin module a genetic locus encoding a toxin that inhibits growth and an antitoxin that neutralizes the activity of the toxin

8

Viruses and Their Replication



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Virophages: Viruses That Parasitize Other Viruses

The simplest definition of a virus is an infectious nucleic acid surrounded by protein. Viruses are also typically much smaller than bacteria. However, giant viruses comparable to some bacterial cells have been discovered. These "megaviruses" can actually be seen under the light microscope, as their diameters reach up to 0.75 μm, and because of their size, they clearly distort the line between a cell and a virus. Megaviruses infect protozoa such as *Amoeba* in diverse aquatic environments.

Virologists have made several exciting discoveries about megaviruses other than their size. For example, for megavirus replication, infected amoeba cells form cytoplasmic "factories" dedicated to virus production, and these factories can be as large as the cell's nucleus. A particularly remarkable discovery was the detection of tiny virus-like particles surrounding intracellular megaviruses. These structures contain DNA surrounded by capsid proteins, and so by definition, they should be viruses. However, the DNA in these particles does not encode proteins required for their replication. With this in mind, how do these virus-like particles reproduce?

Although they cannot replicate in amoeba cells by themselves, these tiny structures hijack the host's megavirus-producing factories for their own benefit. This leads to competition for virus replicating enzymes and the ultimate formation of defective megaviruses, which are unable to infect new host cells. Because of this parasitic activity toward megaviruses, these pesky little structures are called virophages in analogy to the term bacteriophage, which describes viruses that infect *Bacteria*. The transmission electron micrograph here shows active cell infection by Samba megaviruses isolated from a river in the Amazon, along with their associated virophages (arrow). The dark mass in the bottom right is a viral factory (VF), and the inset illustrates the formation of a defective Samba virus that is unable to infect new amoeba cells.

While it is unclear what role(s) virophages play in the biology of the amoeba cell, it is clear that the very existence of the virophage depends on its ability to parasitize the megavirus. Hence, the ability to inactivate megavirus replication and protect host cells from death may well be the selective force that maintains the virophage in this cozy arrangement of three different microbes.

Source: Campos, R.K., et al. 2014. Samba virus: A novel mimivirus from a giant rain forest, the Brazilian Amazon. *Virol. J. 11:* 95.

Throughout this unit we have focused on the growth of bacterial and archaeal cells. Here we shift gears and discuss how viruses "grow," or more accurately, replicate, and how this replication affects cells of all three domains of life.

A **virus** is a genetic element that can replicate only inside a living cell, called the **host cell**. Not considered living entities, viruses rely on the host cell for energy, metabolic intermediates, and protein synthesis, and so they are *obligate intracellular parasites*. However, viruses possess their own genomes and in this sense are independent of the host's genome.

Viruses infect both prokaryotic and eukaryotic cells and are responsible for many infectious diseases of humans and other organisms. The study of viruses is called *virology*, and this chapter covers the basic principles of this science. In Chapter 10 we consider the genomic and diversity aspects of viruses in detail.

I • The Nature of Viruses

8.1 What Is a Virus?

Although viruses are not cells, they nonetheless contain a genome that encodes those functions needed to replicate and they have an extracellular form, called the **virion**, which allows the virus to travel from one host cell to another. Viruses cannot replicate or reproduce unless the virion itself (or its genome, in the case of bacterial viruses) has gained entry into a suitable growing host cell, a process called *infection*.

Viral Components and Activities

The virion of any virus includes a protein shell, called the **capsid**, and the virus genome that the capsid contains. Most bacterial viruses are *naked*, with no further layers, whereas many animal viruses have an outer layer called the *envelope* that consists of a phospholipid bilayer (taken from the host cell membrane) and viral proteins (Figure 8.1). In enveloped viruses, the inner structure of nucleic acid plus capsid protein is called the **nucleocapsid**. The virion protects the viral genome when the virus is outside the host cell, and proteins on the virion surface are important in attaching it to its host cell. The virion may also contain one or more virus-specific enzymes that play a role during infection and replication, as discussed later.

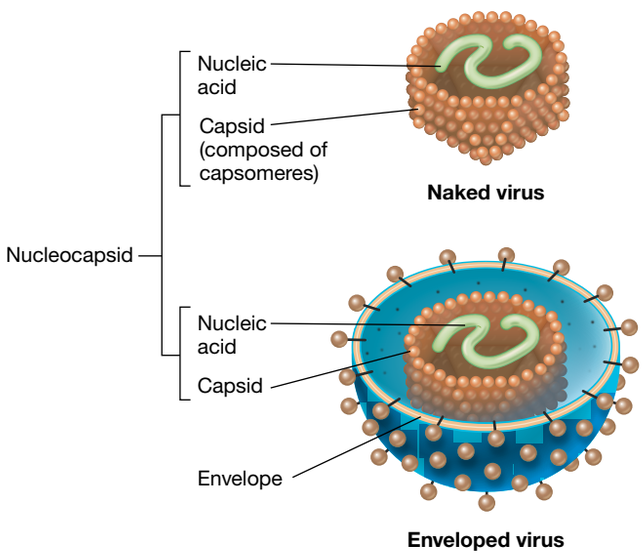


Figure 8.1 Comparison of naked and enveloped virus particles. The envelope originates from host cytoplasmic membrane.

Once inside the host cell, a viral genome can orchestrate one of two quite different events. The virus may replicate and destroy the host in a **virulent** (**lytic**) infection. In a lytic infection, the virus redirects the host cell's metabolism from growth to support virus replication and the assembly of new virions. Eventually, new virions are released, and the process can repeat itself with new host cells. Alternatively, some viruses can cause a *lysogenic* infection; in this case, the host cell is not destroyed but is genetically altered because the viral genome becomes part of the host genome. These types of infection are discussed in detail later in the chapter.

Viral Genomes

All cells contain double-stranded DNA genomes. By contrast, viral genomes consist of either DNA *or* RNA and are further subdivided based on whether the genome is *single-stranded* or *double-stranded*. A very few highly unusual viruses use both DNA and RNA as genetic material, but at different stages of their life cycle (Figure 8.2).

Viral genomes can be either linear or circular, and single-stranded viral genomes may be of either the *plus sense* or *minus sense* in terms of their base sequence. Viral genomes of the plus configuration have the *exact same base sequence* as that of the viral mRNA that will be translated to form viral proteins. By contrast, viral genomes of the minus configuration are *complementary in base sequence* to viral mRNA. This interesting feature of viral genomes requires special genetic information flow processes, and we reserve our discussion of the details of these processes to Chapter 10.

Viral genomes are typically smaller than those of cells. The smallest bacterial genome known is about 139 kilobase pairs, encoding about 110 genes. Most viral genomes encode from a few up to about 350 genes. The smallest viral genomes are those of some small RNA viruses that infect animals. The genomes of these tiny viruses contain fewer than 2000 nucleotides and only two genes. A few very large viral genomes are known, such as the 1.25-Mbp DNA genome of a marine virus called *Megavirus*, which infects protozoans. RNA viruses typically have the smallest genomes and only DNA viruses have genomes encoding more than 40 genes.

Viruses can be classified on the basis of the hosts they infect as well as by their genome structure. Thus, we have bacterial viruses, archaeal viruses, animal viruses, plant viruses, protozoan viruses, and so on. Bacterial viruses are called **bacteriophages** (or simply *phage* for short) and have been intensively studied as model systems for the molecular biology and genetics of virus replication. In this chapter we will use bacteriophages many times to illustrate basic viral principles. Indeed, many of the key tenets of virology were discovered in studies of bacteriophages and subsequently applied to viruses of higher organisms. Because of their frequent medical importance, animal viruses have been extensively studied, whereas plant viruses, although of enormous importance to modern agriculture, have been less well studied.

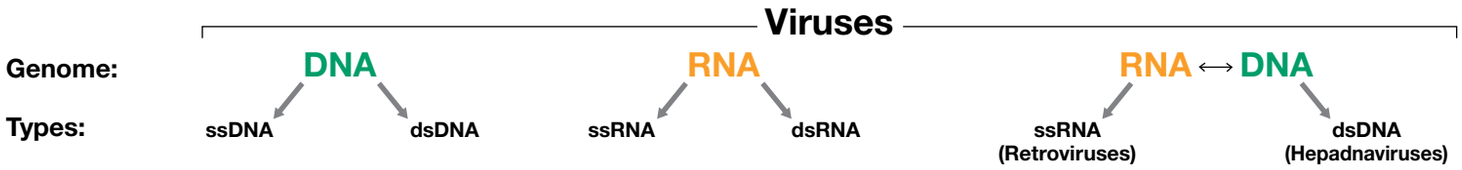


Figure 8.2 Viral genomes. The genomes of viruses can be either DNA or RNA, and some viruses use both at different stages in their replication cycle. However, only one type of genomic nucleic acid is found in the virion of any particular type of virus. Viral genomes can be single-stranded (ss) or double-stranded (ds) and circular or linear.

MINIQUIZ

- How does a virus differ from a cell?
- Why does a virus need a host cell?
- Compared with cells, what is unusual about viral genomes?

8.2 Structure of the Virion

Virions come in many shapes and sizes. Most viruses are smaller than prokaryotic cells, ranging in size from 0.02 to 0.3 µm (20–300 nanometers, nm). Smallpox virus, one of the larger viruses, is about 200 nm in diameter, which is about the size of the smallest known bacterial cells. Poliovirus, one of the smallest viruses, is only 28 nm in diameter, which is about the size of a ribosome, the cell's protein-synthesizing machine (Chapter 4).

Virion Structure

The structures of virions are quite diverse, varying widely in size, shape, and chemical composition (Chapter 10). The nucleic acid of a virion is always surrounded by its capsid (Figure 8.1). The capsid is composed of a number of individual protein molecules called **capsomeres** that are arranged in a precise and highly repetitive pattern around the nucleic acid.

The small size of most viral genomes restricts the number of distinct viral proteins that can be encoded. As a consequence, a few viruses have only a single kind of protein in their capsid. An example is the well-studied tobacco mosaic virus (TMV), which causes disease in tobacco, tomato, and related plants. TMV is a single-stranded RNA virus in which the 2130 copies of the simple capsomere protein are arranged in a helix with dimensions of 18 × 300 nm (Figure 8.3).

The information required for the proper folding and assembly of viral proteins into capsomeres and subsequently into capsids is often embedded within the amino acid sequence of the viral proteins themselves. When this is the case, virion assembly is a spontaneous process and is called *self-assembly*. However, some virus proteins and structures require assistance from host cell folding proteins for proper folding and assembly. For example, the capsid protein of bacteriophage lambda (Section 8.7) requires assistance from the *Escherichia coli* chaperonin GroE (Section 4.11) in order to fold into its active conformation.

Virus Symmetry

Virions are highly symmetric structures. When a symmetric structure is rotated around an axis, the same form is seen again after a certain number of degrees of rotation. Two kinds of symmetry are recognized in viruses, which correspond to the two primary viral shapes, rod and spherical. Rod-shaped viruses have *helical* symmetry while spherical viruses have *icosahedral* symmetry. A typical virus with helical symmetry is TMV (Figure 8.3). The lengths of helical viruses are determined by the length of the nucleic acid, and the width of the helical virion is determined by the size and packaging of the capsomeres.

Viruses with icosahedral symmetry contain 20 triangular faces and 12 vertices and are roughly spherical in shape (Figure 8.4*a*). Axes of symmetry divide the icosahedron into 5, 3, or 2 segments of identical size and shape (Figure 8.4*b*). Icosahedral symmetry is the most efficient arrangement of subunits in a closed shell because it requires the smallest number of capsomeres to build the shell. The simplest arrangement of capsomeres is 3 per triangular face, for a total of 60 capsomeres per virion. However, most viruses have more nucleic acid than can be packed into a shell made from 60 capsomeres and so viruses with some multiple of

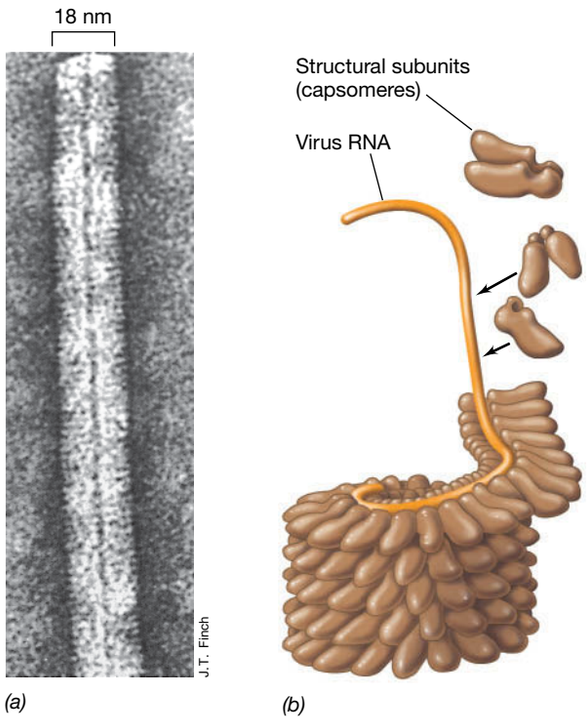


Figure 8.3 The arrangement of RNA and protein coat in a simple virus, tobacco mosaic virus. *(a)* High-resolution electron micrograph of a portion of the tobacco mosaic virus particle. *(b)* Cutaway showing structure of the virion. The RNA forms a helix surrounded by the protein subunits (capsomeres). The center of the virus particle is hollow.

UNIT 2

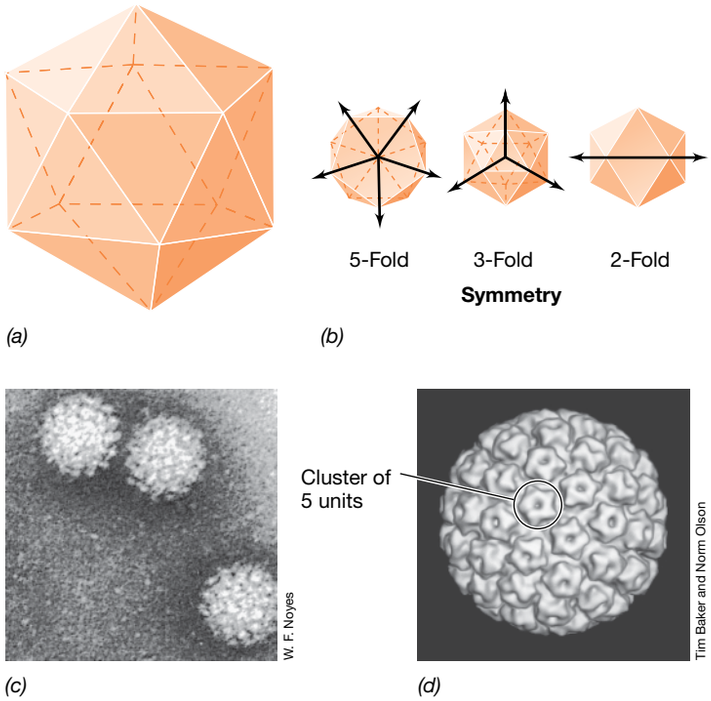


Figure 8.4 Icosahedral symmetry. *(a)* Model of an icosahedron. *(b)* Three views of an icosahedron showing 5-, 3-, or 2-fold symmetry. *(c)* Electron micrograph of human papillomavirus, a virus with icosahedral symmetry. The virion is about 55 nm in diameter. *(d)* Three-dimensional reconstruction of human papillomavirus; a virion contains 360 units arranged in 72 clusters of 5 each.

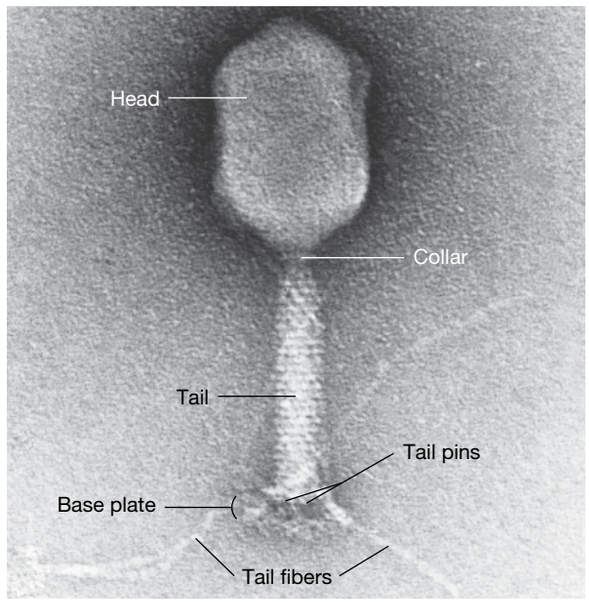


Figure 8.5 Structure of T4, a complex bacteriophage. Transmission electron micrograph of bacteriophage T4 of *Escherichia coli*. The tail components function in attachment of the virion to the host and injection of the nucleic acid (see Figure 8.12). The T4 head is about 85 nm in diameter.

60 capsomeres, such as 180, 240, or 360, are more common. The capsid of the human papillomavirus virus (Figure 8.4*c*), for example, consists of 360 capsomeres, with the capsomeres arranged into 72 clusters of 5 each (Figure 8.4*d*).

The structure of some viruses is highly complex, with the virion consisting of several parts each displaying its own shape and symmetry. The most structurally complex of all viruses are the head-plus-tail bacteriophages that infect *Escherichia coli*, such as phage T4. A T4 virion consists of an icosahedral head plus a helical tail (**Figure 8.5**). Some large viruses that infect eukaryotes are also structurally complex, although in ways quite distinct from the head-plus-tail bacteriophages. Mimivirus and vaccinia virus (see Figure 8.6*d*) are good examples and are discussed in more detail in Chapter 10.

Enveloped Viruses

Enveloped viruses have a lipoprotein membrane surrounding the nucleocapsid (**Figure 8.6**) and can have either RNA or DNA genomes. Most enveloped viruses (for example, Ebola, Figure 8.6*a*, *b*) use proteins on the virion's envelope to attach to and infect animal cells in which the cytoplasmic membrane is directly exposed to the environment. By contrast, plant and bacterial cells are surrounded by a cell wall outside the cytoplasmic membrane, and thus few examples of enveloped viruses are known in these organisms. Typically, the entire virion enters an animal cell during infection, with the envelope, if present, assisting in the infection process by fusing with the host membrane. Enveloped viruses also exit more easily from animal cells. As they pass out of the host cell, they are draped in cytoplasmic membrane material. The viral envelope consists primarily of host cytoplasmic membrane, but some viral surface proteins (Figure 8.6*a*, *b*) become embedded in the envelope as the virus passes out of the cell.

The viral envelope is important in infection, as it is the component of the virion that makes contact with the host cell. The specificity of infection by enveloped viruses and some aspects of their penetration are thus controlled in part by the biochemistry of their envelopes. The virus-specific envelope proteins are critical for both attachment of the virion to the host cell during infection and for release of the virion from the host cell after replication.

Enzymes inside Virions

Viruses do not carry out metabolic processes and are thus metabolically inert. Nonetheless, some viruses carry enzymes in their virions that play important roles in infection. For example, some bacteriophages contain an enzyme that resembles lysozyme (Section 2.4), which is used to make a small hole in the bacterium's peptidoglycan layer to allow nucleic acid from the virion to get into the host cytoplasm. A similar protein is produced in the later stages of infection to lyse the host cell and release new virions. Some animal viruses also contain enzymes that aid in their release from the host. For example, influenza virus (Figure 8.6*c*) has envelope proteins called *neuraminidases* that destroy glycoproteins and glycolipids of animal cell connective tissue, thus liberating the virions (Section 10.9).

RNA viruses carry their own nucleic acid polymerases (RNA-dependent RNA polymerases called *RNA replicases*) that function to replicate the viral RNA genome and produce viral-specific mRNA. Such enzymes are necessary because DNA polymerase cannot make RNA and cells lack enzymes of any type that can make RNA from an RNA template. Retroviruses are unusual RNA animal viruses that replicate via DNA intermediates. Because making

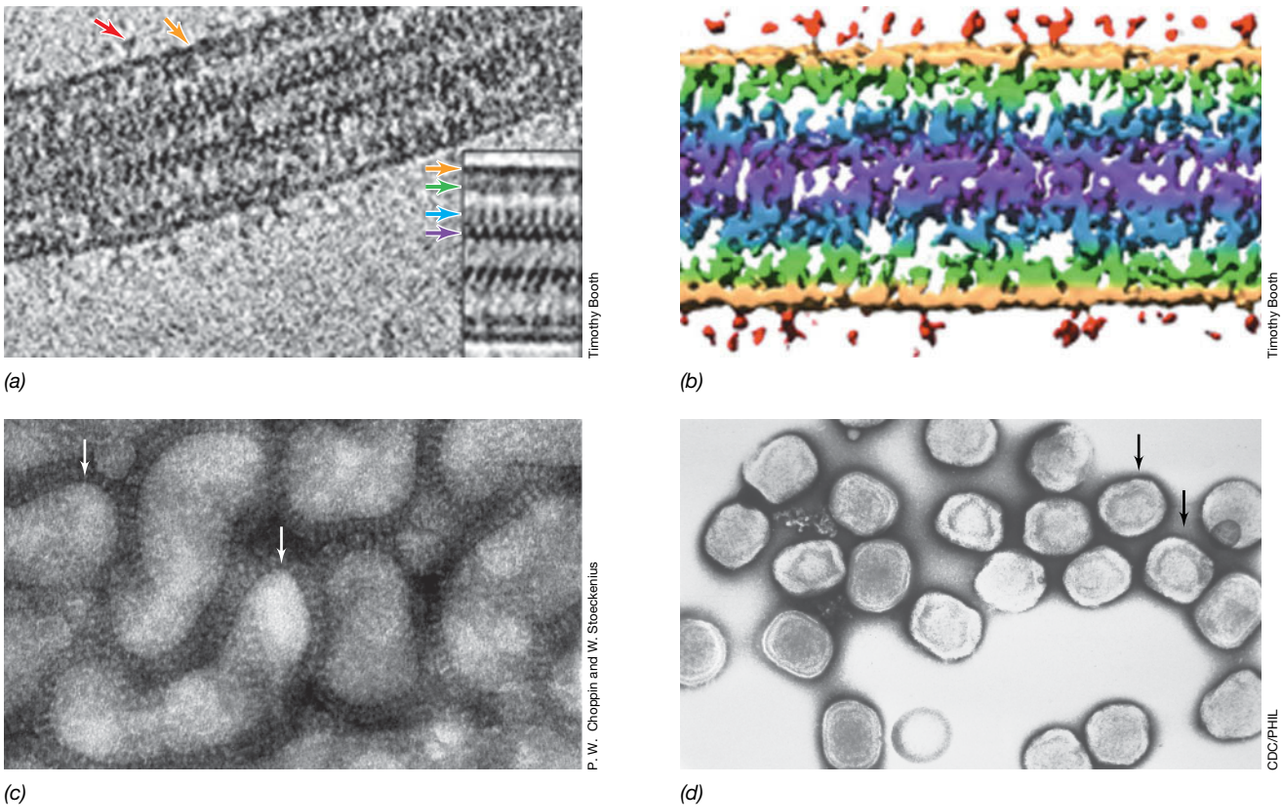


Figure 8.6 Enveloped viruses. *(a)* Cryo-electron micrograph of an Ebola virion. The virions are helical with a diameter of 80 nm. *(b)* Three-dimensional surface representation of an Ebola tomograph. Color-coding (also applies to arrows in part *a*) is as follows: red, spikes of envelope surface glycoproteins; orange, lipid envelope; green, membrane-associated proteins; blue/purple, nucleocapsid proteins. (*c*) Electron micrograph of influenza virus. The virions are about 80 nm in diameter, and can have many shapes. *(d)* Electron micrograph of vaccinia virus, an enveloped icosahedral pox virus about 350 nm wide. The arrows in both *c* and *d* point to the envelopes surrounding the nucleocapsids. Parts *a* and *b* modified from Beniac, D.R., Melito, P.L., deVarennes, S.L., Hiebert, S.L., Rabb, M.J., Lamboo, L.L., Jones, S.M., Booth, T.F. 2012. *PLoS ONE 7*(1): e29608.

DNA from an RNA template is another process cells cannot do, retroviral virions contain an RNA-dependent DNA polymerase called *reverse transcriptase* (Section 8.8). To sum up, although most viruses do not need to carry special enzymes in their virions, those that do absolutely require them for successful infection and replication.

MINIQUIZ

- Distinguish between a capsid and a capsomere. What is a common symmetry for spherical viruses?
- What is the difference between a naked virus and an enveloped virus?
- What kinds of enzymes can be found within the virions of RNA viruses? Why are they there?

8.3 Overview of the Virus Life Cycle

For a virus to replicate, it must induce a living host cell to synthesize all the essential components needed to make new virions. Because of these biosynthetic and energy requirements, dead host cells cannot replicate viruses. During an active viral infection, viral components are assembled into new virions that are released from the cell. Although replication steps are similar in most viruses, a major difference between viral infection of a *prokaryotic* cell and viral infection of a *eukaryotic* cell surrounds the initial step in infection. In cells of *Bacteria* and those *Archaea* in which the infection process has been studied, only the viral nucleic acid enters the host cell. By contrast, in plant and animal cells, the entire virion is taken up. Despite this key difference, the replication of bacterial viruses has been extremely well-studied and so we use them here as a general model of major viral replication events.

A cell that supports the complete replication cycle of a virus is said to be *permissive* for that virus. In a permissive host, the viral replication cycle can be divided into five steps (**Figure 8.7**):

1. *Attachment* (adsorption) of the virion to the host cell
2. *Penetration* (entry, injection) of the virion nucleic acid into the host cell
3. *Synthesis* of virus nucleic acid and protein by host cell machinery as redirected by the virus
4. *Assembly* of capsids and *packaging* of viral genomes into new virions
5. *Release* of new virions from the cell

The growth response during virus replication is illustrated in **Figure 8.8**. The response takes the form of a *one-step growth curve*, so named because a time course of virion numbers in the culture

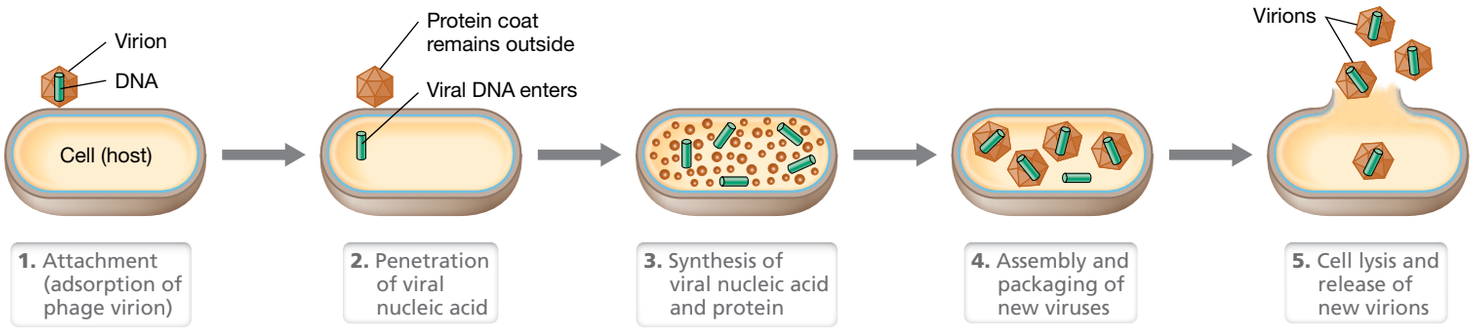


Figure 8.7 The replication cycle of a bacterial virus. The virions and cells are not drawn to scale. The burst size can be a hundred or more virions per host cell.

medium shows essentially no increase during the replication cycle until cells burst and release their newly synthesized virions. In the first few minutes after infection, the virus enters the *eclipse* phase, during which the viral genome and proteins will be replicated and translated, respectively. Once attached to a permissive host cell, a virion is no longer available to infect another cell. This is followed by the entry of viral nucleic acid into the host cell (Figure 8.7). If the infected cell breaks open at this point, the virion no longer exists as an infectious entity since the viral genome is no longer inside its capsid.

The *maturation* phase (Figure 8.8) begins as newly synthesized viral nucleic acid molecules become packaged inside their capsids. During the maturation phase, the number of infectious virions inside the host cell rises dramatically. However, the new virions still cannot be detected in the culture medium unless the cells are artificially lysed to release them. Because newly assembled virions are not yet present outside the cell, the eclipse and maturation periods together comprise the *latent period* of viral infection (Figure 8.8).

At the end of maturation, mature virions are released, either as a result of cell lysis or by budding or excretion, depending on the virus. The number of virions released per cell, called the *burst size*, varies with the particular virus and the particular host cell, and can range from a few to a few thousand. The duration of the virus replication cycle also varies, from 20–60 min (in many bacterial viruses) to 8–40 h (in most animal viruses).

In Sections 8.5 and 8.6 we use a specific example to revisit these stages of the virus replication cycle and examine each in more detail.

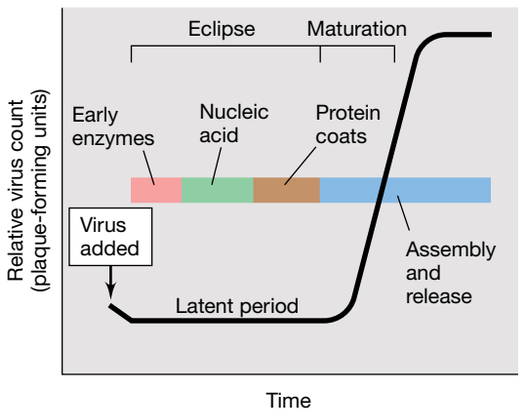


Figure 8.8 One-step growth curve of virus replication. Following adsorption, infectious virions cannot be detected in the growth medium, a phenomenon called *eclipse*. During the latent period, which includes the eclipse and early maturation phases, viral nucleic acid replicates and protein synthesis occurs. During the maturation period, virus nucleic acid and protein are assembled into mature virions and then released.

MINIQUIZ

- What is packaged into capsids during maturation?
- Explain the term burst size.
- Why is the latent period so named?

8.4 Culturing, Detecting, and Counting Viruses

Host cells need to be growing in order for viruses to replicate in them. Pure cultures of bacterial hosts are either inoculated in liquid medium or spread as "lawns" on the surface of agar plates and then inoculated with a virus suspension. Animal viruses are cultivated in *tissue cultures*, which are cells obtained from an animal organ and grown in sterile glass or plastic vessels containing an appropriate culture medium (see Figure 8.10). Tissue culture media are often highly complex, containing a wide assortment of nutrients including blood serum and other highly nutritious substances to feed the animal cells as well as antimicrobial agents to prevent bacterial contamination.

Detecting and Counting Viruses: The Plaque Assay

A viral suspension can be quantified to estimate the number of infectious virions present per volume of fluid, a quantity called the **titer**. This is typically done using a *plaque assay*. When a virus infects host cells growing on a flat surface, a zone of cell lysis called a **plaque** forms and appears as a clear area in the lawn of host cells (Figure 8.9).

With bacteriophages, plaques may be obtained when virions are mixed into a small volume of molten agar containing host bacteria that is spread on the surface of an agar medium (Figure 8.9*a*).

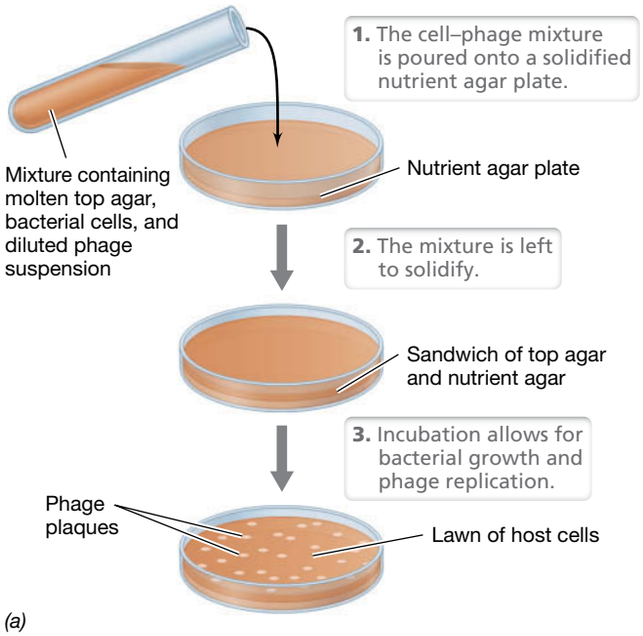


Figure 8.9 Quantification of bacterial virus by plaque assay. *(a)* "Top agar" containing a dilution of virions mixed with permissive host bacteria is poured over a plate of "bottom agar." Infected cells are lysed, forming plaques in the lawn. *(b)* Plaques (about 1–2 mm in diameter) formed by bacteriophage T4.

During incubation, the bacteria grow and form a turbid layer (lawn) that is visible to the naked eye. However, wherever a successful viral infection has occurred, cells are lysed, forming a plaque (Figure 8.9*b*). By counting the number of plaques, one can calculate the titer of the virus sample. The titer is typically expressed as the number of "plaque-forming units" per milliliter rather than an absolute viral number because of variations in plating efficiency, as described below. For replicating animal viruses, a tissue culture is grown and a diluted virus suspension overlaid upon it. As for bacterial viruses, plaques are revealed as cleared zones in the tissue culture cell layer, and from the number of plaques produced, an estimate of the virus titer can be made (**Figure 8.10**).

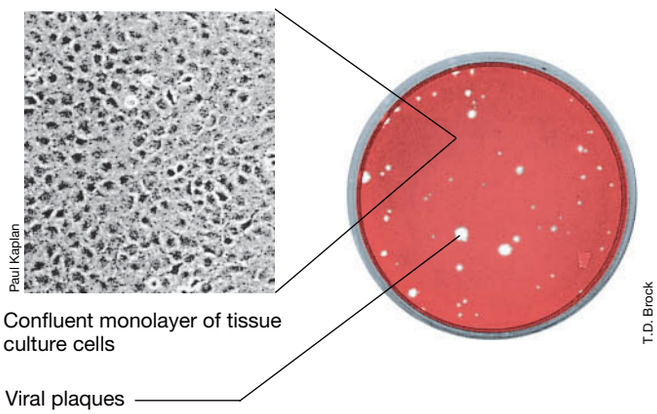


Figure 8.10 Animal cell cultures and viral plaques. The animal cells support replication of the virus, and lysed cells result in plaques.

Plating Efficiency in Estimates of Viral Titers

The concept of *plating efficiency* is important in quantitative virology for all virus types. In any given viral preparation, the number of plaque-forming units is always lower than actual counts of viral particles made microscopically (using an electron microscope). This is because the efficiency with which virions infect host cells is rarely 100% and may often be considerably less. Virions that fail to infect may have assembled incompletely during the maturation process, may contain defective genomes, or may have suffered a spontaneous mutation that prevents them from attaching or otherwise properly replicating. Alternatively, a low plating efficiency may mean that viral growth conditions were not optimal or that some virions were damaged by handling or storage conditions.

Although plating efficiencies of bacterial viruses can often be higher than 50%, with many animal viruses it may be much lower, 0.1% or 1%. Knowledge of plating efficiency is useful in cultivating viruses because it allows the investigator to estimate what a titer needs to be to yield a certain number of plaques. If the titer is extremely low, the viral suspension may need to be concentrated by centrifugation or filtration before being used to infect host cells. This is especially true of animal viruses, as the costs of growing and maintaining tissue cultures can be significant.

MINIQUIZ

- What is meant by a viral titer?
- What is a plaque-forming unit?
- What is meant by the term plating efficiency?

II • The Viral Replication Cycle

Much of our understanding of lytic virus replication comes from the study of bacteriophages that infect *Escherichia coli*. Many RNA as well as DNA bacteriophages replicate in *E. coli* (Table 8.1). Here we choose one, bacteriophage T4, as our model for reviewing the individual stages of the virus life cycle (Figure 8.7) in more detail.

8.5 Attachment and Entry of Bacteriophage T4

The early steps in the life cycle of any bacteriophage are attachment to the surface of its host cell followed by penetration of the host cell outer layer(s) and entry of the viral genome into the cell.

Attachment

A major factor in host specificity of a virus is *attachment*. The virion itself has one or more proteins on its external surface that interact with specific components called *receptors* on the host cell surface. In the absence of its specific receptor, the virus cannot attach to the cell and hence cannot infect. Moreover, if the receptor is altered, for example by mutation, the host may become resistant to virus infection. The host range of a given virus is thus to a major extent determined by the presence of a suitable receptor that the virus can recognize and attach to.

Viral receptors are surface components of the host, such as proteins, carbohydrates, glycoproteins, lipids, or lipoproteins, or cell structures made from these macromolecules (Figure 8.11). The receptors carry out normal functions for the cell; for example, the receptor for phage T1 is an iron-uptake protein (Figure 8.11) and that for bacteriophage lambda functions in maltose uptake. Carbohydrates in the lipopolysaccharide (LPS) outer membrane of gram-negative bacteria are the receptors recognized by bacteriophage T4, a phage that binds to the LPS of *Escherichia coli* (Figure 8.11). Appendages that project from the cell surface, such as flagella and pili, are also common receptors for bacterial viruses. Small icosahedral viruses often bind to the side of these structures, whereas filamentous bacteriophages typically bind at the tip, such as on the pilus (Figure 8.11). Regardless of the receptor used, however, once attachment has occurred, the stage is set for viral infection.

TABLE 8.1 Some bacteriophages of *Escherichia coli*

Bacteriophage	Virion structure	Genome composition[a]	Genome structure	Size of genome[b]
MS2	Icosahedral	ssRNA	Linear	3,600
ϕX174	Icosahedral	ssDNA	Circular	5,400
M13, f1, and fd	Filamentous	ssDNA	Circular	6,400
Lambda	Head & tail	dsDNA	Linear	48,500
T7 and T3	Head & tail	dsDNA	Linear	40,000
T4	Head & tail	dsDNA	Linear	169,000
Mu	Head & tail	dsDNA	Linear	39,000

[a] ss, single-stranded; ds, double-stranded.
[b] In bases (ss genomes) or base pairs (ds genomes). These viral genomes have been sequenced and thus their lengths are known precisely. However, the sequence and length often vary slightly among different isolates of the same virus. Hence, the genome sizes listed here have been rounded off in all cases.

Penetration

Attachment of a virus to its host cell causes changes to both the virus and the host cell surface that result in penetration. Bacteriophages abandon their capsid outside the cell and only the viral genome reaches the cytoplasm. However, entry of the viral genome into a host cell only results in virus replication if the viral genome can be read. Consequently, for the replication of some viruses, for example RNA viruses, specific viral proteins must also enter the host cell along with the viral genome (Section 8.2).

The most intricate viral penetration mechanisms exist with the tailed bacteriophages. Bacteriophage T4 consists of an icosahedral head, within which the viral linear double-stranded DNA is folded, and a long, complex tail, which ends in a series of tail fibers and tail pins that contact the cell surface. Phage T4 virions first attach to *Escherichia coli* cells using their tail fibers (Figure 8.12). The ends of the tail fibers interact specifically with polysaccharides in the cell's LPS layer and then the tail fibers retract, allowing the tail itself to contact the cell wall via the tail pins. The activity of T4 lysozyme then forms a small pore in the peptidoglycan layer and the tail sheath contracts. When this occurs, T4 DNA enters the cytoplasm of the *E. coli* cell through a tail tube in a fashion resembling that of injection by a syringe. By contrast, the T4 capsid remains outside the cell (Figure 8.12). DNA inside bacteriophage heads is under high pressure, and because the interior of a bacterial cell is also under pressure from osmotic forces, the phage DNA injection process takes several minutes to complete.

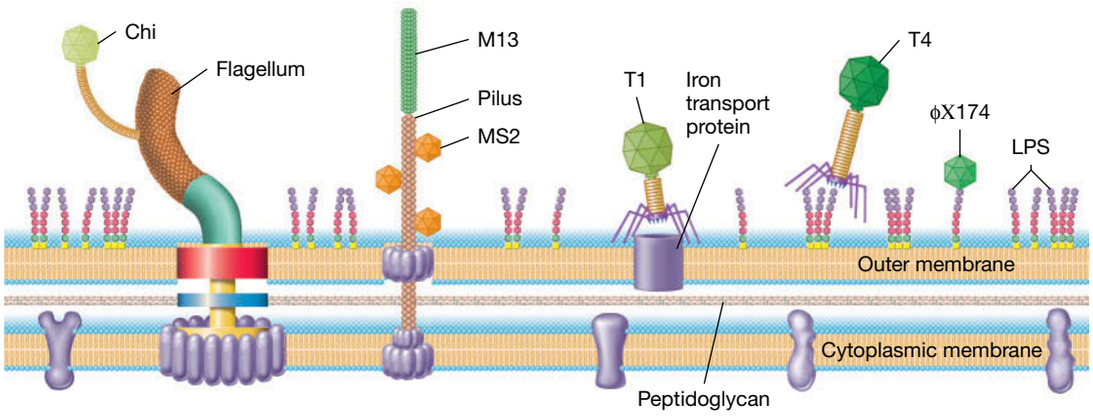


Figure 8.11 Bacteriophage receptors. Examples of the cell receptor sites used by different bacteriophages that infect *Escherichia coli*. All phages depicted except for MS2 are DNA phages.

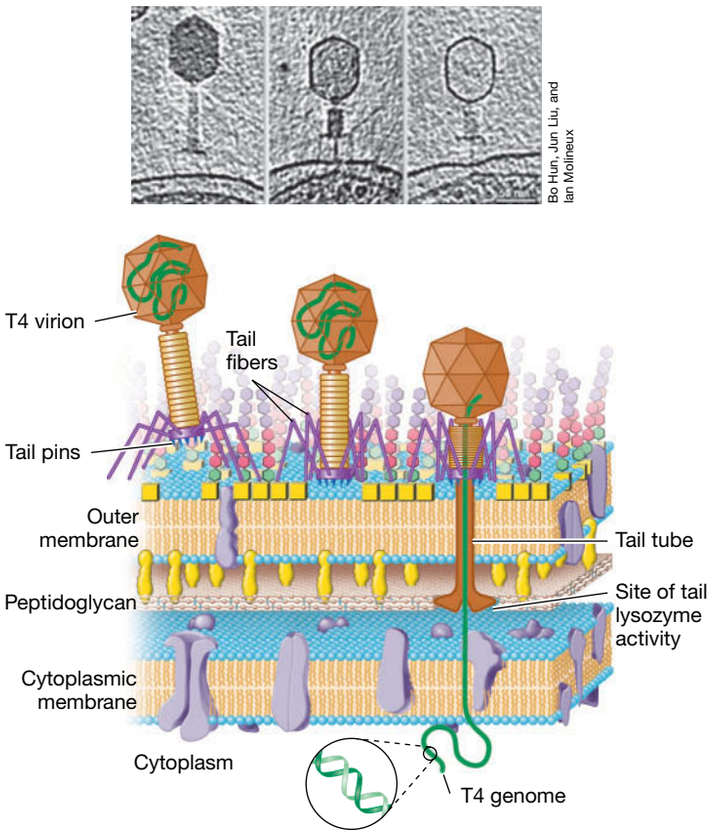


Figure 8.12 Attachment and infection of an *Escherichia coli* cell by bacteriophage T4. The three transmission electron tomographs and the art beneath each depict (left to right) the initial attachment of a T4 virion to the cell outer membrane by tail fiber interactions with lipopolysaccharide (LPS); contact of the cell wall by the tail pins; and contraction of the tail sheath and injection of the T4 genome. The tail tube penetrates the outer membrane, and T4 lysozyme digests a small opening through the *E. coli* cell peptidoglycan layer.

Once a bacteriophage injects its genome into a host cell, a productive infection is not absolutely ensured. Below we consider some mechanisms employed by prokaryotic cells to protect against viral attack and avenues that subsequently evolved in bacteriophage to evade these processes.

Restriction and Modification

Although they lack the immune systems of animals (Chapters 26 and 27), *Bacteria* and *Archaea* possess several weapons against viral attack. Toxin–antitoxin modules (Section 7.11) and an antiviral system called CRISPR (Section 10.13) are two of these mechanisms. Additionally, *Bacteria* and *Archaea* can destroy double-stranded viral DNA through the activity of *restriction endonucleases,* enzymes that cleave foreign DNA at specific sites (Section 12.2). This process is called *restriction* and is a general host mechanism to prevent invasion by viral (or any other foreign) DNA. For such a system to be effective, however, the host must protect its own DNA from restriction enzyme attack. The host accomplishes this by *modification* of its DNA, typically by methylation of nucleotides at the sites where the restriction enzymes cut.

Restriction enzymes are specific for double-stranded DNA, and thus single-stranded DNA viruses and all RNA viruses are unaffected by restriction enzymes. Although host restriction systems confer significant protection from viral attack, some double-stranded DNA viruses have overcome host restriction by modifying their own DNA so it is no longer subject to restriction enzyme attack (**Figure 8.13*a***). Many protective mechanisms are known, but in the *E. coli* bacteriophage T4 this is accomplished by substituting the base *5-hydroxymethylcytosine* in place of cytosine in viral DNA. The hydroxyl group of this modified base is glucosylated, meaning that a molecule of glucose is added (Figure 8.13*a*); DNA with this modification is resistant to cleavage by all known *E. coli* restriction enzymes. By virtue of this viral protection mechanism, copies of the T4 genome are preserved until they are packaged later in the phage replication cycle and released by cell lysis (see next section) to attack uninfected cells of *E. coli*.

MINIQUIZ

- How does attachment contribute to virus–host specificity?
- Why does phage T4 need a lysozyme-like protein in order to infect its host, and what part of T4 enters the host cytoplasm?
- How does *Escherichia coli* try to protect itself from phage attack, and how does T4 protect itself from these weapons?

8.6 Replication of Bacteriophage T4

Drawing on what we already know about T4 phage attachment and penetration from the previous section, we now consider some unusual properties of the T4 genome and examine the steps in its replication cycle.

Genome Replication and Circular Permutation

Once a virus has infected a permissive host cell, the earliest events surround the synthesis of new copies of the viral genome. Because there are many types of viral genomes (Figure 8.2), there are many different schemes for virus genome replication (Section 10.1). In small DNA viruses, the cell's DNA polymerase is needed to replicate the viral genome. However, in more complex DNA viruses such as bacteriophage T4, the virus encodes its own DNA polymerase. Other proteins that function in viral DNA replication such as primases and helicases (Section 4.3) are also encoded by the T4 genome. In fact, T4 produces its own eight-protein DNA replisome complex (Section 4.4) to facilitate phage-specific genome synthesis.

Besides encoding its own replication machinery, the T4 genome has another unusual feature: In a population of T4 virions, although each copy of the genome contains the same set of genes, they are arranged in a different order. This is a phenomenon called *circular permutation*, which is a feature of many virus genomes. The term circular permutation is derived from the fact that DNA molecules that are circularly permuted appear to have been linearized by opening identical circular genomes at different locations. Circularly permuted genomes are also *terminally redundant*, meaning that some DNA sequences are duplicated on both ends of the DNA molecule as a result of the mechanism that generated them.

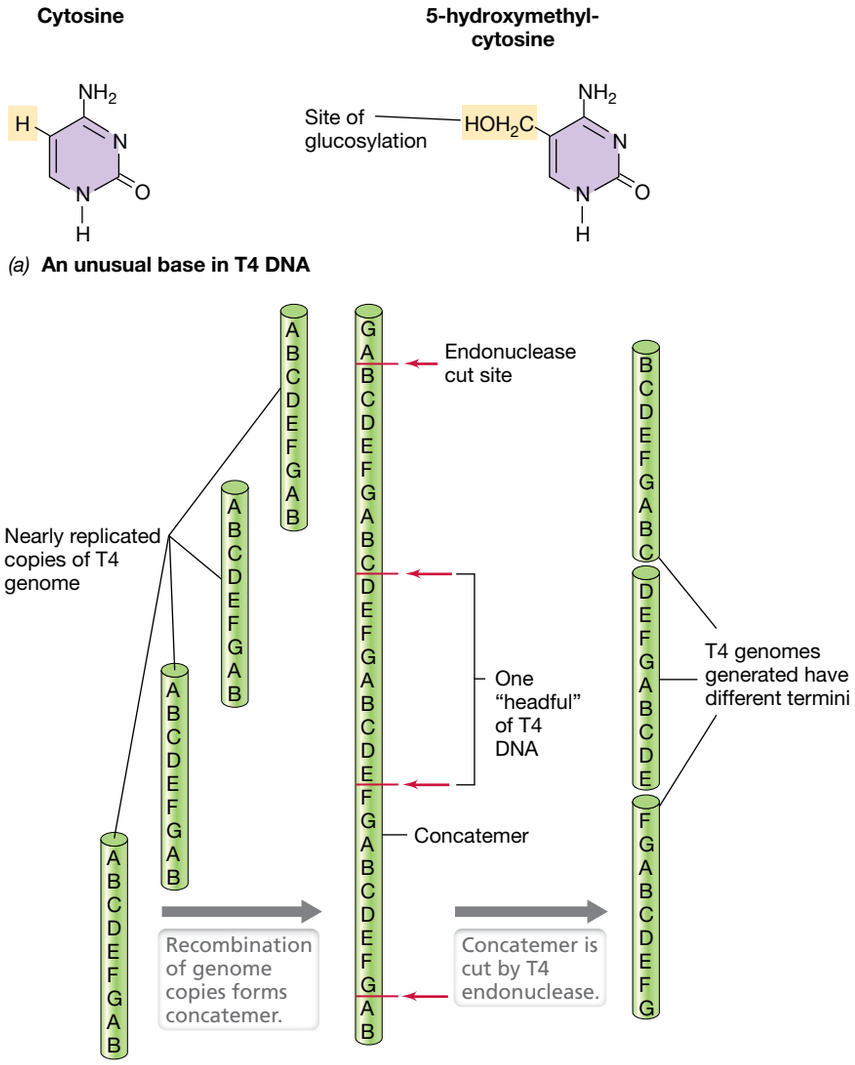


Figure 8.13 Circular permutation and the unique DNA of bacteriophage T4. *(a)* The unique base 5-hydroxymethylcytosine in the DNA of bacteriophage T4. Once this base is glucosylated, the T4 DNA is resistant to restriction enzyme attack. *(b)* Generation of virus-length T4 DNA molecules with permuted sequences by an endonuclease that cuts off constant lengths of DNA from a concatemer regardless of their sequence.

The T4 genome is first replicated as a unit and then several genomic units are recombined end to end to form a long DNA molecule called a **concatemer** (Figure 8.13*b*). When the T4 DNA is packaged into capsids, the concatemer is not cut at a specific sequence; instead linear segments of DNA just long enough to fill a phage head are generated. This is called *headful packaging*, and is common among bacteriophages. However, because the T4 head holds slightly more than a genome length, the headful mechanism generates terminal repeats of about 3–6 kbp at each end of the DNA molecule (Figure 8.13*b*).

Transcription and Translation

Shortly after infection, T4 DNA is transcribed and translated, and the process of new virion synthesis begins. In less than half an hour, the process culminates in the release of new virions from the lysed cell. The major events are summarized in Figure 8.14.

Within a minute after T4 DNA enters the host cytoplasm, the synthesis of host DNA and RNA ceases and transcription of specific phage genes begins, thus inhibiting normal host growth. Translation of viral mRNA also begins quickly, and within 4 min of infection, phage DNA replication has already begun. The T4 genome encodes three major sets of proteins called **early proteins**, **middle proteins**, and **late proteins**, the terms referring to the general order of their appearance in the cell. Early proteins include enzymes for the synthesis and glucosylation of the unusual T4 base 5-hydroxymethylcytosine (Figure 8.13*a*), enzymes that function in the T4 replisome to produce copies of the phage-specific genome, and early proteins that modify host RNA polymerase. By contrast, middle and late proteins include additional RNA polymerase–modifying proteins, and virion structural and release proteins. These include, in particular, viral head and tail proteins and the enzymes required to liberate new virions from the cell (Figure 8.14).

The T4 genome does not encode its own RNA polymerase; instead, T4-specific proteins modify the specificity of the host RNA polymerase so that it recognizes only phage promoters (recall that promoters are the regions upstream of a structural gene where RNA polymerase binds to initiate transcription, ↬ Section 4.5). These modification proteins are encoded by T4 early genes and are transcribed by the host RNA polymerase. Host transcription is shut down shortly after this by a phage-encoded antisigma factor (↬ Section 6.15) that binds to the host RNA polymerase sigma factor and prevents it from recognizing promoters on host genes. This effectively switches the activity of host RNA polymerase from transcribing host genes to transcribing T4 genes. Later in the infection process, other phage proteins modify the host RNA polymerase so it now recognizes T4 middle gene promoters. Finally, transcription of T4 late genes begins, and this requires a new T4-encoded sigma factor that directs host RNA polymerase to promoters for these genes only. At this point, viral assembly can begin.

Packaging the T4 Genome and Virion Assembly and Release

The bacteriophage T4 DNA genome is forcibly pumped into a preassembled capsid using an energy-linked packaging motor. The motor components are encoded by viral genes, but host cell metabolism is needed to produce the proteins and supply the ATP required for the pumping process. The packaging process can be divided into three stages (Figure 8.15*a*). First, precursors of the bacteriophage head called *proheads* are assembled but remain empty. Proheads contain temporary "scaffolding proteins" as well as head structural proteins. Second, a packaging motor

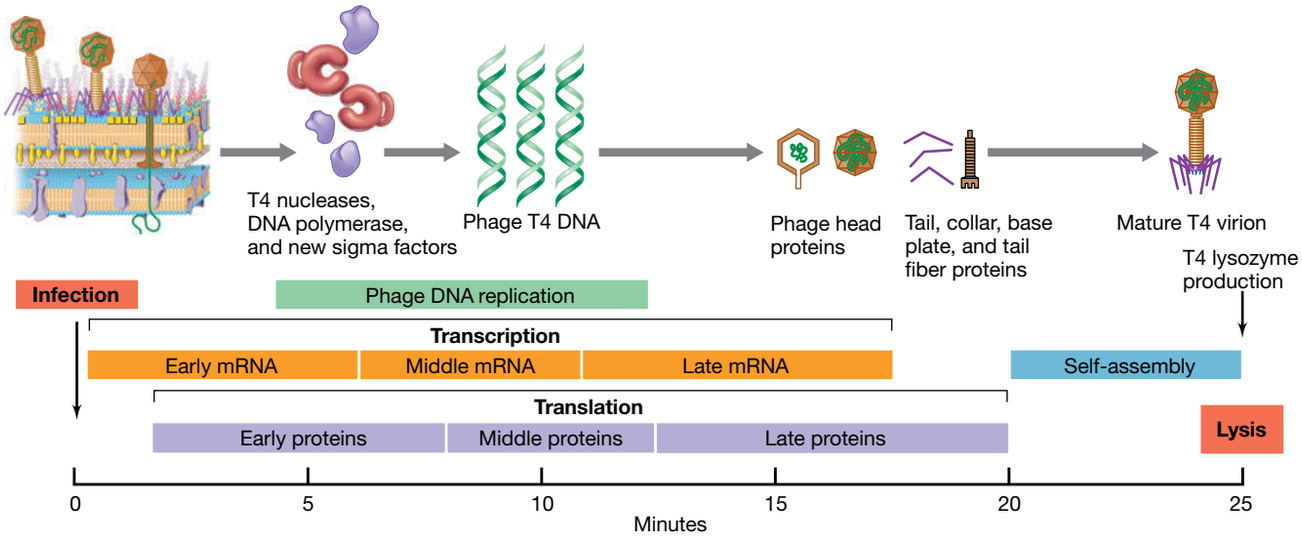
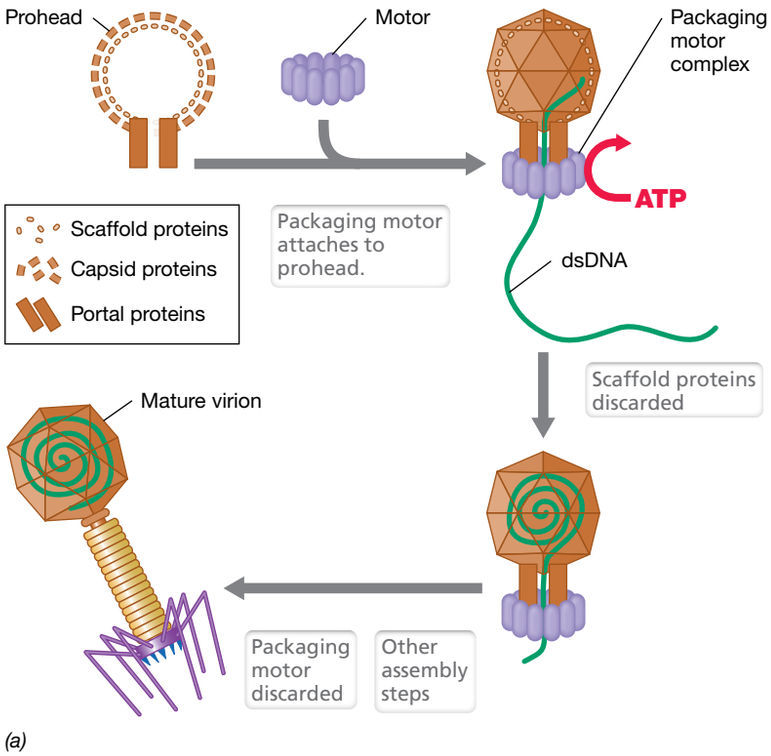


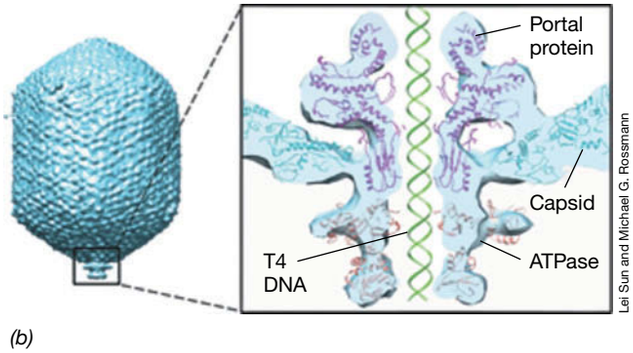
Figure 8.14 Time course of events in phage T4 infection. Following injection of DNA, early and middle mRNAs are produced that encode nucleases, T4 DNA polymerase, new phage-specific sigma factors, and other proteins needed for DNA replication. Late mRNAs encode virion structural proteins and T4 lysozyme, which are needed to lyse the cell and release new virions.

is assembled at the opening to the prohead (Figure 8.15*b*). The double-stranded linear T4 DNA genome (Figure 8.13*b*) is then pumped into the prohead under pressure using ATP as the driving force. The prohead expands when pressurized by the entering DNA and the scaffolding proteins are simultaneously discarded. Third, the packaging motor itself is discarded and the capsid head is sealed.

After the head has been filled, the T4 tail, tail fibers, and the other components of the virion are added, primarily by spontaneous reactions (self-assembly, Figures 8.14 and 8.15). The phage genome encodes a pair of very late enzymes that combine to breach the two major barriers to virion release: the host cytoplasmic membrane and peptidoglycan layer. Once these structures are compromised, the cell breaks open by osmotic lysis and the newly



(a)



(b)

Lei Sun and Michael G. Rossmann

Figure 8.15 Packaging of DNA into a T4 phage head. *(a)* Proheads are assembled from capsid and portal proteins, both of which remain in the mature virion. As the head fills with DNA (driven by ATP hydrolysis), it expands and becomes more angular. Once the head is filled, the packaging motor detaches and the tail components are added. *(b)* Cryo-electron micrograph reconstruction of the T4 prohead and packaging motor. The blowup shows proteins within the motor and the prohead and their contact with DNA.

synthesized virions are released. After each replication cycle, which takes only about 25 min (Figure 8.14), over 100 new virions are released from each host cell (the *burst size*, Section 8.3), and these are now free to infect neighboring host cells.

MINIQUIZ

- What is a concatemer?
- Give one example each of T4 early, middle, and late proteins.
- What is required to package the T4 genome into its phage head?

8.7 Temperate Bacteriophages and Lysogeny

Bacteriophage T4 is a virulent virus and once infection begins, it always kills its host. However, some double-stranded DNA bacterial viruses, although capable of a virulent cycle, can also infect their host and establish a long-term stable relationship. These viruses are called **temperate viruses**.

Temperate viruses can enter into a state called **lysogeny**. In this state, most virus genes are not transcribed and instead, the virus genome is replicated in synchrony with the host chromosome and passed to daughter cells at cell division. A cell that harbors a temperate virus is therefore called a **lysogen**. While lysogen growth is controlled by its local environment and nutritional profile, the lysogenic state may confer new genetic properties—a condition called *lysogenic conversion*. We will see several examples in later chapters of pathogenic bacteria whose virulence (ability to cause disease) is at least in part linked to a lysogenic bacteriophage.

The Replication Cycle of a Temperate Phage

Two well-characterized temperate bacteriophages are lambda and P1. The life cycle of a temperate bacteriophage is shown in Figure 8.16. During lysogeny, the temperate virus genome is either integrated into the bacterial chromosome (lambda) or can exist in the cytoplasm as a plasmid (P1). In either case, the viral DNA, now called a **prophage**, replicates along with the host cell as long as the genes that activate the phage virulent pathway are repressed.

Maintenance of the lysogenic state is due to a phage-encoded *repressor protein*. Normally, low-level transcription of repressor genes and their subsequent translation maintains the repressor at a low level in the cell. However, if the phage repressor is inactivated or if its synthesis is in some way prevented, the prophage can be induced into the lytic stage. If induction occurs while the viral DNA is incorporated into the bacterial chromosome, the viral DNA is excised and phage genes are transcribed and translated; new virions are then produced, and the host cell is lysed (Figure 8.16). Various cell stress conditions, especially damage to host cell DNA, can induce a prophage to enter the lytic pathway. In contrast to this process, the viral "decision" to proceed to lysogeny or the lytic pathway upon initial viral infection is another matter altogether, and has been particularly well studied in bacteriophage lambda. We explore this story now.

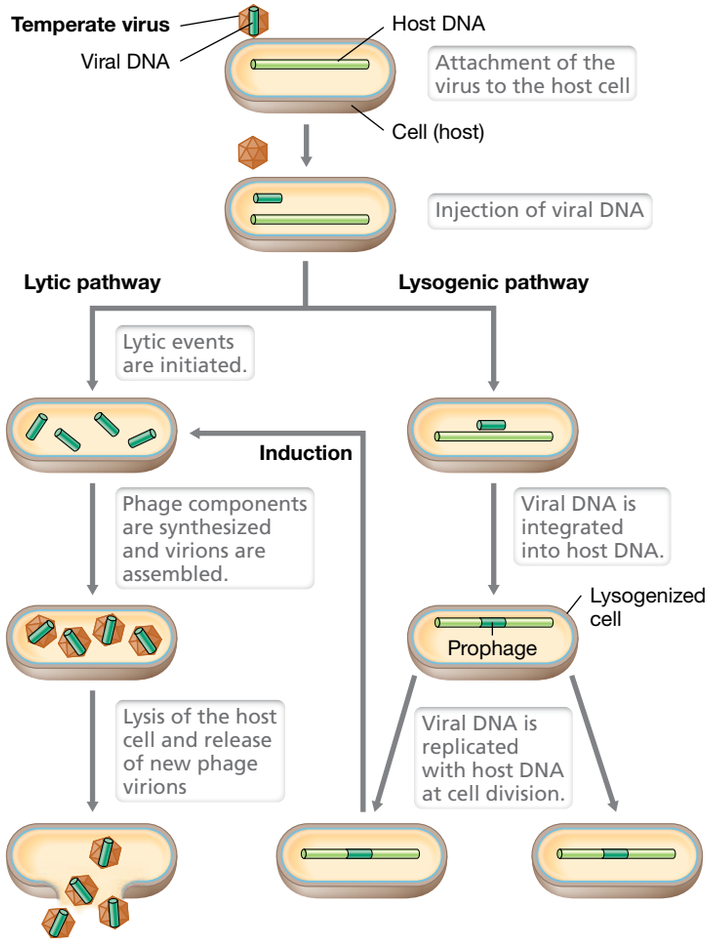
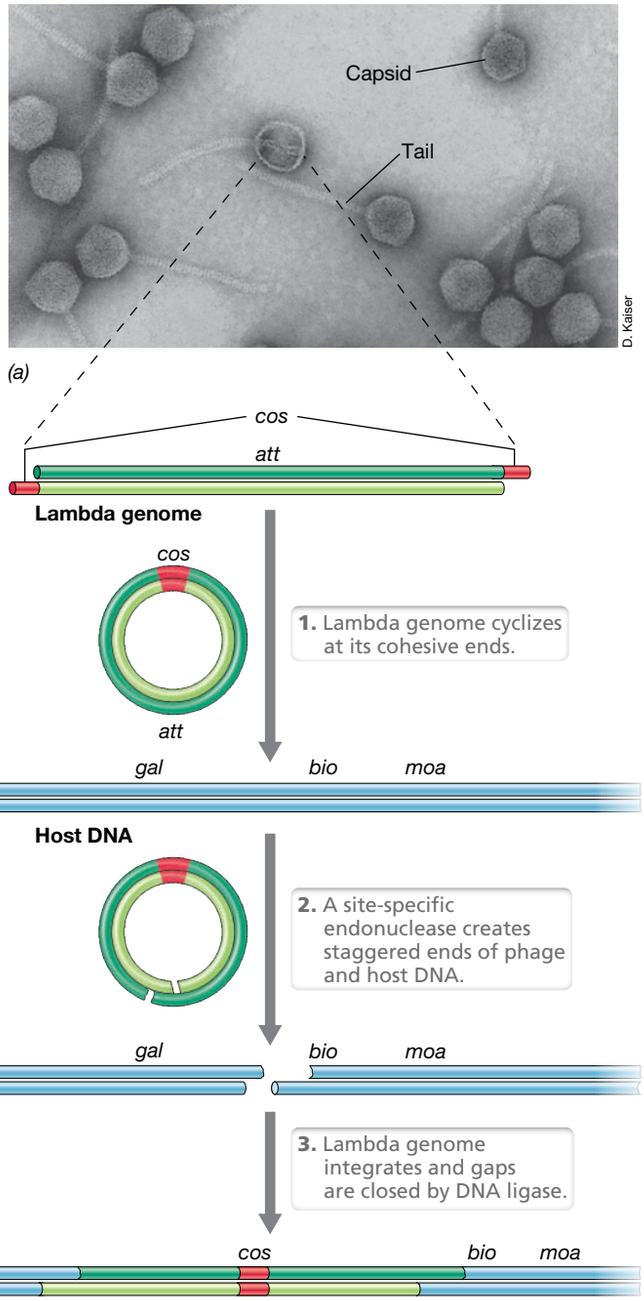


Figure 8.16 Consequences of infection by a temperate bacteriophage. The alternatives upon infection are replication and release of mature virions (lysis) or lysogeny, often by integration of the virus DNA into the host DNA, as shown here. The lysogen can be induced to produce mature virions and lyse.

Bacteriophage Lambda

Bacteriophage lambda, which infects *Escherichia coli*, is a double-stranded DNA virus with a head and tail (Figure 8.17*a*). At the 5′ end of each DNA strand of the linear lambda genome is a single-stranded region 12 nucleotides long. These single-stranded "cohesive" ends are complementary in base sequence; when lambda DNA enters the host cell, they base-pair to form the *cos* site and cyclize (circularize) the genome (Figure 8.17*b*).

If lambda enters the lytic pathway, long, linear concatemers of genomic DNA are synthesized by a mechanism called **rolling circle replication**. In this process, one strand in the circular lambda genome is nicked and is "rolled out" as a template for synthesis of the complementary strand (Figure 8.17*c*). The double-stranded concatemer is then cut into genome-sized lengths at the *cos* sites and the resulting genomes packaged into lambda phage heads. Once the tail has been added and mature lambda virions have been assembled (Figure 8.17*a*), cell lysis occurs and the virions are released. In its role as a lytic phage, lambda can also package a few chromosomal genes from its lysed host in newly synthesized virions and then transfer these to a second host cell,



(b) **Integration of lambda DNA into the host**

(c) **Rolling circle replication of lambda genome**

Figure 8.17 Bacteriophage lambda: virions, integration of viral DNA and rolling circle replication. *(a)* Transmission electron micrograph of phage lambda virions. The head of each virion is about 65 nm in diameter and contains linear dsDNA. *(b)* Lambda DNA integrates at specific attachment (*att*) sites on both the host and phage genomes. Host genes near *att* include *gal*, galactose utilization; *bio*, biotin synthesis; and *moa*, molybdenum cofactor synthesis. Lambda integrase is required, and specific pairing of the complementary ends results in integration of lambda DNA. *(c)* During rolling circle replication, as one strand (dark green) rolls out, it is both replicated at its opposite end and serves as a template for synthesis of the complementary strand.

a process called *transduction*. Transduction is an important means of horizontal gene transfer in nature and is also an important tool in bacterial genetics (Section 11.7).

Instead of the lytic pathway, if lambda takes the lysogenic route, its genome integrates into the *E. coli* chromosome. This requires a protein called *lambda integrase*, a phage-encoded enzyme that recognizes the phage and bacterial genome attachment sites (*att* in Figure 8.17*b*) and facilitates integration of the lambda genome. From this relatively stable state, certain events such as host DNA damage can initiate the lytic cycle once again. After such a trigger, a lambda excision protein excises the lambda genome from the host chromosome, transcription of lambda DNA begins, and lytic events unfold.

We now consider how these opposing processes of lysis and lysogeny are controlled upon initial infection of an *E. coli* cell by a phage lambda virion.

Lysis or Lysogeny: Regulation of the Lambda Lifestyle

Whether lysis or lysogeny occurs in a lambda infection depends in large part on the levels of two key repressor proteins that can accumulate in the cell following infection: the *lambda repressor*, also called the *cI protein*, and a second repressor called *Cro*. In a nutshell, the first repressor to accumulate will control the outcome of the infection.

If genes encoding the cI protein are rapidly transcribed following infection and cI accumulates, it represses the transcription of all other lambda-encoded genes, including Cro. When this happens, the lambda genome integrates into the host's genome and becomes a prophage (**Figure 8.18**). The host continues to grow, yielding more lysogens, until an event that triggers lysis is encountered. Cro, on the other hand, represses expression of a protein called cII whose function is to activate the synthesis of cI. Hence, following infection, if cI is present at insufficient levels to repress expression of phage-specific genes, Cro can accumulate in the cell; if this happens, lambda travels the lytic pathway.

Control of these alternative lifestyles—lysis or lysogeny—of lambda has been likened to a "genetic switch," where a defined series of events must occur for one pathway to be favored over the other. Although infection of an *E. coli* cell by a lambda virion typically results in the lytic cycle, as we have said, lytic events can be switched off if sufficient concentrations of cII are present to ensure adequate levels of cI (Figure 8.18). But how does this come about? Levels of protein cII are controlled by the relative activity of a protease in the cell that slowly degrades cII and by levels of yet another protein, cIII, whose function is to stabilize cII and protect it from protease attack. We thus have a cascade of regulatory

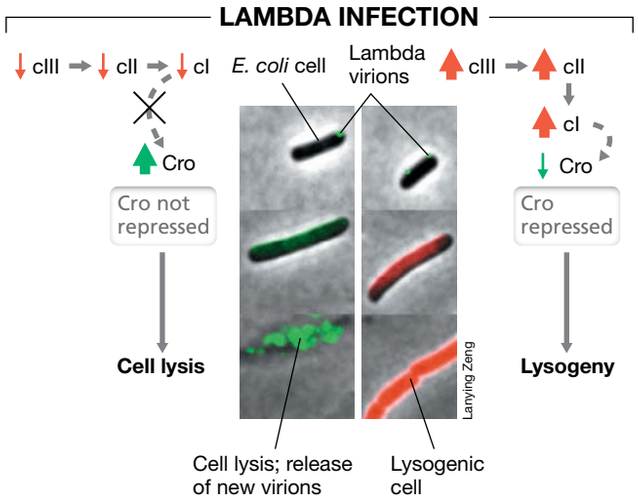


Figure 8.18 Regulation of lytic and lysogenic events in phage lambda. The photomicrographs show time courses of cells of *Escherichia coli* following a course of lytic (left panel, green) or lysogenic (right panel, red) events, as controlled by various repressors. The colors originate from genetically engineered lambda phage that trigger the production of specific fluorescent proteins when either lytic genes (green) or lysogenic genes (red) are expressed. Lytic cells are killed whereas *E. coli* lysogens continue to grow and divide.

TABLE 8.2 Representative viral diseases of humans

Disease	*Virus*	*Genome DNA or RNA*[a]	*Size*[b]
Cold sores/genital herpes	Herpes simplex	dsDNA	152,000
Smallpox	Variola major	dsDNA	190,000
Polio	Poliovirus	ssRNA (+)	7,500
Rabies	Rabies virus	ssRNA (–)	12,000
Influenza	Influenza A virus	ssRNA (–)	13,600
Measles	Measles virus	ssRNA (–)	15,900
Ebola hemorrhagic fever	Ebola virus	ssRNA (–)	19,000
Severe acute respiratory syndrome (SARS)	SARS virus	ssRNA (+)	29,800
Infant diarrhea	Rotavirus	dsRNA	18,600
Acquired immunodeficiency syndrome (AIDS)	Human immunodeficiency virus (HIV)	ssRNA/dsDNA (a retrovirus) (+)	9,700

[a] ss, single-stranded; ds, double-stranded. +, plus-strand virus; –, negative-strand virus (Section 8.1).
[b] In bases (ss genomes) or base pairs (ds genomes). These viral genomes have been sequenced and thus their lengths are known precisely. However, the sequence and length often vary slightly among different isolates of the same virus. Hence, the genome sizes listed here have been rounded off in all cases.

events here: cIII controls cII, which in turn controls cI. But even this is not the end of the story. Several other proteins not described here also play a role in the lambda lytic/lysogenic "decision," and hence the progress of a lambda infection is a highly intricate series of events.

Indeed, this tiny bacteriophage employs some of the most elaborate regulatory systems known in virology. One of the few lambda proteins expressed during lysogeny prevents the growing lysogen from entering dormancy during stressful conditions (↔ Section 7.11). This regulatory mechanism helps ensure the continued spread of the lambda virus in a mixed population of *Escherichia coli* lysogens and nonlysogens.

MINIQUIZ

- What is a lysogen and what is a prophage?
- How does DNA replication in lambda differ from that of its host?
- What commits lambda to the lytic versus the lysogenic pathway?

8.8 An Overview of Animal Virus Infection

The major tenets of virology—presence of a capsid to carry the viral DNA or RNA genome, infection and takeover of host metabolic processes, and assembly and release from the cell—are universal, regardless of the nature of the host. Like bacterial viruses, animal viruses are classified by their genomes, with the majority of important human viral diseases caused by RNA viruses (Table 8.2). However, two key differences between bacterial and animal viruses are that (1) the entire virion of animal viruses (rather than just the nucleic acid) enters the host cell, and (2) eukaryotic cells contain a nucleus, where many animal viruses replicate. We explore some aspects of animal viruses here.

Viral Infection of Animal Cells

While a large number of animal viruses exist (↔ Section 10.14), most animal viruses that have been studied in detail are those that can replicate in cell cultures (Section 8.4 and Figure 8.10). To initiate infection, animal viruses also bind to specific host cell receptors. These virus receptors are typically animal cell surface proteins used in cell–cell contact or that function in the immune system. For example, the receptors for poliovirus and for HIV (the causative agent of AIDS) are normally used in intercellular communication between human cells. In multicellular organisms, cells in different tissues or organs often express different proteins on their cell surfaces. Consequently, viruses that infect animals often infect only certain tissues. For example, viruses that cause the common cold infect only cells of the upper respiratory tract.

Once an animal virus is bound to a receptor, its entry into a host cell generally occurs by fusion with the cytoplasmic membrane or by endocytosis (Figure 8.19). After entry into the cell, animal viruses must eventually lose their outer coat to deliver their genetic cargo to the cytosol. Some enveloped animal viruses are uncoated at the host cytoplasmic membrane, releasing the nucleocapsid into the cytoplasm, while naked and enveloped animal viruses that enter via endocytosis are uncoated in the host cytoplasm. If the viral genome is DNA, the genome passes through the nuclear membrane to the nucleus for replication. Conversely, the genomes of most RNA-based viruses are replicated or converted to DNA by viral enzymes within the nucleocapsid. We will focus shortly on the unique mode of replication of retroviruses, highly unusual RNA animal viruses with significant medical implications (Table 8.2).

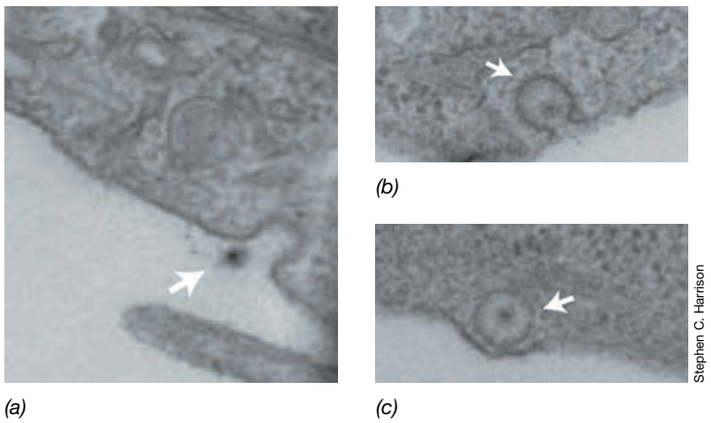


Figure 8.19 Rotavirus cell entry. Electron micrographs of rotavirus virions illustrating the stages of cell entry. The arrows point to the virion. *(a)* Virion bound to the cell surface. *(b)* Engulfment of virion by the cell membrane. *(c)* Compartmentalization of the virion. Modified from Abdelhakim, A.H., Salgado, E.N., Fu, X., Pasham, M., Nicastro, D., Kirchhausen, T., and Harrison, S.C. 2014. *PLoS Pathogens 10:* e1004355.

Virion Assembly and Infection Outcomes

Virion assembly and morphogenesis occurs once viral mRNAs corresponding to the nucleocapsid have been translated by the host's machinery. After viral genome copies are packaged within their outer coat, many animal viruses must be enveloped (see Figure 8.22). This typically occurs when the virion exits the animal cell through budding or cell lysis. During this process the virus may pick up part of the cell's cytoplasmic membrane and use it as part of the viral envelope.

Unlike a bacteriophage infection, in which one of only *two* outcomes—lysis or lysogeny—is possible depending on the virus, other events are possible in an animal virus infection. If an animal virus initially evades the immune system, animal viruses can catalyze at least four different outcomes (Figure 8.20). A *virulent infection* results in lysis of the host cell; this is the most common outcome. By contrast, in a *latent infection*, the viral DNA exists in the host's genome and virions are not produced; this leaves the host cell unharmed unless and until an event triggers the virulent pathway. With some enveloped animal viruses, release of virions, which occurs by a kind of budding process, may be slow, and the host cell may not be lysed (and thus not killed); instead it continues to grow and produce more virions. Such infections are called *persistent infections*. Finally, certain animal viruses can convert a normal cell into a tumor cell, a process called *transformation* (Figure 8.20).

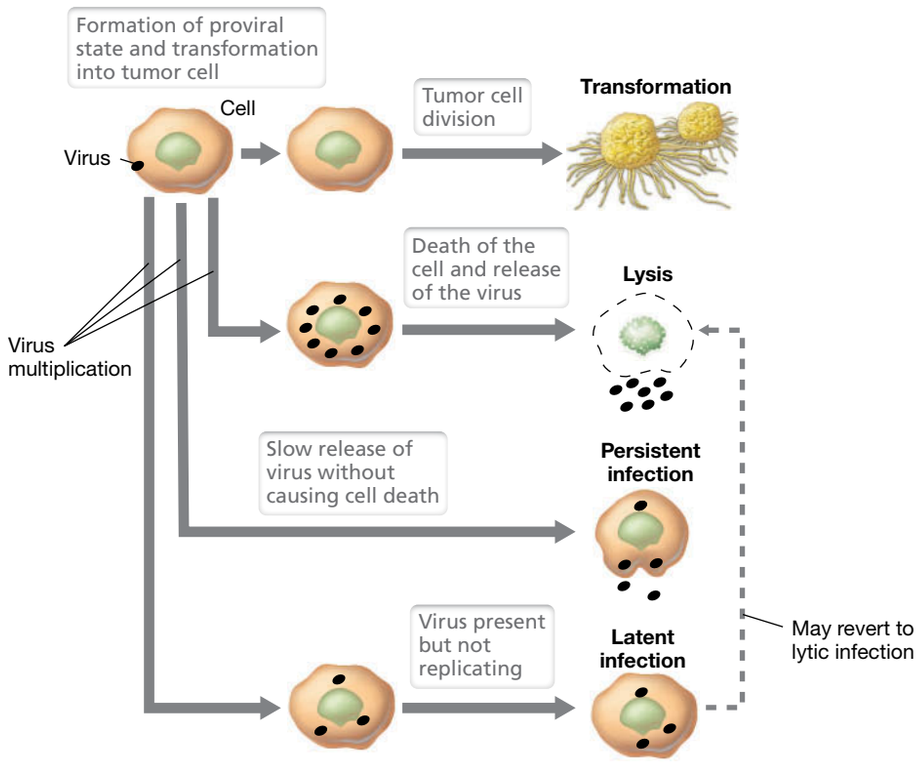


Figure 8.20 Possible effects that animal viruses may have on cells they infect. Most animal viruses are lytic, and only a very few are known to cause cells to transform and become cancerous.

Retroviruses and Reverse Transcriptase

Retroviruses are structurally complex animal viruses that contain an RNA genome. However, unlike other RNA viruses, the genome is replicated inside the host cell by way of a *DNA* intermediate. The prefix *retro* means "backward," and the term *retrovirus* refers to the fact that these viruses transfer information from RNA to DNA (in contrast to genetic information flow in cells, which occurs from DNA to RNA). Retroviruses use the enzyme **reverse transcriptase** to carry out this unusual process. Retroviruses were the first viruses shown to cause cancer, and the human immunodeficiency virus (HIV) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS).

Retroviruses are enveloped viruses and carry several enzymes within the virion (Figure 8.21*a*). These include *reverse transcriptase*, *integrase*, and a retroviral-specific *protease*. The genome of the retrovirus is unique and consists of two identical single-stranded RNAs of the plus sense (Section 8.1). The genome contains the genes *gag* (structural proteins), *pol* (reverse transcriptase and integrase), and *env* (envelope proteins) (Figure 8.21*b*). At each end of the retrovirus genome are repeated sequences that are essential for viral replication.

The replication of a retrovirus begins with the virion entering the host cell where the envelope is removed and reverse transcription begins in the nucleocapsid (Figure 8.22). A single strand of DNA is produced and then reverse transcriptase uses this as a template to make a complementary strand; double-stranded DNA is the final product. The dsDNA is released from the nucleocapsid and enters the host nucleus along with the integrase protein, and the integrase facilitates the incorporation of the retroviral DNA into the host genome.

UNIT 2

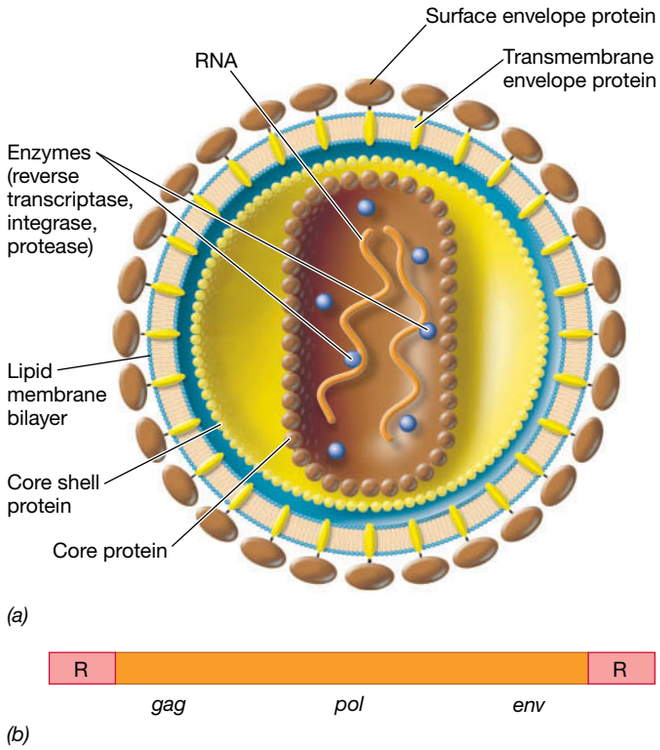


Figure 8.21 Retrovirus structure and function. *(a)* Structure of a retrovirus. *(b)* Genetic map of a typical retrovirus genome. Each end of the genomic RNA contains direct repeats (R).

The retroviral DNA is now a **provirus**. The provirus remains in the host genome indefinitely, and proviral DNA can be transcribed by the host RNA polymerase to form copies of the retroviral RNA genome and mRNA. Eventually, nucleocapsids are assembled that contain two copies of the retroviral RNA genome and are enveloped as they bud through the host cell cytoplasmic membrane (Figure 8.22). From here, the mature retrovirus virions are free to infect neighboring cells.

As exemplified by the retroviruses and some of the other viruses we have considered in this chapter, viruses are truly fascinating microbes. On the one hand, the growth (replication) of any virus is completely dependent on the growth and activities of its host cell; on the other hand, viruses can control the growth, survival, and genetic properties of their hosts. We revisit the viruses in Chapter 10 with a focus on viral genomics as the criterion by which we reveal the enormous genetic diversity of these important microbes. Although our discussion of viruses in this chapter has covered some of their remarkable characteristics, we have only scratched the surface.

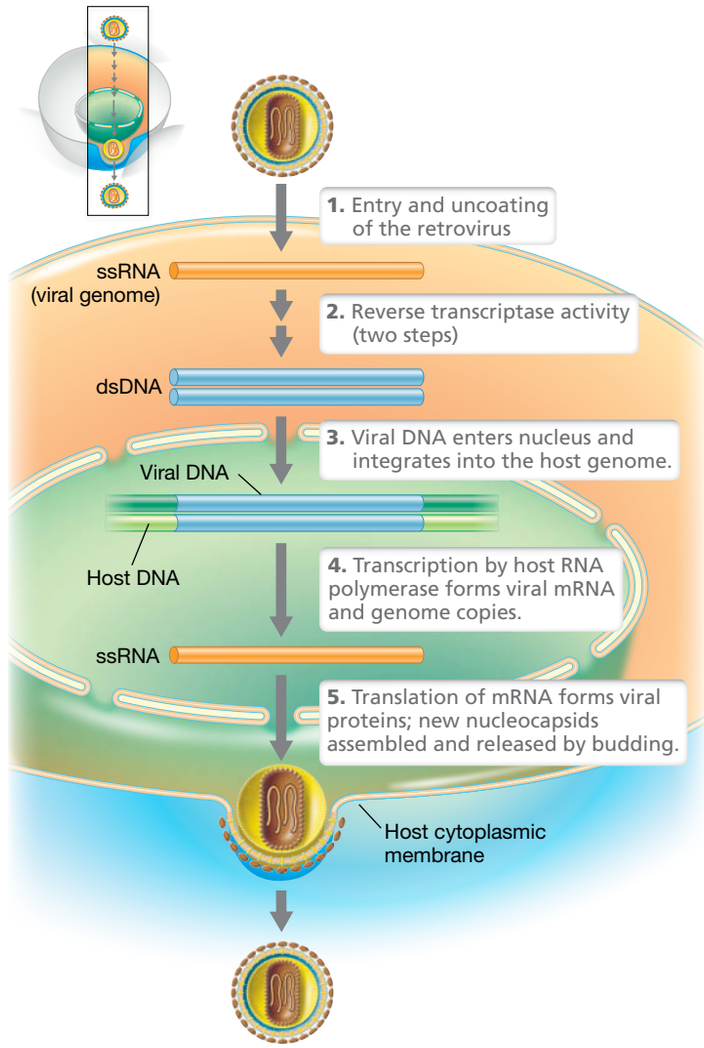


Figure 8.22 Replication of a retrovirus. The virion carries two identical copies of the RNA genome (orange). Reverse transcriptase, carried in the virion, makes single-stranded DNA from viral RNA and then double-stranded DNA that integrates into the host genome as a provirus. Transcription and translation of proviral genes leads to the production of new virions that are then released by budding.

MINIQUIZ

- Contrast the ways in which animal and bacterial viruses enter their hosts.
- What is the difference between a persistent and a latent viral infection?
- Why are retroviruses so named? What is required to carry out this process?

Chapter Review

I • The Nature of Viruses

8.1 A virus is an obligate intracellular parasite that requires a suitable host cell for replication. A virion is the extracellular form of a virus and contains either an RNA or a DNA genome inside a protein shell. Once the virus is inside the cell, the viral nucleic acid, and sometimes viral enzymes, redirects host metabolism to support virus replication. Viruses are classified by the characteristics of their genome and hosts. Bacteriophages infect bacterial cells.

Q Briefly describe the two approaches a bacteriophage can take to trigger an infection once inside its host cell.

8.2 In the virion of a naked virus, only nucleic acid and protein are present; the entire unit is called the nucleocapsid. Enveloped viruses have one or more lipoprotein layers surrounding the nucleocapsid. The nucleocapsid is arranged in a symmetric fashion, with the icosahedron being a common morphology. Although virus particles are metabolically inert, one or more key enzymes are present within the virion in some viruses.

Q Where does the envelope surrounding animal viruses originate?

8.3 The virus replication cycle can be divided into five major stages: attachment (adsorption), penetration (uptake of the entire virion or injection of the nucleic acid only), protein and nucleic acid synthesis, assembly and packaging, and virion release.

Q Why does a one-step growth curve differ in shape from that of a bacterial growth curve?

8.4 Viruses can replicate only in their correct host cells. Bacterial viruses have proved useful as model systems because their host cells are easy to grow and manipulate in culture. Many animal viruses can be grown in cultured animal cells. Viruses can be quantified (titered) by a plaque assay. Plaques are clearings that develop on lawns of host cells, and in analogy to bacterial colonies, arise from the viral infection of a single cell.

Q Draw a diagram of the one-step growth curve of virus replication, and briefly explain why the growth curve differs from a typical bacterial growth curve.

II • The Viral Replication Cycle

8.5 The attachment of a virion to a host cell is a highly specific process. Recognition proteins on the virus recognize specific receptors on the host cell. Sometimes the entire virion enters the host cell, whereas in other cases, as with most bacteriophages, only the viral genome enters. Host cells employ restriction enzymes in attempts to destroy viral and other foreign DNA, but T4 has chemically modified its DNA to make it resistant to such attack. Cells also modify their own DNA to protect it from their own restriction enzymes.

Q What is required for a bacteriophage T4 virion to attach to an *Escherichia coli* cell?

8.6 Bacteriophage T4 contains a double-stranded DNA genome that is both circularly permuted and terminally redundant. T4 encodes its own DNA polymerase and several other replication proteins. After a T4 virion penetrates a host cell, viral genes are expressed and regulated so as to redirect the host synthetic machinery to make viral nucleic acid and protein. Early viral genes encode viral genome replication events; middle and late viral genes encode structural proteins and capsid assembly. Once T4 components have been synthesized, new virions are made, primarily by self-assembly, and the virions are released after lysis of the host cell.

Q Bacteriophage T4 lacks its own RNA polymerase. How do T4 genes get expressed or converted to mRNA? What host barriers must be broken before release of virions from the host cell?

8.7 Some bacteriophages are temperate, meaning that they can initiate lytic events or integrate into the host genome as a prophage. Integration initiates a state called lysogeny in which the virus does not destroy the cell. A well-studied lysogenic virus of *Escherichia coli* is phage lambda; this phage uses an intricate regulatory system to govern whether the lytic or lysogenic state is initiated following infection.

Q What enzyme is required to form a prophage, and what are the two main transcriptional repressors that control whether lambda proceeds to lysogeny or lysis?

8.8 There are animal viruses with all known modes of viral genome replication. Many animal viruses are enveloped, picking up portions of host membrane as they leave the cell. Viral infection of animal host cells can result in cell lysis, but latent or persistent infections are also common, and a few animal viruses can cause cancer. Retroviruses like the AIDS virus are RNA viruses that employ the enzyme reverse transcriptase to replicate their RNA genome through a DNA intermediate. The DNA can integrate into the host chromosome where it can later be transcribed to yield viral mRNA and genomic RNA.

Q Why can it be said that the retrovirus genome is unique in all of biology?

Application Questions

1. What causes the viral plaques that appear on a bacterial lawn to stop growing larger?
2. The promoters on genes encoding early proteins in viruses like T4 have a different sequence than the promoters on genes encoding late proteins in the same virus. Explain how this benefits the virus.
3. Under some conditions, it is possible to obtain nucleic acid–free protein coats (capsids) of certain viruses. Under the electron microscope, these capsids look very similar to complete virions. What does this tell you about the role of the virus nucleic acid in the virus assembly process? Would you expect such particles to be infectious?

Chapter Glossary

Bacteriophage a virus that infects bacterial cells
Capsid the protein shell that surrounds the genome of a virus particle
Capsomere the subunit of a virus capsid
Concatemer two or more linear nucleic acid molecules joined covalently in tandem
Early protein a protein synthesized soon after virus infection and before replication of the virus genome
Enveloped in reference to a virus, having a lipoprotein membrane surrounding the virion
Host cell a cell inside which a virus replicates
Late protein a protein, typically a structural protein, synthesized late in virus infection
Lysogen a bacterium containing a prophage
Lysogeny a state in which the viral genome is replicated in step with the genome of the host
Lytic pathway the type of virus infection that leads to virus replication and destruction of the host cell
Middle protein a protein with either a structural or catalytic function synthesized after the early proteins in a virus infection
Nucleocapsid the complex of nucleic acid and capsid (shell) proteins of a virus
Plaque a zone of lysis or growth inhibition caused by virus infection of a bacterial lawn or other culture of sensitive host cells
Prophage the lysogenic form of a bacteriophage (see *provirus*)
Provirus the genome of a temperate or latent animal virus when it is replicating in step with the host chromosome
Retrovirus a virus whose RNA genome is replicated via a DNA intermediate
Reverse transcriptase the retroviral enzyme that can produce DNA from an RNA template
Rolling circle replication a DNA replication mechanism in which one strand is nicked and unrolled for use as a template to synthesize a complementary strand
Temperate virus a virus whose genome can replicate along with that of its host without causing cell death, in a state called lysogeny (bacterial viruses) or latency (animal viruses)
Titer the number of infectious virions per volume of fluid in a viral suspension
Virion the infectious virus particle; the viral genome surrounded by a protein coat and sometimes other layers
Virulent virus a virus that lyses or kills the host cell after infection
Virus a genetic element containing either RNA or DNA surrounded by a protein capsid and that replicates only inside host cells

Microbial Systems Biology

9

microbiologynow

DNA Sequencing in the Palm of Your Hand

DNA sequencing technologies are revolutionizing microbiology at a remarkable pace. Innovations in next-generation sequencing have even tackled the issues of cost and portability. The world's first mobile nucleic acid sequencer—the MinION—is a palm-sized device that possesses 2000 tiny pore-containing proteins called nanopores. As single strands of nucleic acid travel through the nanopores, individual nucleotides are identified based on changes in electrical current. These current changes are relayed to a computer through a USB connection, which also powers the MinION. This miniature but mighty machine can display nucleic acid sequences from critical field samples in real time on a computer screen.

The utility of the MinION was clearly on display during the 2014–2015 Ebola virus hemorrhagic fever outbreak in West Africa. Scientists traveled to Guinea with three MinIONs in their luggage, a feat in itself as most DNA sequencers are too large and delicate to travel in baggage. Once in Guinea, scientists were able to survey the spread of different Ebola virus strains by analyzing unique nucleotide sequences present in each strain's genome. In as little as 48 hours after sample collection, Ebola virus genomes from 14 patients were determined using MinION sequencing. The photo here shows a researcher loading a patient's sample onto a MinION set up in a mobile field laboratory (inset).

Because the Ebola genome mutates on average every two weeks, the astonishing turnaround time provided by the MinION allowed epidemiologists to track geographical movements of different strains of the virus. This real-time analysis indicated that two major viral strains were the cause of Ebola persistence and that cross-border transmission between Sierra Leone and Guinea severely prolonged the outbreak. Traditional sequencing methods would not have supported such surveillance, as it requires weeks to obtain results after shipment of samples to remote laboratories.

While field biologists have envisioned a myriad of uses for the MinION, developers are currently attempting to modify it to operate from a smartphone instead of a computer. Also in its immediate future is outer space—NASA plans on testing the MinION on the International Space Station. And, because of its size, relatively low cost, and ease of use, the next frontier for the MinION will undoubtedly be the classroom.

Source: Quick, J., et al. 2016. Real-time, portable genome sequencing for Ebola surveillance. *Nature 530:* 228–232. Photo credits: ©EMLab/Tommy Trenchard 2016.