5

Microbial Growth

The curved bacterium *Caulobacter* has been a model for studying the cell division process, including how shape-determining proteins such as crescentin (shown here stained red) give cells their distinctive shape.

I Bacterial Cell Division

In the last two chapters we discussed cell structure and function (Chapter 3) and the principles of microbial nutrition and metabolism (Chapter 4). Before we begin our study of the biosynthesis of macromolecules in microorganisms (Chapters 6 and 7), we consider microbial growth. Growth is the ultimate process in the life of a cell—one cell becoming two.

5.1 Cell Growth and Binary Fission

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. There are many reasons why understanding how microbial cells grow is important. For example, many practical situations call for the control of microbial growth, in particular, bacterial growth. Knowledge of how microbial populations can rapidly expand is useful for designing methods to control microbial growth, whether the methods are used to treat a life-threatening infectious disease or simply to disinfect a surface. We will study these control methods in Chapter 26. Knowledge of the events surrounding bacterial growth also allows us to see how these processes are related to cell division in higher organisms. As we will see, there are many parallels.

Bacterial cell growth depends upon a large number of cellular reactions of a wide variety of types. Some of these reactions transform energy. Others synthesize small molecules—the building blocks of macromolecules. Still others provide the various cofactors and coenzymes needed for enzymatic reactions. However, the key reactions of cell synthesis are polymerizations that make macromolecules from monomers. As macromolecules accumulate in the cytoplasm of a cell, they are assembled into new structures, such as the cell wall, cytoplasmic membrane, flagella, ribosomes, enzyme complexes, and so on, eventually leading to the process of cell division itself.

In a growing rod-shaped cell, elongation continues until the cell divides into two new cells. This process is called **binary fission** ("binary" to express the fact that two cells have arisen from one). 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 partition 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 variations in this general pattern. In some bacteria, such as *Bacillus subtilis*, a septum forms without cell wall constriction, while in the budding bacterium *Caulobacter*, constriction occurs but no septum is formed. But in all cases, 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 5.9).

During one generation, all cellular constituents increase proportionally; cells are thus said to be in *balanced growth*. Each daughter cell receives a chromosome and sufficient copies of ribosomes and all other macromolecular complexes, monomers, and inorganic ions to exist as an independent cell. 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 (see Figure 5.3).

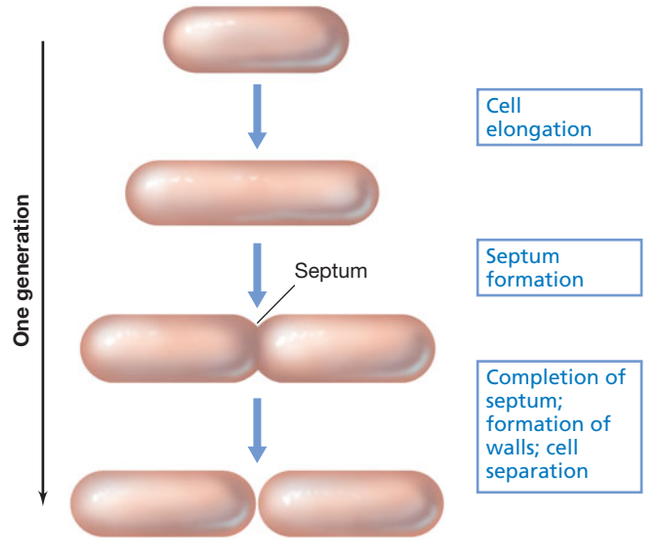


Figure 5.1 Binary fission in a rod-shaped prokaryote. Cell numbers double every generation.

The time required for a generation in a given bacterial species is highly variable and is dependent on nutritional and genetic factors, and 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 grow much slower. In nature it is likely that microbial cells grow much slower than their maximum rate because rarely are all conditions and resources necessary for optimal growth present at the same time.

MiniQuiz

- Define the term generation. What is meant by the term generation time?

5.2 Fts Proteins and Cell Division

A series of proteins present in all *Bacteria*, called *Fts proteins*, are essential for cell division. The acronym *Fts* stands for *f*ilamentous *t*emperature *s*ensitive, which describes the properties of cells that have mutations in the genes that encode Fts proteins. Such cells do not divide normally, but instead form long filamentous cells that fail to divide. **FtsZ**, a key Fts protein, has been well studied in *Escherichia coli* and several other bacteria, and much is known concerning its important role in cell division.

FtsZ is found in all prokaryotes, including the *Archaea*; FtsZ-type proteins have even been found in mitochondria and chloroplasts, further emphasizing the evolutionary ties of these organelles to the *Bacteria*. Interestingly, the protein FtsZ is related to tubulin, the important cell-division protein in eukaryotes (↻ Section 20.5). However, most other Fts proteins are

found only in species of *Bacteria* and not in *Archaea*, so our discussion here will be restricted to the *Bacteria*. Among *Bacteria*, the gram-negative *E. coli* and the gram-positive *Bacillus subtilis* have been the model species.

Fts Proteins and Cell Division

Fts proteins interact to form a cell-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 prescribes what will eventually become the cell-division plane. In a cell of *E. coli* about 10,000 FtsZ molecules polymerize to form the ring, and the ring attracts other divisome proteins, including *FtsA* and *ZipA* (**Figure 5.2**). ZipA is an anchor that connects the FtsZ ring to the cytoplasmic membrane and stabilizes it. FtsA, a protein related

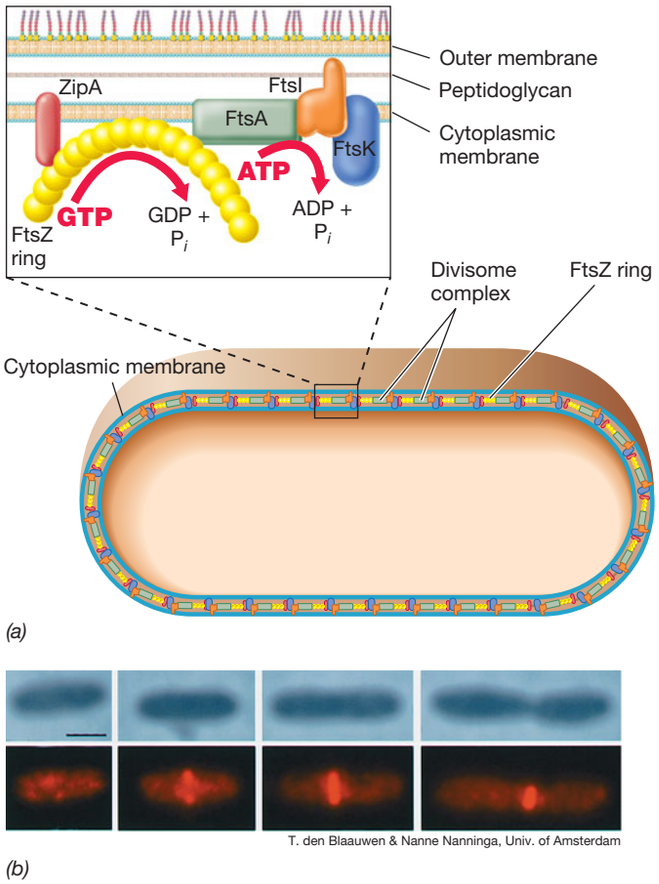


Figure 5.2 The FtsZ ring and cell division. *(a)* Cutaway view of a rod-shaped cell showing the ring of FtsZ molecules around the division plane. Blowup shows the arrangement of individual divisome proteins. ZipA is an FtsZ anchor, FtsI is a peptidoglycan biosynthesis protein, FtsK assists in chromosome 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.

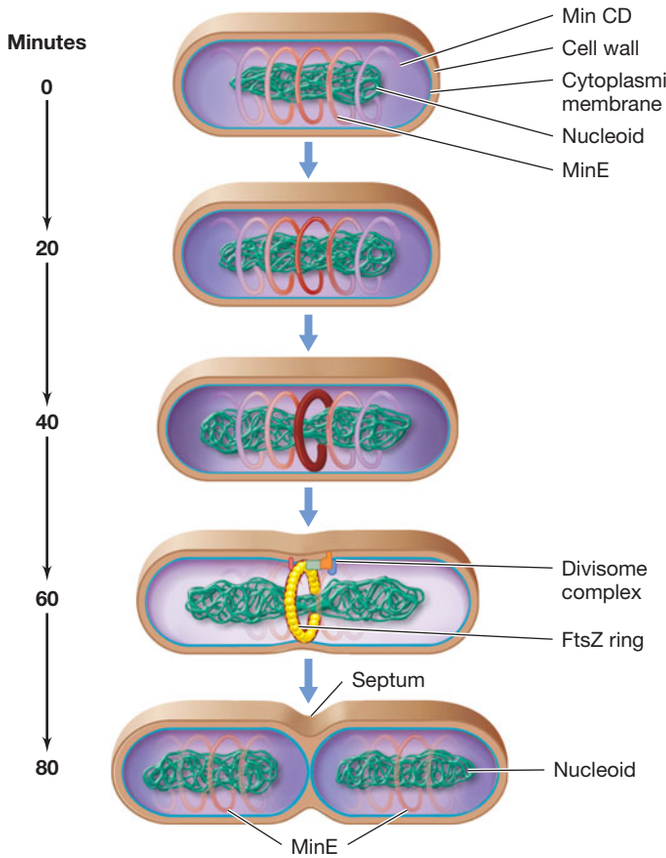


Figure 5.3 DNA replication and cell-division events. The protein MinE directs formation of the FtsZ ring and divisome complex at the cell-division plane. Shown is a schematic for cells of *Escherichia coli* growing with a doubling time of 80 min. MinC and MinD (not shown) are most abundant at the cell poles.

to actin, also helps to connect the FtsZ ring to the cytoplasmic membrane and has an additional role in recruiting other divisome proteins. The divisome forms well after elongation of a newborn cell has already begun. For example, in cells of *E. coli* the divisome forms about three-quarters of the way into cell division. However, before the divisome forms, the cell is already elongating and DNA is replicating (see Figure 5.3).

The divisome also contains Fts proteins needed for peptidoglycan synthesis, such as FtsI (Figure 5.2). 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 5.4). 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 it reaches twice its original length. Following this, the elongated cell divides, yielding two daughter cells (Figure 5.1).

DNA Replication, Min Proteins, and Cell Division

As we noted, DNA replicates before the FtsZ ring forms (**Figure 5.3**). The ring forms in the space between the duplicated nucleoids because, before the nucleoids segregate, they effectively block formation of the FtsZ ring. Location of the actual cell

midpoint by FtsZ is facilitated by a series of proteins called *Min* proteins, especially MinC, MinD, and MinE. MinD forms a spiral structure on the inner surface of the cytoplasmic membrane and oscillates back and forth from pole to pole; MinD is also required to localize MinC to the cytoplasmic membrane. Together, MinC and D inhibit cell division by preventing the FtsZ ring from forming. MinE also oscillates from pole to pole, sweeping MinC and D aside as it moves along. Because MinC and MinD dwell longer at the poles than elsewhere in the cell during their oscillation cycle, on average the center of the cell has the lowest concentration of these proteins. As a result, the cell center is the most permissive site for FtsZ ring assembly, and the FtsZ ring thus defines the division plane. In this way, the Min proteins ensure that the divisome forms only at the *cell center* and not at the cell poles (Figure 5.3).

As cell elongation continues and septum formation begins, the two copies of the chromosome are pulled apart, each to its own daughter cell (Figure 5.3). 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 5.2 and 5.3).

Properly functioning Fts proteins are essential for cell division. Much new information on cell division in *Bacteria* and *Archaea* has emerged in recent years, and genomic studies have confirmed that at least FtsZ is a key and universal cell-division protein. There is great practical interest in understanding bacterial cell division in great detail 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

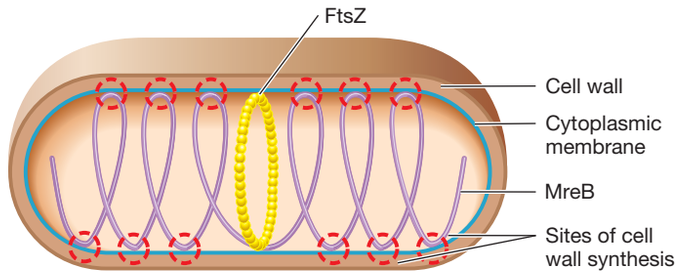
- When does the bacterial chromosome replicate in the binary fission process?
- How does FtsZ find the cell midpoint of a rod-shaped cell?

5.3 MreB and Determinants of Cell Morphology

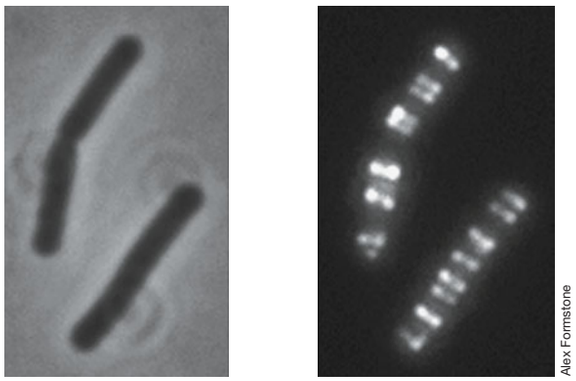
Just as specific proteins direct cell *division* in prokaryotes, other specific proteins specify cell *shape*. Interestingly, these shape-determining proteins show significant homology to key cytoskeletal proteins in eukaryotic cells. As more is learned about these proteins, it has become clear that, like eukaryotes, prokaryotes also contain a cell cytoskeleton, one that is both dynamic and multifaceted.

Cell Shape and Actinlike Proteins in Prokaryotes

The major shape-determining factor in prokaryotes is a protein called *MreB*. MreB forms a simple cytoskeleton in cells of *Bacteria* and probably in *Archaea* as well. MreB forms spiral-shaped bands around the inside of the cell, just underneath the cytoplasmic membrane (**Figure 5.4**). Presumably, the MreB cytoskeleton defines cell shape by recruiting other proteins that orchestrate cell wall growth in a specific pattern. Inactivation of the gene encoding MreB in rod-shaped bacteria



(*a*)



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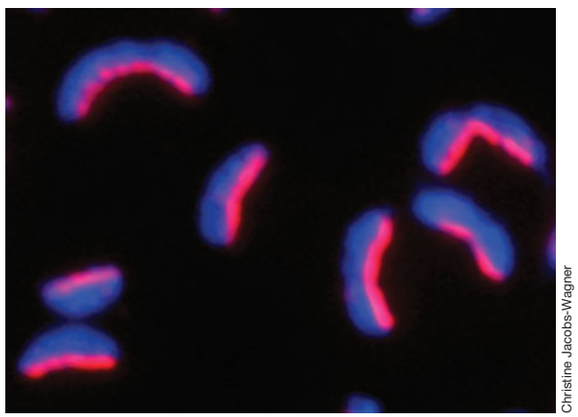


(*c*)

Figure 5.4 MreB and crescentin as determinants of cell morphology. *(a)* The cytoskeletal protein MreB is an actin analog that winds as a coil through the long axis of a rod-shaped cell, making contact with the cytoplasmic membrane in several locations (red dashed circles). These are sites of new cell wall synthesis. *(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).

causes the cells to become coccoid (coccus-shaped). Interestingly, naturally coccoid bacteria lack the gene that encodes MreB and thus lack MreB. This indicates that the "default" shape for a bacterium is a sphere (coccus). Variations in the arrangement of MreB filaments in cells of nonspherical bacteria are probably responsible for the common morphologies of prokaryotic cells (Figure 3.1).

Besides cell shape, MreB plays other important roles in the bacterial cell; in particular, it assists in the segregation of the replicated chromosome such that one copy is distributed to each daughter cell. Other actinlike proteins also play a role in this regard. *Par proteins*, for example, are a series of proteins that function in an analogous fashion to the mitotic apparatus of eukaryotic cells, separating chromosomes and plasmids to the poles of the cell during the division process. Par proteins bind to the origin of replication of the bacterial chromosome. After the origin has been replicated, the Par proteins partition the two origins to opposite cell poles and then physically push or pull the two chromosomes apart.

Mechanism of MreB

How does MreB define a cell's shape? The answer is not entirely clear, but experiments on cell division and its link to cell wall synthesis have yielded two important clues. First, the helical structures formed by MreB (Figure 5.4) are not static, but instead can rotate within the cytoplasm of a growing cell. Second, newly synthesized peptidoglycan (Section 5.4) is associated with the MreB helices at points where the helices contact the cytoplasmic membrane (Figure 5.4). It thus appears that MreB functions to localize synthesis of new peptidoglycan and other cell wall components to specific locations along the cylinder of a rod-shaped cell during growth. This would explain the fact that new cell wall material in an elongated rod-shaped cell forms at several points along its long axis rather than from a single location at the FtsZ site outward, as in spherical bacteria (see Figure 5.5). By rotating within the cell cylinder and initiating cell wall synthesis where it contacts the cytoplasmic membrane, MreB would direct new wall synthesis in such a way that a rod-shaped cell would elongate only along its long axis.

Crescentin

Caulobacter crescentus, a vibrio-shaped species of *Proteobacteria* (Section 17.16), produces a shape-determining protein called *crescentin* 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. The arrangement and localization of crescentin filaments are thought to somehow impart the characteristic curved morphology to the *Caulobacter* cell (Figure 5.4*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. 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 (Section 8.13). Although thus far crescentin has been found only in *Caulobacter*, proteins similar to crescentin have been found in other helically shaped cells, such as *Helicobacter*. This suggests that these proteins may be necessary for the formation of curved cells.

Archaeal Cell Morphology and the Evolution of Cell Division and Cell Shape

Although less is known about how cell morphology is controlled in *Archaea* than in *Bacteria*, the genomes of most *Archaea* contain genes that encode MreB-like proteins. Thus, it is likely that these function in *Archaea* as they do in *Bacteria*. Along with the finding that FtsZ also exists in *Archaea*, it appears that there are strong parallels in cell-division processes and morphological determinants in all prokaryotes.

How do the determinants of cell shape and cell division in prokaryotes compare with those in eukaryotes? Interestingly, the protein MreB is structurally related to the eukaryotic protein actin, and FtsZ is related to the eukaryotic protein tubulin. In eukaryotic cells actin assembles into structures called *microfilaments* that function as scaffolding in the cell cytoskeleton and in cytokinesis, whereas tubulin forms *microtubules* that are important in eukaryotic mitosis and other processes (Sections 7.1, 20.1, and 20.5). 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 are also part of the eukaryotic cytoskeleton and are fairly widespread among *Bacteria*. It thus appears that most of the proteins that control cell division and cell shape in eukaryotic cells have their evolutionary roots in prokaryotic cells, cells that preceded them on Earth by billions of years (Figure 1.6).

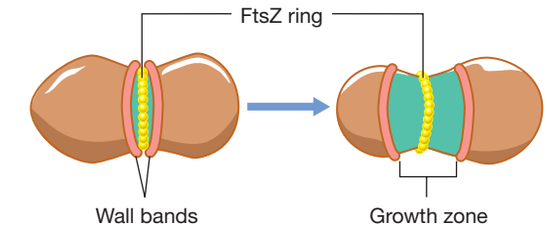
MiniQuiz

- What eukaryotic protein is related to MreB? What does this protein do in eukaryotic cells?
- What is crescentin and what does it do?

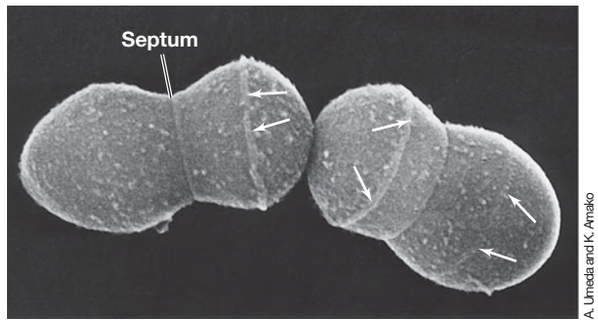
5.4 Peptidoglycan Synthesis and Cell Division

In the previous section we considered some of the key events in binary fission and learned that a major feature of the cell-division process is the production of new cell wall material. In most cocci, cell walls grow in opposite directions outward from the FtsZ ring (**Figure 5.5**), whereas the walls of rod-shaped cells grow at several locations along the length of the cell (Figure 5.4). However, in both cases preexisting peptidoglycan has to be severed to allow newly synthesized peptidoglycan to be inserted. How does this occur?

Beginning at the FtsZ ring (Figures 5.2 and 5.3), small gaps in the wall are made by enzymes called *autolysins*, enzymes that function like lysozyme (Section 3.6) to hydrolyze the β-1,4 glycosidic bonds that connect *N*-acetylglucosamine and *N*-acetylmuramic acid in the peptidoglycan backbone. New cell wall material is then added across the gaps (Figure 5.5*a*). The junction between new and old peptidoglycan forms a ridge on the cell surface of gram-positive bacteria called a *wall band*



(a)



(b)

Figure 5.5 **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 5.4). *(b)* Scanning electron micrograph of cells of *Streptococcus hemolyticus* showing wall bands (arrows). A single cell is about 1 μm in diameter.

(Figure 5.5*b*), analogous to a scar. It is of course essential in peptidoglycan synthesis that new cell wall precursors (*N*-acetylmuramic acid/*N*-acetylglucosamine/tetrapeptide units, see Figure 5.7) be spliced into existing peptidoglycan in a coordinated and consistent manner in order to prevent a breach in peptidoglycan integrity at the splice point; a breach could cause spontaneous cell lysis, called *autolysis*.

Biosynthesis of Peptidoglycan

We discussed the general structure of peptidoglycan in Section 3.6. The peptidoglycan layer can be thought of as a stress-bearing fabric, much like a thin sheet of rubber. Synthesis of new peptidoglycan during growth requires the controlled cutting of preexisting peptidoglycan by autolysins along with the simultaneous insertion of peptidoglycan precursors. A lipid carrier molecule called *bactoprenol* (**Figure 5.6**) plays a major role in this process.

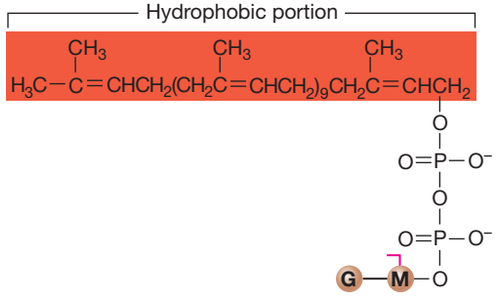
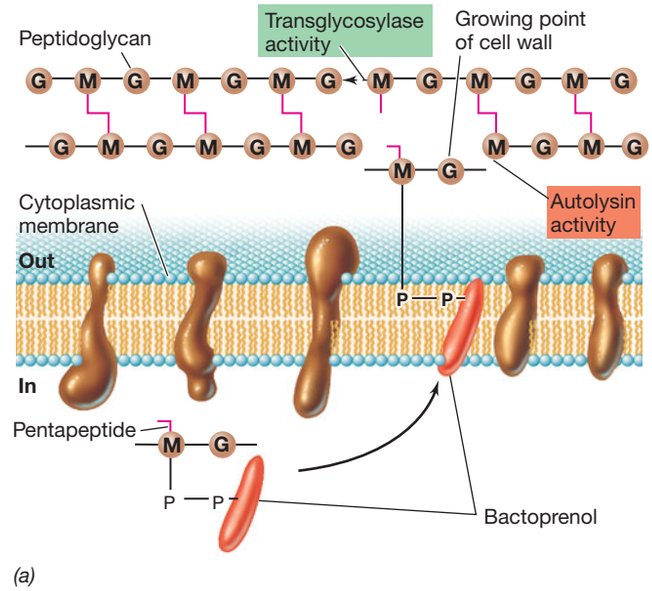
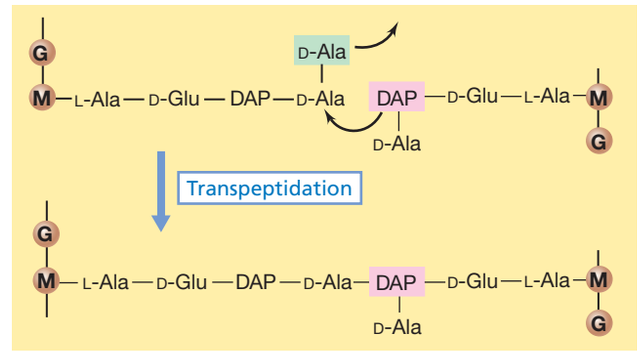


Figure 5.6 **Bactoprenol (undecaprenol diphosphate).** This highly hydrophobic molecule carries cell wall peptidoglycan precursors through the cytoplasmic membrane.



(a)



(b)

Figure 5.7 **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.

Bactoprenol is a hydrophobic C_{55} alcohol that bonds to a *N*-acetylglucosamine/*N*-acetylmuramic acid/pentapeptide peptidoglycan precursor (**Figure 5.7*a***). Bactoprenol transports peptidoglycan precursors across the cytoplasmic membrane by rendering them sufficiently hydrophobic to pass through the membrane interior. Once in the periplasm, bactoprenol interacts with enzymes called *transglycosylases* that insert cell wall precursors into the growing point of the cell wall and catalyze glycosidic bond formation (Figure 5.7*b*).

Transpeptidation

The final step in cell wall synthesis is **transpeptidation**. Transpeptidation forms the peptide cross-links between muramic acid residues in adjacent glycan chains (↻ Section 3.6 and Figures 3.16 and 3.17). 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 (Figure 5.7*b*; see also Figure 3.17). Initially, there are *two* D-alanine residues at the end of the peptidoglycan precursor, but only *one* remains in the final molecule as the other D-alanine molecule is removed during the transpeptidation reaction (Figure 5.7*b*). This reaction, which is exergonic, supplies the energy necessary to drive the reaction forward (transpeptidation occurs outside the cytoplasmic membrane, where ATP is unavailable). In *E. coli*, the protein FtsI (Figure 5.2*a*) is the key protein in transpeptidation at the division septum, while a separate transpeptidase enzyme cross-links peptidoglycan elsewhere in the growing cell. In gram-positive bacteria, where a glycine interbridge is common, cross-links occur across the interbridge, typically from an L-lysine of one peptide to a D-alanine on the other (Section 3.6 and Figure 3.17).

Transpeptidation and Penicillin

Transpeptidation is medically noteworthy because it is the reaction inhibited by the antibiotic penicillin. Several penicillin-binding proteins have been identified in bacteria, including the previously mentioned FtsI (Figure 5.2*a*). When penicillin is bound to penicillin-binding proteins the proteins lose their catalytic activity. In the absence of transpeptidation, the continued activity of autolysins (Figure 5.7) so weakens the cell wall that the cell eventually bursts.

Penicillin has been a successful drug in clinical medicine for at least two reasons. First, humans are *Eukarya* and therefore lack peptidoglycan; the drug can thus be administered in high doses and is typically nontoxic. And second, most pathogenic bacteria contain peptidoglycan and are thus potential targets of the drug. Nevertheless, the continual and excessive use of penicillin since it became commercially available following World War II has selected for resistant mutants of many common pathogens previously susceptible to this drug. Many of these are widespread in human and other animal populations because they make variants of penicillin-binding proteins that are catalytically active but no longer bind penicillin. In these cases, cell wall synthesis occurs uninterrupted in the presence of the drug, and other drugs need to be used to thwart the infection.

MiniQuiz

- What are autolysins and why are they necessary?
- What is the function of bactoprenol?
- What is transpeptidation and why is it important?

II Population Growth

As we mentioned earlier, microbial growth is defined as an increase in the *number* of cells in a population. So we now move on from considering the growth and division of an individual cell to consider the dynamics of growth in bacterial populations.

5.5 The Concept of Exponential 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. Generation times vary widely among microorganisms. In general, most bacteria have shorter generation times than do most microbial eukaryotes. The generation time of a given organism in culture is dependent on the growth medium and the incubation conditions used. Many bacteria have minimum generation times of 0.5–6 h under the best of growth conditions, but a few very rapidly growing organisms are known whose doubling times are less than 20 min and a few slow-growing organisms whose doubling times are as long as several days or even weeks.

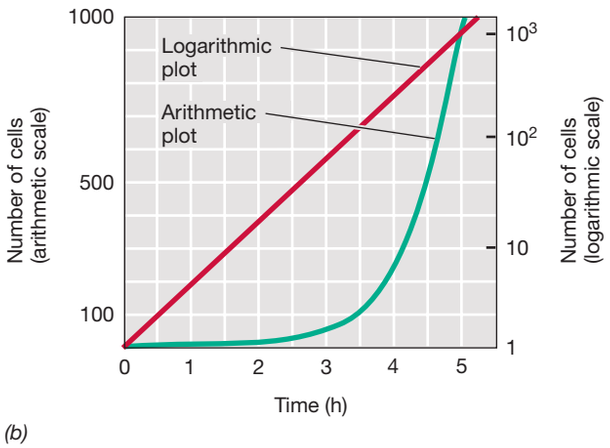
Exponential Growth

A growth experiment beginning with a single cell having a doubling time of 30 min is presented in **Figure 5.8**. This pattern of population increase, where the number of cells doubles during 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 5.8*b*).

By contrast, when the number of cells is plotted on a logarithmic ($\log_{10}$) scale and time is plotted arithmetically (a *semilogarithmic* graph), as shown in Figure 5.8*b*, the points fall on a straight line. This straight-line function reflects the fact that the cells are growing exponentially and the population is doubling in a constant time interval. Semilogarithmic graphs are also convenient to use to estimate the generation time of a microbial culture from a set of growth data. Generation times may be read directly from

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^{19})

(a)



(b)

Figure 5.8 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.

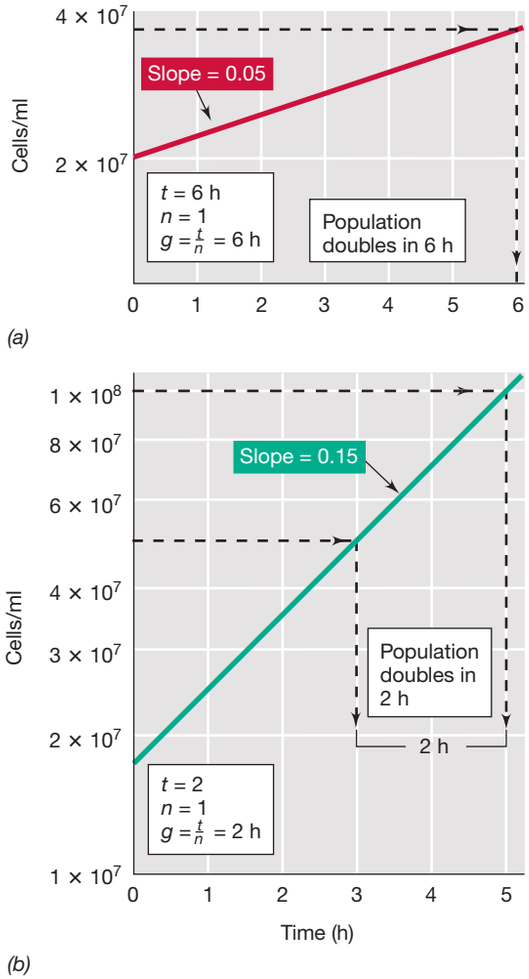


Figure 5.9 Calculating microbial growth parameters. Method of estimating the generation times (*g*) of exponentially growing populations with generation times of *(a)* 6 h and *(b)* 2 h from data plotted on semilogarithmic graphs. The slope of each line is equal to 0.301/*g*, and *n* is the number of 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.

the graph as shown in **Figure 5.9**. 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 5.9*b*).

The 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 growth, this results in an explosive increase in cell numbers. For example, in the experiment in Figure 5.8, 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 considerably higher, 256 cells per 30 min, and between 5.5 and 6 h of growth it is 2048 cells per 30 min (Figure 5.8). Thus in an actively growing bacterial culture, cell numbers can get very large very quickly.

Consider the following practical implication of exponential growth. For a nonsterile and nutrient-rich food product such as milk to stand at room temperature for a few hours during the early stages of exponential growth, when total bacterial cell numbers are relatively low, is not detrimental. However, when cell numbers are initially much higher, standing for the same length of time leads to spoilage of the milk. The lactic acid bacteria responsible for milk spoilage contaminate milk during its collection. These harmless organisms grow only very slowly at refrigeration temperatures (~4°C), and only after several days of slow growth at this temperature are the effects of spoilage (rancid milk) noticeable. However, at room temperature or above, growth is greatly accelerated. Thus two bottles of milk that have expiration dates one week apart will contain considerably different bacterial cell numbers and have different outcomes if they are left at room temperature overnight; the fresher milk with still relatively low cell numbers may have no off taste while the older milk with much higher cell numbers is spoiled.

MiniQuiz

- Why does exponential growth lead to large cell populations in so short a period of time?
- What is a *semilogarithmic* plot and what information can we derive from it?

5.6 The Mathematics of Exponential Growth

The increase in cell number in an exponentially growing bacterial culture approaches 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 5.8*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 can be expressed mathematically 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 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 a 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 Relationship of *N* and N_0 to *n*

The equation $N = N_0 2^n$ can be expressed in terms of n as follows:

$$N = N_0 2^n$$

$$\log N = \log N_0 + n \log 2$$

$$\log N - \log N_0 = n \log 2$$

$$n = \frac{\log N - \log N_0}{\log 2} = \frac{\log N - \log N_0}{0.301}$$

$$= 3.3\ (\log N - \log N_0)$$

With this simple formula, we can calculate generation times in terms of measurable quantities, N and N_0. As an example, consider actual growth data from the graph in Figure 5.9*b*, in which $N = 10^8$, $N_0 = 5 \times 10^7$, and $t = 2$:

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

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.

Other Growth Expressions

Besides determination of the generation time of an exponentially growing culture by inspection of graphical data (Figure 5.9*b*), g can be calculated from the slope of the straight-line function obtained in a semilogarithmic plot of exponential growth. The slope is equal to 0.301 n/t (or 0.301/g). In the above example, 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 term 0.301/g is called the *specific growth rate*, abbreviated k.

Another useful growth expression is the reciprocal of the generation time, called the *division rate*, abbreviated ν. The division rate is equal to $1/g$ and has units of reciprocal hours (h^{-1}). Whereas g is a measure of the *time* it takes for a population to double in cell number, ν is a measure of the *number of generations* per unit of time in an exponentially growing culture. The slope of the line relating log cell number to time (Figure 5.9) is equal to $\nu/3.3$.

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

MiniQuiz

- Distinguish between the terms specific growth rate and generation time.
- If in 8 h an exponentially growing cell population increases from 5×10^6 cells/ml to 5×10^8 cells/ml, calculate g, n, ν, and k.

5.7 The Microbial Growth Cycle

The data presented in Figures 5.8 and 5.9 reflect only part of the growth cycle of a microbial population, the part called *exponential growth*. For several reasons, an organism growing in an enclosed vessel, such as a tube or a flask (a growth condition called a **batch culture**), cannot grow exponentially indefinitely. Instead, a typical *growth curve* for the population is obtained, as illustrated in **Figure 5.10**. The growth curve describes an entire growth cycle, and includes the lag phase, exponential phase, stationary phase, and death phase.

Lag Phase

When a microbial culture is inoculated into a fresh medium, growth usually begins only after a period of time called the *lag phase*. This interval may be brief or extended, depending on the history of the inoculum and the growth conditions. If an exponentially growing culture is transferred into the same medium under the same conditions of growth (temperature, aeration, and the like), there is no lag and exponential growth begins immediately. However, if the inoculum is taken from an old

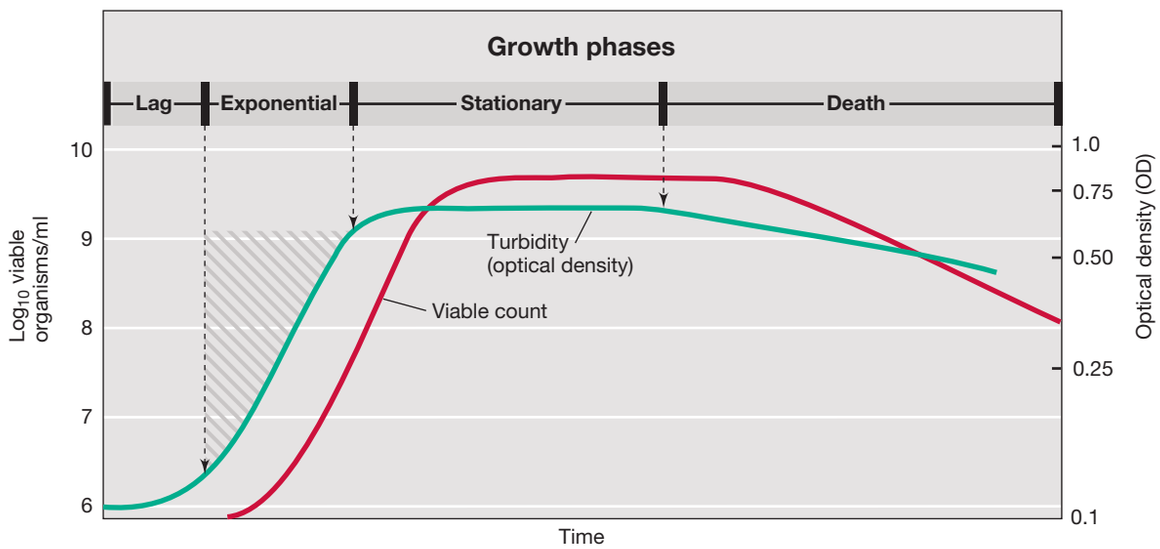


Figure 5.10 **Typical growth curve for a bacterial population.** A viable count measures the cells in the culture that are capable of reproducing. Optical density (turbidity), a quantitative measure of light scattering by a liquid culture, increases with the increase in cell number.

(stationary phase) culture and transferred into the same medium, there is usually a lag even if all the cells in the inoculum are alive. This is because the cells are depleted of various essential constituents and time is required for their biosynthesis. A lag also ensues when the inoculum consists of cells that have been damaged (but not killed) by significant temperature shifts, radiation, or toxic chemicals because of the time required for the cells to repair the damage.

A lag is also observed when a microbial population is transferred from a rich culture medium to a poorer one; for example, from a complex medium to a defined medium (↻ Section 4.2). To grow in any culture medium the cells must have a complete complement of enzymes for synthesis of the essential metabolites not present in that medium. Hence, upon transfer to a medium where essential metabolites must be biosynthesized, time is needed for production of the new enzymes that will carry out these reactions.

Exponential Phase

As we saw in the previous section, during the *exponential phase* of growth each cell divides to form two cells, each of which also divides to form two more cells, and so on, for a brief or extended period, depending on the available resources and other factors. Cells in exponential growth are typically in their healthiest state and hence are most desirable for studies of their enzymes or other cell components.

Rates of exponential growth vary greatly. The rate of exponential growth is influenced by environmental conditions (temperature, composition of the culture medium), as well as by genetic characteristics of the organism itself. In general, prokaryotes grow faster than eukaryotic microorganisms, and small eukaryotes grow faster than large ones. This should remind us of the previously discussed concept of surface-to-volume ratio. Recall that small cells have an increased capacity for nutrient and waste exchange compared with larger cells, and this metabolic advantage can greatly affect their growth and other properties (↻ Section 3.2).

Stationary Phase

In a batch culture (tube, flask bottle, Petri dish), exponential growth is limited. Consider the fact that a single cell of a bacterium with a 20-min generation time would produce, if allowed to grow exponentially in a batch culture for 48 h, a population of cells that weighed 4000 times the weight of Earth! This is particularly impressive when it is considered that a single bacterial cell weighs only about one-trillionth (10^{-12}) of a gram.

Obviously, this scenario is impossible. Something must happen to limit the growth of the population. Typically, either one or both of two situations limit growth: (1) an essential nutrient of the culture medium is used up, or (2) a waste product of the organism accumulates in the medium and inhibits growth. Either way, exponential growth ceases and the population reaches the *stationary phase*.

In the stationary phase, there is no net increase or decrease in cell number and thus the growth rate of the population is zero. Although the population may not grow during the stationary phase, many cell functions can continue, including energy metabolism and biosynthetic processes. Some cells may even divide during the stationary phase but no net increase in cell number occurs. This is because some cells in the population grow, whereas others die, the two processes balancing each other out. This is a phenomenon called *cryptic growth*.

Death Phase

If incubation continues after a population reaches the stationary phase, the cells may remain alive and continue to metabolize, but they will eventually die. When this occurs, the population enters the *death phase* of the growth cycle. In some cases death is accompanied by actual cell lysis. Figure 5.10 indicates that the death phase of the growth cycle is also an exponential function. Typically, however, the rate of cell death is much slower than the rate of exponential growth.

The phases of bacterial growth shown in Figure 5.10 are reflections of the events in a *population* of cells, not in individual cells. Thus the terms lag phase, exponential phase, stationary phase, and death phase have no meaning with respect to individual cells but only to cell populations. Growth of an individual cell is a necessary 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 what phase of the growth curve in Figure 5.10 are cells dividing in a regular and orderly process?
- Under what conditions does a lag phase not occur?
- Why do cells enter stationary phase?

5.8 Continuous Culture: The Chemostat

Our discussion of population growth thus far has been confined to batch cultures. A batch culture is continually being altered by the metabolic activities of the growing organisms and is therefore a *closed* system. In the early stages of exponential growth in batch cultures, conditions may remain relatively constant. But in later stages, when cell numbers become quite large, the chemical and physical composition of the culture medium changes dramatically.

For many studies in microbiology it is useful to be able to keep cultures under constant conditions for long periods. For example, if one is studying a physiological process such as the synthesis of a particular enzyme, the ready availability of exponentially growing cells may be very convenient. This is only possible with a *continuous culture device*. Unlike a batch culture, a continuous culture is an *open* system. The continuous culture vessel maintains a constant volume to which fresh medium is added at a constant rate while an equal volume of spent culture medium (containing cells) is removed at the same rate. Once such a system is in equilibrium, the chemostat volume, cell number, and nutrient status remain constant, and the system is said to be in *steady state*.

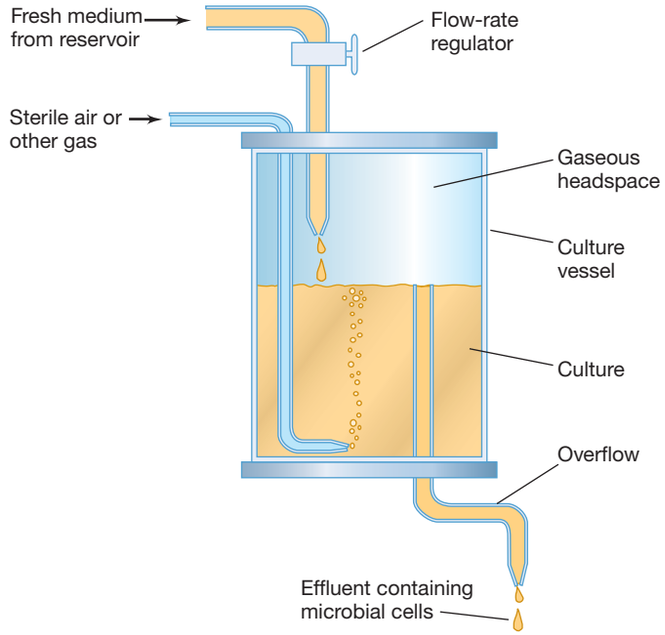


Figure 5.11 **Schematic for a continuous culture device (chemostat).** The population density is controlled by the concentration of limiting nutrient in the reservoir, and the growth rate is controlled by the flow rate. Both parameters can be set by the experimenter.

The Chemostat

The most common type of continuous culture device is the **chemostat** (**Figure 5.11**). In the chemostat, both growth rate and cell density of the culture can be controlled independently and simultaneously. Two factors govern growth rate and cell density respectively. These are: (1) the *dilution rate*, which is the rate at which fresh medium is pumped in and spent medium is removed; and (2) the *concentration of a limiting nutrient*, such as a carbon or nitrogen source, present in the sterile medium entering the chemostat vessel.

In a batch culture, the nutrient concentration can affect both growth rate and growth yield (**Figure 5.12**). At very low concentrations of a given nutrient, the growth rate is submaximal because the nutrient cannot be transported into the cell fast enough to satisfy metabolic demand. At moderate or higher nutrient levels, however, the growth rate plateaus, but the final cell density may continue to increase in proportion to the concentration of nutrients in the medium up to some fixed limit (Figure 5.12). In a chemostat, by contrast, growth rate and growth yield are controlled independently: The growth rate is set by the dilution rate, while the cell yield (number/milliliter) is controlled by the limiting nutrient. Independent control of these two growth parameters is impossible in a batch culture because it is a closed system where growth conditions are constantly changing with time.

Varying Chemostat Parameters

The effects on bacterial growth of varying the dilution rate and concentration of growth-limiting nutrient in a chemostat are shown in **Figure 5.13**. As seen, there are rather wide limits over

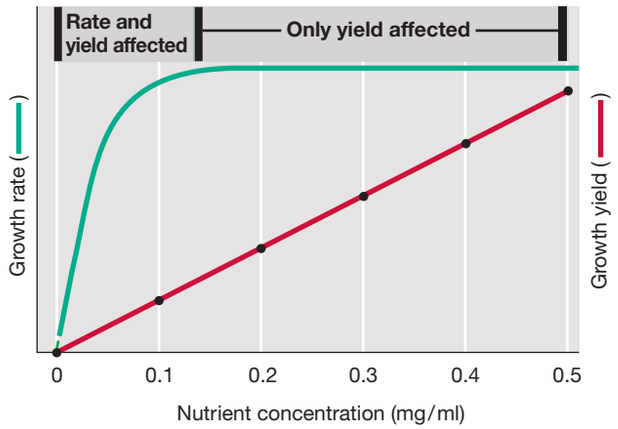


Figure 5.12 **The effect of nutrients on growth.** Relationship between nutrient concentration, growth rate (green curve), and growth yield (red curve) in a batch culture (closed system). Only at low nutrient concentrations are both growth rate and growth yield affected.

which the dilution rate controls growth rate, although at both very low and very high dilution rates the steady state breaks down. At too high a dilution rate, the organism cannot grow fast enough to keep up with its dilution and is washed out of the chemostat. By contrast, at too low a dilution rate, cells may die from starvation because the limiting nutrient is not being added fast enough to permit maintenance of cell metabolism. However, between these limits, different growth rates can be achieved by simply varying the dilution rate.

Cell density in a chemostat is controlled by a limiting nutrient, just as it is in a batch culture (Figure 5.12). If the concentration of this nutrient in the incoming medium is raised, with the dilution rate remaining constant, the cell density will increase while the

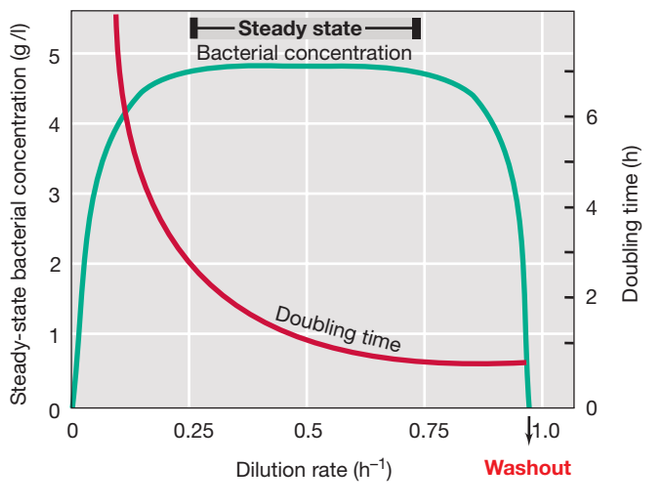


Figure 5.13 **Steady-state relationships in the chemostat.** The dilution rate is determined from the flow rate and the volume of the culture vessel. Thus, with a vessel of 1000 ml and a flow rate through the vessel of 500 ml/h, the dilution rate would be 0.5 h^{-1}. Note that at high dilution rates, growth cannot balance dilution, and the population washes out. Note also that although the population density remains constant during steady state, the growth rate (doubling time) can vary over a wide range.

growth rate remains the same. Thus, by adjusting the dilution rate and nutrient level accordingly, the experimenter can obtain 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 low, moderate, or high rates.

Experimental Uses of the Chemostat

A practical advantage to the chemostat is that a cell population may be maintained in the exponential growth phase for long periods, days or even weeks. Because exponential phase cells are usually most desirable for physiological experiments, such cells can be available at any time when grown in a chemostat. Moreover, repetition of experiments can be done with the knowledge that each time the cell population will be as close to being the same as possible. For some applications, such as the study of a particular enzyme, enzyme activities may be significantly lower in stationary phase cells than in exponential phase cells, and thus chemostat-grown cultures are ideal. In practice, after a sample is removed from the chemostat, a period of time is required for the vessel to return to its original volume and for steady state to be reachieved. Once this has occurred, the vessel is ready to be sampled once again.

The chemostat has been used in microbial ecology as well as in microbial physiology. For example, because the chemostat can easily mimic the low substrate concentrations that often prevail in nature, it is possible to prepare mixed or pure bacterial populations in a chemostat and study the competitiveness of different organisms at particular nutrient concentrations. Using these methods together with the powerful tools of phylogenetic stains and gene tracking (Chapters 16 and 23), experimenters can monitor changes in the microbial community in the chemostat as a function of different growth conditions. Such experiments often reveal interactions within the population that are not obvious from growth studies in batch culture.

Chemostats have also been used for enrichment and isolation of bacteria. From a natural sample, one can select a stable population under the nutrient and dilution-rate conditions chosen and then slowly increase the dilution rate 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!

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 growth rate of the organism?
- Do pure cultures have to be used in a chemostat?

III Measuring Microbial Growth

Population growth is measured by tracking changes in the number of cells or changes in the level of some cellular component. The latter could be protein, nucleic acids, or the dry weight of the cells themselves. We consider here two common measures of cell growth: cell counts and turbidity, the latter of which is a measure of cell mass.

5.9 Microscopic Counts

A total count of microbial numbers can be achieved using a microscope to observe and enumerate the cells present in a culture or natural sample. The method is simple, but the results can be unreliable.

The most common total count method is the microscopic cell count. Microscopic counts can be done on either samples dried on slides or on samples in liquid. Dried samples can be stained to increase contrast between cells and their background (↻ Section 2.2). With liquid samples, specially designed counting chambers are used. In such a counting chamber, a grid with squares of known area is marked on the surface of a glass slide (**Figure 5.14**). When the coverslip is placed on the chamber, each square on the grid has a precisely measured 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 5.14).

A second method of enumerating cells in liquid samples is with a flow cytometer. This is a machine that employs a laser beam and complex electronics to count individual cells. Flow cytometry is

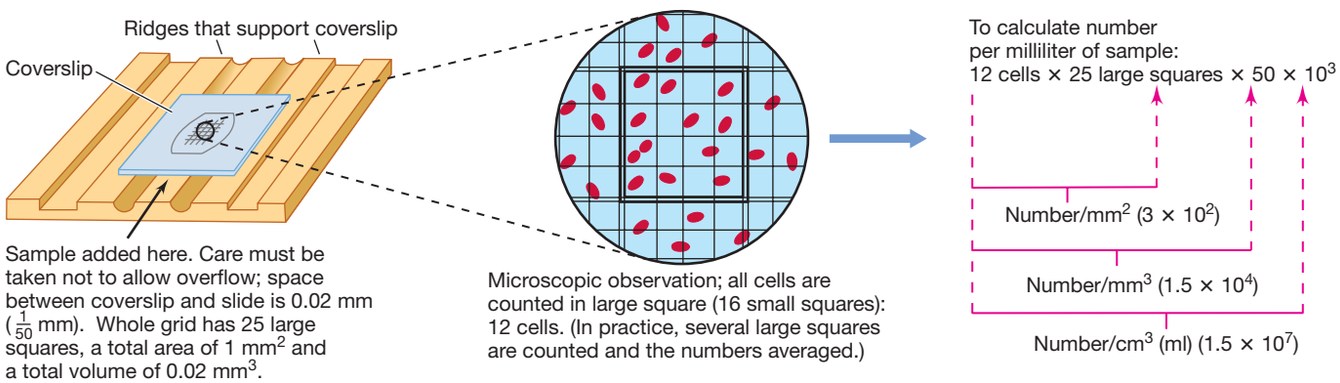


Figure 5.14 Direct microscopic counting procedure using the Petroff–Hausser counting chamber.
A phase-contrast microscope is typically used to count the cells to avoid the necessity for staining.

rarely used for the routine counting of microbial cells, but has applications in the medical field for counting and differentiating blood cells and other cell types from clinical samples. It has also been used in microbial ecology to separate different types of cells for isolation purposes.

Microscopic counting is a quick and easy way of estimating microbial cell number. However, it has several limitations: (1) Without special staining techniques (↻ Section 22.3), dead cells cannot be distinguished from live cells. (2) Small cells are difficult to see under the microscope, and some cells are inevitably missed. (3) Precision is difficult to achieve. (4) A phase-contrast microscope is required if the sample is not stained. (5) Cell suspensions of low density (less than about 10^6 cells/milliliter) have few if any bacteria in the microscope field unless a sample is first concentrated and resuspended in a small volume. (6) Motile cells must be immobilized before counting. (7) Debris in the sample may be mistaken for microbial cells.

In microbial ecology, total cell counts are often performed on natural samples using stains to visualize the cells. The stain DAPI (↻ Section 2.2 and Figure 2.6*c*) stains all cells in a sample because it reacts with DNA. By contrast, fluorescent stains that are highly specific for certain organisms or groups of related organisms can be prepared by attaching the 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 measure cell numbers of each domain in a given sample; the use of these stains will be discussed in Section 16.9. If cells are present at low densities, for example in a sample of open ocean water, this problem can be overcome by first concentrating cells on a filter and then counting them after staining. Because they are easy to do and often yield useful information, microscopic cell counts are very common in microbial studies of natural environments. www.microbiologyplace.com **Online Tutorial 5.1: Direct Microscopic Counting Procedure**

MiniQuiz

- What are some of the problems that can arise when unstained preparations are used to make total cell counts of samples from natural environments?

5.10 Viable Counts

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, we can use a viable counting method. To do this, we typically determine the number of cells in a sample capable of forming colonies on a suitable agar medium. For this reason, the viable count is also called a **plate count**. The assumption made in the viable counting procedure is that each viable cell can grow and divide to yield one colony. Thus, colony numbers are a reflection of cell numbers.

There are at least two ways of performing a plate count: the *spread-plate method* and the *pour-plate method* (**Figure 5.15**). 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. The plate is then incubated until colonies appear, and the number of colonies is counted. The surface of the plate must not be too moist because the added liquid must soak in so the cells remain stationary. Volumes greater than about 0.1 ml are avoided in this method because the excess

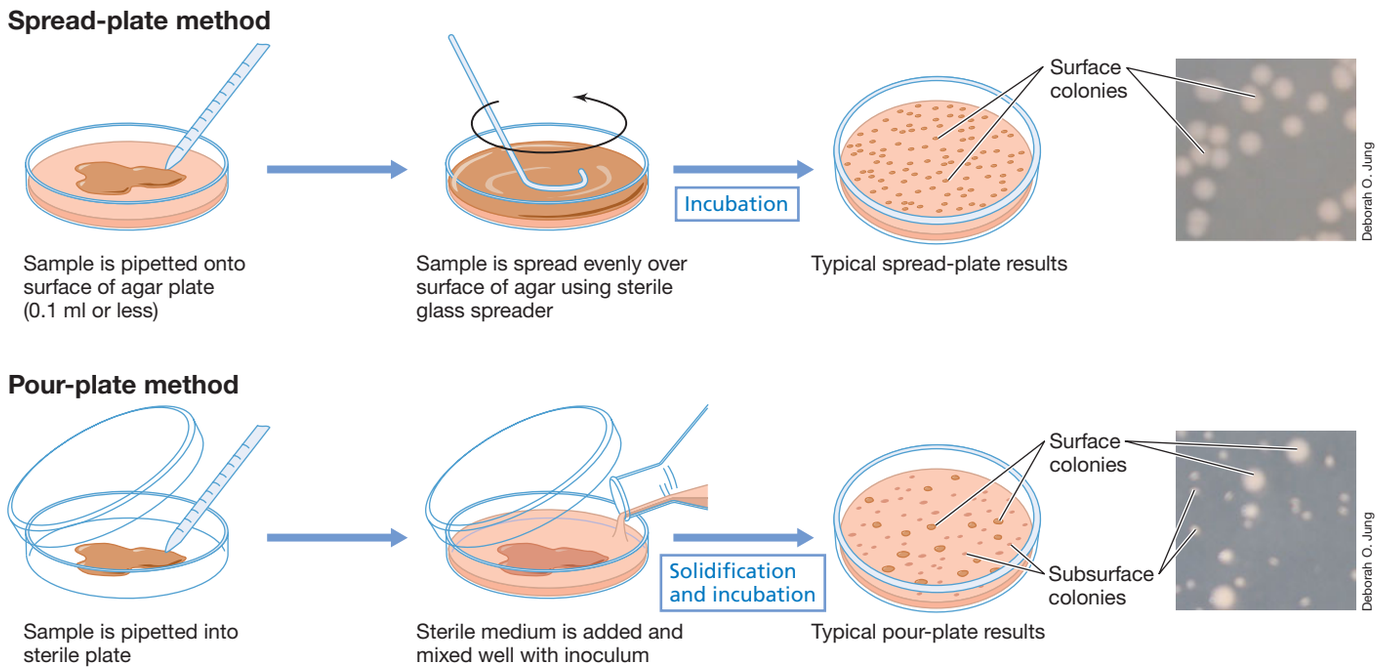


Figure 5.15 **Two methods for the viable count.** In the pour-plate method, colonies form within the agar as well as on the agar surface. On the far right are photos of colonies of *Escherichia coli* formed from cells plated by the spread-plate method (top) or the pour-plate method (bottom).

liquid does not soak in and may cause the colonies to coalesce as they form, making them difficult to count.

In the pour-plate method (Figure 5.15), a known volume (usually 0.1–1.0 ml) of culture is pipetted into a sterile Petri plate. Melted agar medium, tempered to just about gelling temperature, is then added and mixed well by gently swirling the plate on the benchtop. Because the sample is mixed with the molten agar medium, a larger volume can be used than with the spread plate. However, with this method the organism to be counted must be able to withstand brief exposure to the temperature of molten agar (~45–50°C). Here, colonies form throughout the medium and not just on the agar surface as in the spread-plate method. The plate must therefore be examined closely to make sure all colonies are counted. If the pour-plate method is used to enumerate cells from a natural sample, another problem may arise; any debris in the sample must be distinguishable from actual bacterial colonies or the count will be erroneous.

Diluting Cell Suspensions before Plating

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 have between 30 and 300 colonies.

To obtain the appropriate colony number, the sample to be counted 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 5.16**). 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 5.16).

Sources of Error in Plate Counting

The number of colonies obtained in a viable count experiment 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 used, 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.

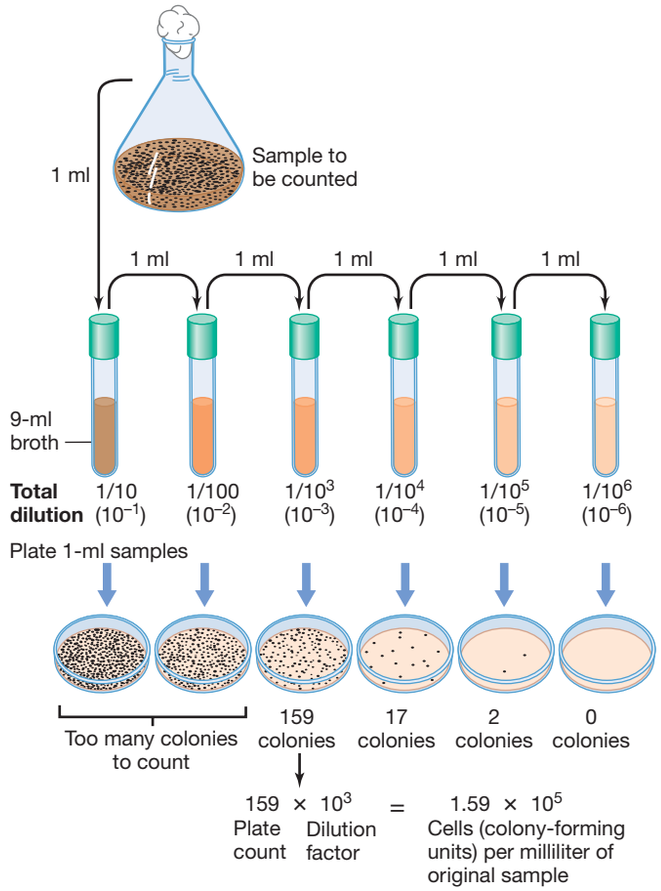


Figure 5.16 Procedure for viable counting using serial dilutions of the sample and the pour-plate method. The sterile liquid used for making dilutions can simply be water, but a solution of mineral salts or actual growth medium may yield a higher recovery. The dilution factor is the reciprocal of the dilution.

Viable counts can be subject to rather large errors for several reasons. These include plating inconsistencies, such as inaccurate pipetting of a liquid sample, a nonuniform sample (for example, a sample containing cell clumps), insufficient mixing, and other factors. 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. Note also that if two or more cells are in a clump, they will grow to form only a single colony. So if a sample contains many cell clumps, a viable count of that sample may be erroneously low. Data are often expressed as the number of *colony-forming units* obtained rather than the actual number of viable cells, because a colony-forming unit may contain one or more cells.

Despite the difficulties associated with viable counting, the procedure gives the best estimate of the number of viable cells in a sample and so is widely used in many areas 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.

Targeted Plate Counts

The use of highly selective culture media and growth conditions in viable counting procedures allows one to target only particular species, or in some cases even a single species, in a mixed population of microorganisms present in the sample. For example, a complex medium containing 10% NaCl is very useful in isolating species of *Staphylococcus* from skin, because the salt inhibits growth of most other bacteria. In practical applications such as in the food industry, viable counting on both complex and selective media allows for both quantitative and qualitative assessments of the microorganisms present in a food product. That is, with a single sample one medium may be employed for a total count and a second medium used to target a particular organism, such as a specific pathogen. Targeted counting is common in wastewater and other water analyses. For instance, enteric bacteria originate from feces and are easy to target 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 is unsafe for human contact.

The Great Plate Count Anomaly

Direct microscopic counts of natural samples typically reveal far more organisms 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. Some microbiologists have referred to this as "the great plate count anomaly."

Why do plate counts show lower numbers of cells than direct microscopic counts? One obvious factor is that microscopic methods count dead cells, whereas viable methods by definition will 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 4.1 and 4.2). Thus, one medium and set of growth conditions can at best be expected to support the growth of only a subset of the total microbial community. If this subset makes up, for example, 10^6 cells/g in 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.

Plate count results can thus carry a large caveat. Targeted plate counts using highly selective media, as in, for example, the microbial analysis of sewage or food, can often yield quite reliable data since the physiology of the targeted organisms are known. 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.

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}.
- What is the "great plate count anomaly"?

5.11 Turbidimetric Methods

During exponential growth, all cellular components increase in proportion to the increase in cell numbers. Thus, instead of measuring changes in cell number over time, one could instead measure the increase in protein, DNA, or dry weight of a culture as a barometer of growth. However, since cells are actual objects instead of dissolved substances, cells scatter light, and a rapid and quite useful method of estimating cell numbers based on this property is *turbidity*.

A suspension of cells looks cloudy (turbid) to the eye because cells scatter light passing through the suspension. The more cells that are present, the more light is scattered, and hence the more turbid the suspension. What is actually assessed in a turbidimetric measurement is total cell mass. However, because cell mass is proportional to cell number, turbidity can be used as a measure of cell numbers and can also be used to follow an increase in cell numbers of a growing culture.

Optical Density

Turbidity is measured with a spectrophotometer, an instrument that passes light through a cell suspension and measures the unscattered light that emerges; the more cells that are present in the cell suspension, the more turbid it will be (**Figure 5.17**). A spectrophotometer employs a prism or diffraction grating to generate incident light of a specific wavelength (Figure 5.17*a*). Commonly used wavelengths for bacterial turbidity measurements include 480 nm (blue), 540 nm (green), 600 nm (orange), 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 called *optical density* (*OD*) at the wavelength specified, for example, OD_{540} for spectrophotometric measurements at 540 nm (Figure 5.17). The term *absorbance* (A), for example A_{540}, is also commonly used, but it should be understood that it is light scattering, not absorbance per se, that is being measured in turbidimetric measurements of microbial growth.

Relating OD to Cell Numbers

For unicellular organisms, OD 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), dry weight, or protein content to turbidity. As can be seen in such a plot, proportionality only holds within limits (Figure 5.17*c*). Thus, at high cell concentrations, light scattered away from the spectrophotometer's photocell by one cell can be scattered back by another. To the photocell, this is as if light had never been scattered in the first place. At such high cell densities, the one-to-one correspondence between cell number and turbidity deviates from linearity, and OD measurements become less accurate. However, up to this limit, turbidity measurements can be accurate measures of cell number or dry weight. Also, because different organisms differ in size and shape, equal cell numbers of two different bacterial species will not necessarily yield the same OD. Thus, to relate OD to actual cell numbers, a standard curve relating these two

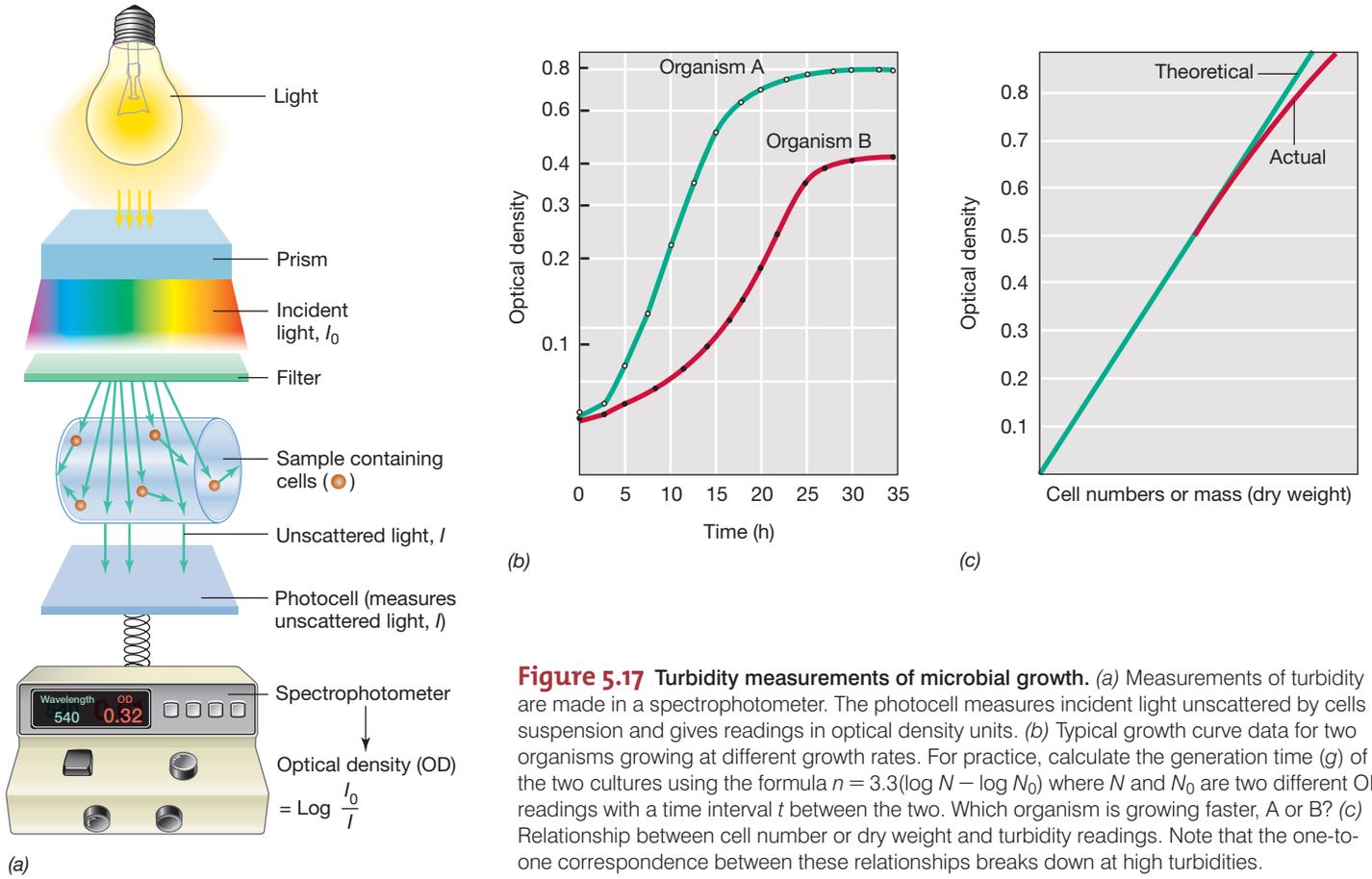


Figure 5.17 Turbidity measurements of microbial growth. *(a)* Measurements of turbidity are made in a spectrophotometer. The photocell measures incident light unscattered by cells in suspension and gives readings in optical density units. *(b)* Typical growth curve data for two organisms growing at different growth rates. For practice, calculate the generation time (*g*) 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.

parameters must be made for each different organism grown routinely in the laboratory.

Turbidity measurements have the virtue of being quick and easy to perform. Turbidity measurements can typically be made without destroying or significantly disturbing the sample. For these reasons, turbidity measurements are widely employed to monitor growth of microbial cultures. The same sample can be checked repeatedly and the measurements plotted on a semilogarithmic plot versus time. From these, it is easy to calculate the generation time and other parameters of the growing culture (Figure 5.17*b*).

Turbidity measurements are sometimes problematic. Although many microorganisms grow in even suspensions in liquid medium, many others do not. Some bacteria 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 grow in films on the sides of tubes or other growth vessels, mimicking in laboratory culture how they actually grow in nature (see the Microbial Sidebar "Microbial Growth in the Real World: Biofilms"). Thus for ODs to accurately reflect cell mass (and thus cell numbers) in a liquid culture, clumping and biofilms have to be minimized. This can often 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 biofilms.

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.

IV Temperature and Microbial Growth

The activities of microorganisms including growth are greatly affected by the chemical and physical state of their environment. Many environmental factors can be considered. However, four key factors control the growth of all microorganisms: temperature, pH, water availability, and oxygen; we consider each of these here. Some other factors can potentially affect the growth of microorganisms, such as pressure and radiation. These more specialized environmental factors will be considered later in this book when we encounter microbial habitats in which they play major roles. However, it is important to remember that for the successful culture of any microorganism, both medium and growth conditions must be suitable.

Microbial Growth in the Real World: Biofilms

In this chapter we have discussed several ways in which microbial growth can be measured, including microscopic methods, viable counts, and measurements of light scattering (turbidity) by cells suspended in a liquid culture. The turbidimetric measures of bacterial growth assume that cells remain evenly distributed in their liquid growth medium. Under these conditions, the optical density of a culture is proportional to the log of the number of cells in suspension (Figure 1). This floating lifestyle, called *planktonic,* is the way some bacteria, for example, organisms that inhabit the water column of a lake, actually live in nature. However, many other microorganisms are *sessile*, meaning that they grow attached to a surface. These attached cells can then develop into **biofilms**.

Humans encounter bacterial biofilms on a daily basis, for example, when cleaning out a pet's water bowl that has been sitting unattended for a few days or when you sense with your tongue the "film" that develops on your unbrushed teeth.

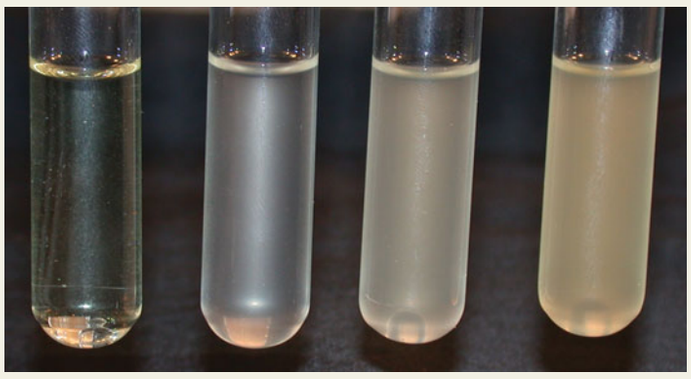
A biofilm is an attached polysaccharide matrix containing bacterial cells. Biofilms form in stages: (1) reversible attachment of planktonic cells, (2) irreversible attachment of the same cells, (3) cell growth and production of polysaccharide, and (4) further development to form the tenacious and nearly impenetrable mature biofilm. In the early stages of biofilm formation, the attachment of bacterial cells to a surface triggers biofilm-specific gene expression. Genes that encode proteins that produce cell surface polysaccharides are transcribed, and the increased amount of slime facilitates attachment of more cells. Eventually, through growth and recruitment, entire microbial communities develop within the slimy polysaccharide matrix.

Bacterial biofilms can dramatically affect humans. For example, bacterial infections are often linked to pathogens that develop in biofilms during the disease process. The genetic disease cystic fibrosis (CF) is characterized by development of a biofilm containing *Pseudomonas aeruginosa* and other bacteria in the lungs of CF patients (Figure 2). The biofilm matrix, which contains alginate and other polysaccharides as well as bacterial DNA, greatly reduces the ability of antimicrobial agents, such as antibiotics, to penetrate, and thus bacteria within the biofilm may be unaffected by the drugs. Bacterial biofilms have also been implicated in difficult-to-treat infections of implanted medical devices, such as replacement heart valves and artificial joints.

Biofilms are also a major problem in industry. Microbial biofilms can cause fouling of equipment and the contamination of products, especially if the flowing liquid contains good microbial substrates, such as in milk. Biofilms can also do long-term damage to water distribution facilities and other public utilities. Biofilms that develop in bulk storage containers, such as fuel storage tanks, can contaminate the fuel and cause souring from chemicals, such as hydrogen sulfide (H_2S), excreted by the biofilm bacteria.

Biofilms are a common form of bacterial growth in nature. Not only does the biofilm offer protection from harmful chemicals, the thick matrix of the biofilm provides a barrier to grazing by protists and prevents bacterial cells from being washed away into a less-favorable habitat. So, while optical densities give us a laboratory picture of the perfectly suspended bacterial culture, in the "real" world bacterial growth in the biofilm state is often observed.

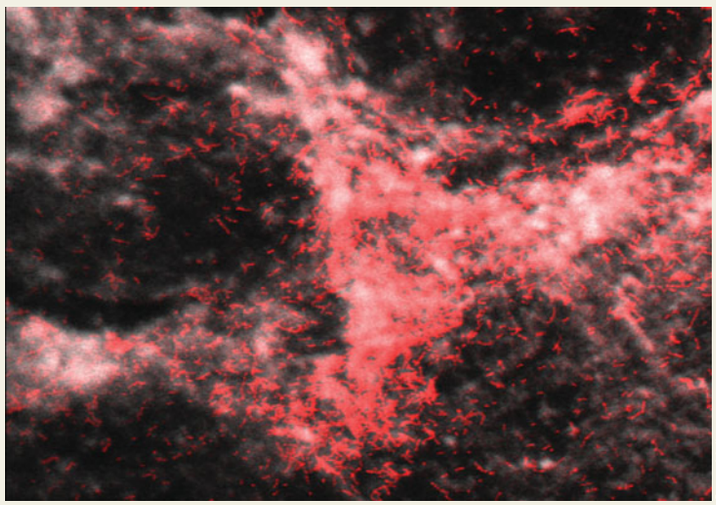
We examine biofilms in more detail in our focus on surfaces as microbial habitats in Sections 23.4 and 23.5.



Deborah O. Jung

OD_{540} 0 0.18 0.45 0.68

Figure 1 **Liquid cultures of *Escherichia coli.*** In these cultures cells are in a planktonic state and are evenly suspended in the medium. The increasing (left to right) optical density (OD_{540}) of each culture is shown below the tube. Optical density is a measure of light scattering and was measured at 540 nm here as described in Figure 5.17*a*.



Soren Molin

Figure 2 **Fluorescently stained cells of *Pseudomonas aeruginosa.*** The cells were from a sputum sample of a cystic fibrosis patient. The red cells are *P. aeruginosa* and the white material is alginate, a polysaccharide-like material that is produced by cells of *P. aeruginosa*.

5.12 Effect of Temperature on Growth

Temperature is probably *the* most important environmental factor affecting the growth and survival 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 for growth vary greatly among different microorganisms and usually reflect the temperature range and average temperature of their habitats.

Cardinal Temperatures

Temperature affects microorganisms in two opposing ways. As temperatures rise, chemical and enzymatic reactions in the cell proceed at more rapid rates and growth becomes faster; however, above a certain temperature, cell components may be irreversibly damaged. Thus, as the temperature is increased within a given range, growth and metabolic function increase up to a point where denaturation reactions set in. Above this point, cell functions fall to zero. 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 (**Figure 5.18**). These three temperatures, called the **cardinal temperatures**, are characteristic for any given microorganism.

The cardinal temperatures of different microorganisms differ widely; some organisms have temperature optima as low as 4°C and some higher than 100°C. The temperature range throughout which microorganisms grow is even wider than this, from below freezing to well above the boiling point of water. However, no single organism can grow over this whole temperature range, as the range for any given organism is typically 25–40 degrees.

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, as previously discussed, the cytoplasmic membrane must be in a semifluid state for transport (↻ Section 3.5) and other important functions to take place. An organism's minimum temperature may well be governed by membrane functioning; 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. The growth temperature *optimum* reflects a state in which all or most cellular components are functioning at their maximum rate and is typically closer to the maximum than to the minimum (see Figure 5.19).

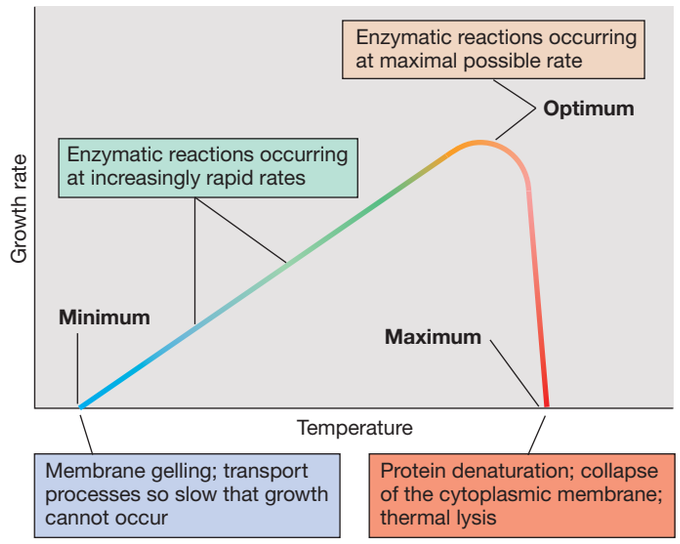


Figure 5.18 The cardinal temperatures: minimum, optimum, and maximum. The actual values may vary greatly for different organisms (see Figure 5.19).

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 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 5.19**).

Mesophiles are widespread in nature. They are found in warm-blooded animals and in terrestrial and aquatic environments in temperate and tropical latitudes. Psychrophiles and thermophiles are found in unusually cold and unusually hot environments, respectively. Hyperthermophiles are found in extremely hot habitats such as hot springs, geysers, and deep-sea hydrothermal vents.

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, near the high end for prokaryotes (Figure 5.19).

We now turn to the interesting cases of microorganisms growing at very low or very high temperatures, some of the physiological problems they face, and some of the biochemical solutions they have evolved to survive under 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?

5.13 Microbial Life in the Cold

Because humans live and work on the surface of Earth where temperatures are generally moderate, it is natural to consider very hot and very cold environments as "extreme." However, many microbial habitats are very hot or very cold. The organisms that live in these environments are therefore called **extremophiles** (↻ Section 2.8 and Table 2.1). Interestingly, in most cases these organisms have evolved to grow *optimally* at their environmental temperature. We consider the biology of these fascinating organisms here and in the next section.

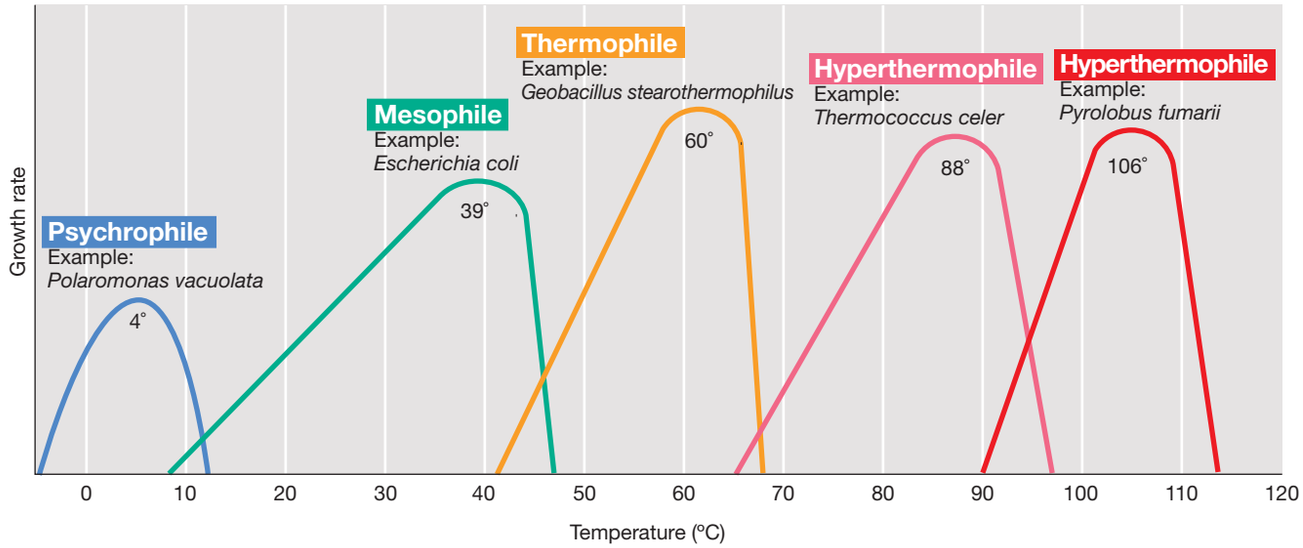


Figure 5.19 **Temperature and growth response in different temperature classes of microorganisms.**
The temperature optimum of each example organism is shown on the graph.

Cold Environments

Much of Earth's surface is cold. The oceans, which make up over half of Earth's surface, have an average temperature of 5°C, and the depths of the open oceans have constant temperatures of 1–3°C. Vast land areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer (**Figure 5.20**). These cold environments are not sterile, as viable microorganisms can be found growing at any low-temperature environment in which some liquid water remains. Salts and other solutes, for example, depress the freezing point of water and allow microbial growth to occur below the freezing point of pure water, 0°C. But even in frozen materials there are often small pockets of liquid water where solutes have concentrated and microorganisms can metabolize and grow. Within glaciers, for example, there exists a network of liquid water channels in which prokaryotes thrive and reproduce.

In considering cold environments, 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 a period of ice cover in the winter, but the time that the water is at 0°C is relatively brief. Such highly variable environments are less favorable habitats for cold-active microorganisms than are the constantly cold environments characteristic of polar regions, high altitudes, and the depths of the oceans. For example, lakes in the Antarctic McMurdo Dry Valleys contain a permanent ice cover several meters thick (Figure 5.20*d*). The water column below the ice in these lakes remains at 0°C or colder year round and is thus an ideal habitat for cold-active microorganisms.

Psychrophilic Microorganisms

As noted earlier, organisms with low temperature optima are called *psychrophiles*. A psychrophile is defined as an organism with an optimal growth temperature of 15°C or lower, a maximum growth temperature below 20°C, and a minimal growth temperature at 0°C or lower. Organisms that grow at 0°C but have optima of 20–40°C are called **psychrotolerant**.

Psychrophiles are found in environments that are constantly cold and may be rapidly killed by warming, even to as little as 20°C. For this reason, their laboratory study requires that great care be taken to ensure that they never warm up during sampling, transport to the laboratory, isolation, or other manipulations. In open ocean waters, where temperatures remain constant at about 3°C, various cold-active *Bacteria* and *Archaea* are present, although only a relatively few have been isolated in laboratory culture. Temperate environments, which warm up in summer, cannot support the heat-sensitive psychrophiles because they cannot survive the warming.

Psychrophilic microbial communities containing algae and bacteria grow in dense masses within and under sea ice (frozen seawater that forms seasonally) in polar regions (Figures 5.20*a*, *b*), and are also often present on the surfaces of snowfields and glaciers at such densities that they impart a distinctive coloration to the surface (**Figure 5.21*a***). The common snow alga *Chlamydomonas nivalis* is an example of this, its spores responsible for the brilliant red color of the snow surface (Figure 5.21*b*). This green 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.

In addition to snow algae, several psychrophilic bacteria have been isolated, mostly from marine sediments or sea ice, or from Antarctica. Some of these, particularly isolates from sea ice such as *Polaromonas* (Figure 5.20*c*), have very low growth temperature optima and maxima (4°C and 12°C, respectively). A species of the sea ice bacterium *Psychromonas* grows at −12°C, the lowest temperature for any known bacterium. But even this is

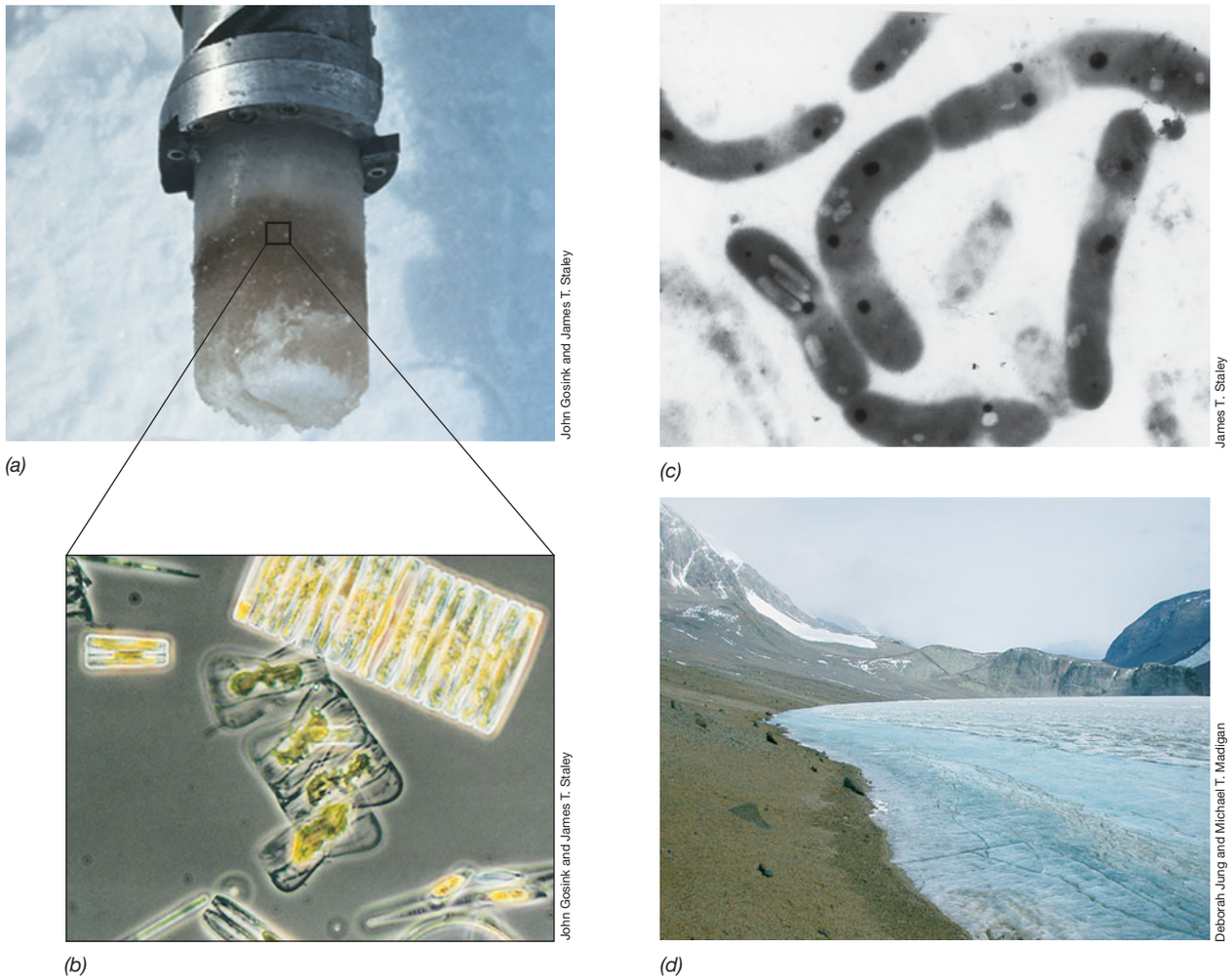


Figure 5.20 Antarctic microbial habitats and microorganisms. *(a)* A core of frozen seawater from McMurdo Sound, Antarctica. The core is about 8 cm wide. Note the dense coloration due to pigmented microorganisms. *(b)* Phase-contrast micrograph of phototrophic microorganisms from the core shown in part 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. *(d)* Photo of the surface of Lake Bonney, McMurdo Dry Valleys, Antarctica. Like many other Antarctic lakes, Lake Bonney, which is about 40 m deep, remains permanently frozen and has an ice cover of about 5 m. The water column of Lake Bonney remains near 0°C and contains both oxic and anoxic zones; thus both aerobic and anaerobic microorganisms inhabit the lake. However, no higher eukaryotic organisms inhabit Dry Valley lakes, making them uniquely microbial ecosystems.

unlikely to be the lower temperature limit for bacterial growth, which is probably closer to −20°C. Pockets of liquid water can exist at −20°C, and studies have shown that enzymes from cold-active bacteria can still function under such conditions. Growth rates at such cold temperatures would likely be extremely low, with doubling times of months, or even years. But if an organism can grow, even if only at a very slow rate, it can remain competitive and maintain a population in its habitat.

Psychrotolerant Microorganisms

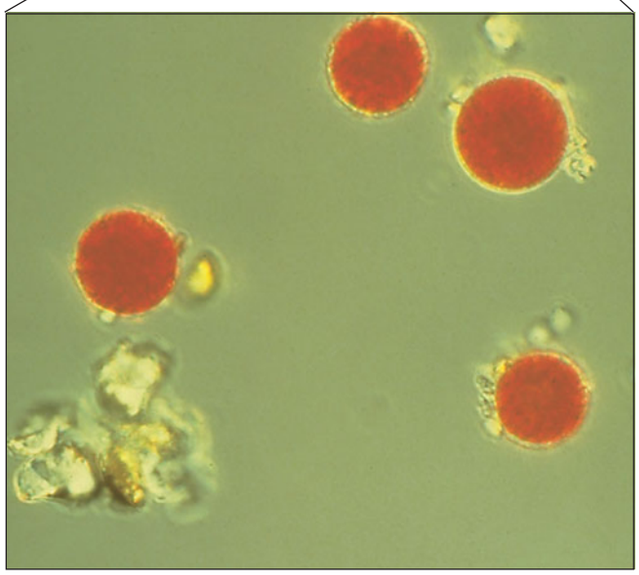
Psychrotolerant microorganisms are more widely distributed in nature than are psychrophiles and can be isolated from soils and water in temperate climates, as well as from meat, milk and other dairy products, cider, vegetables, and fruit stored at refrigeration temperatures (~4°C). As noted, psychrotolerant microorganisms grow best at a temperature between 20 and 40°C. Moreover, although psychrotolerant microorganisms do grow at 0°C, most do not grow very well at that temperature, and one must often wait several weeks before visible growth is seen in laboratory cultures. Various *Bacteria*, *Archaea*, and microbial eukaryotes are psychrotolerant.

Molecular Adaptations to Psychrophily

Psychrophiles produce enzymes that function 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 is clearly linked to protein structure. For example, several cold-active enzymes show greater amounts of α-helix and lesser amounts of β-sheet secondary structure (Section 6.21) than do enzymes that are inactive in the cold.

Katherine M. Brock

(a)



T. D. Brock

(b)

Figure 5.21 **Snow algae.** *(a)* Snow bank in the Sierra Nevada, California, with red coloration caused by the presence of snow algae. Pink snow such as this is common on summer snow banks at high altitudes throughout the world. *(b)* Photomicrograph of red-pigmented spores of the snow alga *Chlamydomonas nivalis*. The spores germinate to yield motile green algal cells. Some strains of snow algae are true psychrophiles but many are psychrotolerant, growing best at temperatures above 20°C. From a phylogenetic standpoint, *C. nivalis* is a green alga, and these organisms are covered in Section 20.20.

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 than their mesophilic and thermophilic counterparts (↻ Figure 6.29 for structures of amino acids). Moreover, cold-active proteins tend to have lower numbers of weak bonds, such as hydrogen and ionic bonds, and fewer specific interactions between regions (domains) compared with proteins from organisms that grow best at higher temperatures. Collectively, these molecular features probably help these enzymes remain flexible and functional under cold conditions.

Another feature of psychrophiles is that compared to mesophiles, transport processes (↻ Section 3.5) function optimally at low temperature. This is an indication that the cytoplasmic membranes of psychrophiles are structurally modified in such a way that low temperatures do not inhibit membrane functions. Cytoplasmic membranes from psychrophiles tend to have a higher content of unsaturated and shorter-chain fatty acids. This helps the membrane remain in a semifluid state at low temperatures (membranes composed of predominantly saturated or long-chain fatty acids would become stiff and waxlike at low temperatures). In addition, the lipids of some psychrophilic bacteria contain polyunsaturated fatty acids, something very uncommon in prokaryotes. For example, the psychrophilic bacterium *Psychroflexus* contains fatty acids with up to five double bonds. These fatty acids remain more flexible at low temperatures than saturated or monounsaturated fatty acids.

Other molecular adaptations to cold include "cold-shock" proteins and cryoprotectants. Cold-shock proteins are a series of proteins that have several functions including helping the cell maintain other proteins in an active form under cold conditions or binding to specific mRNAs and facilitating their translation. These mRNAs include, in particular, those that encode other cold-functional proteins, most of which are not produced when the cell is growing near its temperature optimum. 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.

Freezing

Although temperatures below −20°C prevent microbial growth, such temperatures, or even much colder ones, do not necessarily cause microbial death. Microbial cells can continue to metabolize at temperatures far beneath that which will support growth. For example, microbial respiration as measured by CO_2 production has been shown in tundra soils at temperatures as low as −39°C. Thus, enzymes continue to function at temperatures far below those that allow for cell growth.

The medium in which cells are suspended also affects their sensitivity to freezing. If cryoprotectants such as glycerol or dimethyl sulfoxide (DMSO) are added to a cell suspension, this depresses the freezing point and prevents ice crystal formation. To freeze cells for long-term preservation, cells are typically suspended in

growth medium containing 10% DMSO or glycerol and quickly frozen at −80°C (ultracold-freezer temperature) or −196°C (liquid nitrogen temperature). Properly prepared frozen cells that do not thaw and refreeze can remain viable for decades or even longer.

We now travel to the other end of the thermometer and look at microorganisms growing at high temperatures.

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?

5.14 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 present are typically highly adapted to their environmental temperature.

Thermal Environments

Organisms whose growth temperature optimum exceeds 45°C are called *thermophiles* and those whose optimum exceeds 80°C are called *hyperthermophiles* (Figure 5.19). Temperatures as high as these are found only in certain areas. For example, the surface of soils subject to full sunlight can be heated to above 50°C at midday, and some surface soils may become warmed to even 70°C. Fermenting materials such as compost piles and silage can also reach temperatures of 70°C. However, the most extensive and extreme high-temperature environments in nature are associated with volcanic phenomena. These include, in particular, hot springs.

Many hot springs have temperatures at or near boiling, and steam vents (fumaroles) may reach 150–500°C. Hydrothermal vents in the bottom of the ocean can have temperatures of 350°C or greater (↻ Section 23.12). Hot springs exist 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 1–2°C over many years. In addition, different springs have different chemical compositions and pH values. Above 65°C, only prokaryotes are present (**Table 5.1**), but the diversity of *Bacteria* and *Archaea* may be extensive.

Hyperthermophiles in Hot Springs

In boiling hot springs (**Figure 5.22**), a variety of hyperthermophiles are typically present, including both chemoorganotrophic and chemolithotrophic species. Growth of natural populations of hyperthermophiles can be studied very simply by immersing a microscope slide into a spring and then retrieving it a few days later; microscopic examination reveals colonies of prokaryotes that have developed from single cells that attached to and grew on the glass surface (Figure 5.22*b*). Scrapings of cell material can then be used for molecular analyses. Such ecological studies of organisms living in boiling springs have shown that growth rates are often rapid; doubling times as short as 1 h have been recorded.

Table 5.1 *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
Prokaryotes	
Bacteria	
Cyanobacteria	73
Anoxygenic phototrophs	70–73
Chemoorganotrophs/chemolithotrophs	95
Archaea	
Chemoorganotrophs/chemolithotrophs	122

Cultures of many hyperthermophiles have been obtained, and a variety of morphological and physiological types of both *Bacteria* and *Archaea* are known. Phylogenetic studies using ribosomal RNA (rRNA) gene sequencing have shown great evolutionary diversity among these hyperthermophiles as well. Some hyperthermophilic *Archaea* have growth-temperature optima above 100°C, while no species of *Bacteria* are known to 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. Such organisms typically originate from undersea hot springs (hydrothermal vents). The most heat-tolerant of all known *Archaea* is *Methanopyrus*, a methanogenic organism capable of growth at 122°C.

Thermophiles

Many thermophiles (optima 45–80°C) are also present in hot springs and other thermal environments. In hot springs, as boiling water overflows the edges of the spring and flows away from the source, it gradually cools, setting up a thermal gradient. Along this gradient, various microorganisms grow, with different species

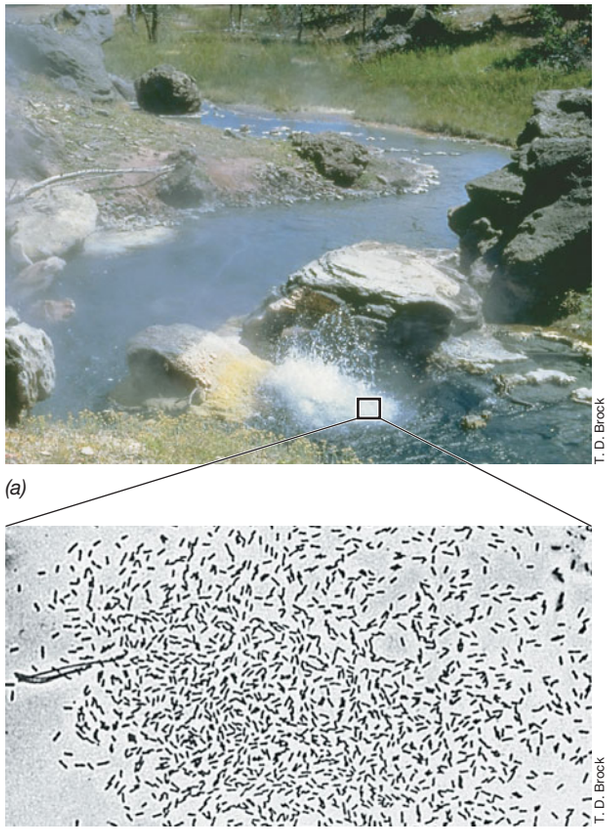


Figure 5.22 **Growth of hyperthermophiles in boiling water.** *(a)* Boulder Spring, a small boiling spring in Yellowstone National Park. This spring is superheated, having a temperature 1–2°C above the boiling point. The mineral deposits around the spring consist mainly of silica and sulfur. *(b)* Photomicrograph of a microcolony of prokaryotes that developed on a microscope slide immersed in such a boiling spring.

growing in the different temperature ranges (**Figure 5.23**). By studying the species distribution along such thermal gradients and by examining hot springs and other thermal habitats at different temperatures around the world, it has been possible to determine the upper temperature limits for each type of organism (Table 5.1). From this information we can conclude that (1) prokaryotic organisms are able to grow at far higher temperatures than are eukaryotes, (2) the most thermophilic of all prokaryotes are certain species of *Archaea*, and (3) nonphototrophic organisms can grow at higher temperatures than can phototrophic organisms.

Thermophilic prokaryotes have also been found in artificial thermal environments, such as hot water heaters. The domestic or industrial hot water heater has a temperature of 60–80°C and is therefore a favorable habitat for the growth of thermophilic prokaryotes. Organisms resembling *Thermus aquaticus*, a common hot spring thermophile, have been isolated from domestic and industrial hot water heaters. Electric power plants, hot water discharges, and other artificial thermal sources also provide sites where thermophiles can grow. Many of these organisms can be readily isolated using complex media incubated at the temperature of the habitat from which the sample originated.

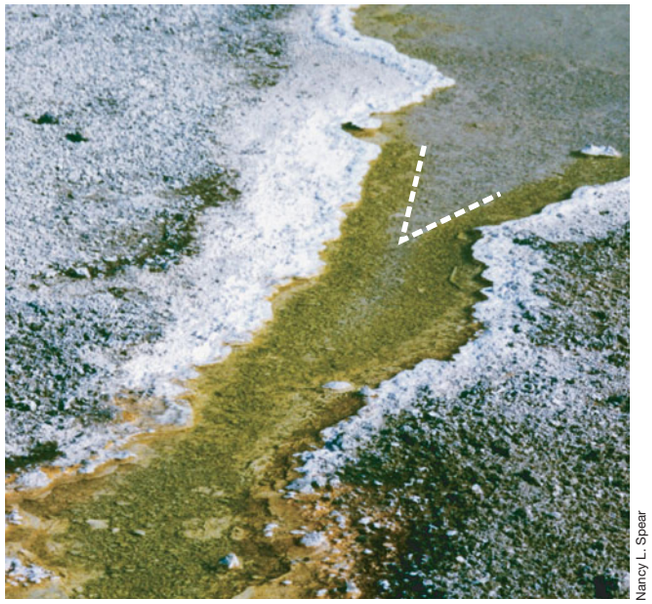


Figure 5.23 **Growth of thermophilic cyanobacteria in a hot spring in Yellowstone National Park.** Characteristic V-shaped pattern (shown by the dashed white lines) formed by cyanobacteria at the upper temperature for phototrophic life, 70–74°C, in the thermal gradient formed from a boiling hot spring. The pattern develops because the water cools more rapidly at the edges than in the center of the channel. The spring flows from the back of the picture toward the foreground. The light-green color is from a high-temperature strain of the cyanobacterium *Synechococcus*. As water flows down the gradient, the density of cells increases, less thermophilic strains enter, and the color becomes more intensely green.

Protein Stability at High Temperatures

How do thermophiles and hyperthermophiles survive at high temperature? First, their enzymes and other proteins are much more heat-stable than are those of mesophiles and actually function *optimally* at high temperatures. How is heat stability achieved? Amazingly, studies of several heat-stable enzymes have shown that they often differ very little in amino acid sequence from heat-sensitive forms of the enzymes that catalyze the same reaction in mesophiles. It appears that critical amino acid substitutions at only a few locations in the enzyme allow the protein to fold in such a way that it is heat-stable.

Heat stability of proteins in hyperthermophiles is also bolstered by an increased number of ionic bonds between basic and acidic amino acids and their often highly hydrophobic interiors; the latter property is a natural resistance to unfolding in an aqueous cytoplasm. Finally, solutes such as di-inositol phosphate, diglycerol phosphate, and mannosylglycerate are produced at high levels in certain hyperthermophiles, and these may also help stabilize their proteins against thermal degradation.

Membrane Stability at High Temperatures

In addition to enzymes and other macromolecules in the cell, the cytoplasmic membranes of thermophiles and hyperthermophiles must be heat-stable. We mentioned earlier that psychrophiles

have membrane lipids rich in unsaturated fatty acids, making the membranes semifluid and functional at low temperatures. Conversely, thermophiles typically have lipids rich in saturated fatty acids. This feature allows the membranes to remain stable and functional at high temperatures. Saturated fatty acids form a stronger hydrophobic environment than do unsaturated fatty acids, which helps account for 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 (↻ Figures 3.6*c* and 3.7*b*) bonded by ether linkage to glycerol phosphate. In addition, however, the architecture of the cytoplasmic membranes of hyperthermophiles takes a unique twist: The membrane forms a lipid *monolayer* rather than a lipid *bilayer* (↻ Figure 3.7*e*). This structure prevents the membrane from melting (peeling apart) at the high growth temperatures of hyperthermophiles. We consider other aspects of heat stability in hyperthermophiles, including that of DNA stability, in Chapter 19.

Thermophily and Biotechnology

Thermophiles and hyperthermophiles are interesting for more than just basic biological reasons. These organisms offer some major advantages for industrial and biotechnological processes, many of which can be run more rapidly and efficiently at high temperatures. For example, enzymes from thermophiles and hyperthermophiles are widely used in industrial microbiology. Such enzymes can catalyze biochemical reactions at high temperatures and are in general more stable than enzymes from mesophiles, thus prolonging the shelf life of purified enzyme preparations.

A classic example of a heat-stable enzyme of great importance to biology is the DNA polymerase isolated from *T. aquaticus*. *Taq polymerase*, as this enzyme is known, has been used to automate the repetitive steps in the polymerase chain reaction (PCR) technique (↻ Section 6.11), an extremely important tool for biology. Several other uses of heat-stable enzymes and other heat-stable cell products are also known or are being developed for industrial applications.

MiniQuiz

- Which domain of prokaryotes includes species with optima of >100°C? What special techniques are required to culture them?
- What is the structure of membranes of hyperthermophilic *Archaea*, and why might this structure be useful for growth at high temperature?
- What is *Taq* polymerase and why is it important?

V Other Environmental Factors Affecting Growth

Temperature has a major effect on the growth of microorganisms. But many other factors do as well, chief among these being pH, osmolarity, and oxygen.

5.15 Acidity and Alkalinity

Acidity or alkalinity of a solution is expressed by its **pH** on a scale on which neutrality is pH 7 (**Figure 5.24**). pH values less than 7 are *acidic* and those greater than 7 are *alkaline*. It is important to remember that pH is a logarithmic function—a change of one pH unit corresponds to a 10-fold change in hydrogen ion (H^+) concentration. Thus, vinegar (pH near 2) and household ammonia (pH near 11) differ in hydrogen ion concentration by a billionfold.

Every microorganism has a pH range within which growth is possible and typically shows a well-defined growth pH optimum. Most organisms show a growth range of 2–3 pH units. Most natural environments have a pH between 4 and 9, and organisms with optima in this range are most commonly encountered. Only a few species can grow at pH values of lower than 3 or greater than 9. Some terms used to describe organisms that grow best in particular pH ranges are shown in **Table 5.2**.

Acidophiles

Organisms that grow optimally at a pH value in the range termed *circumneutral* (pH 5.5 to 7.9) are called **neutrophiles** (Table 5.2). 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, including in particular the genus *Acidithiobacillus*. An even more restricted group grow best below pH 2 and those with pH optima below 1 are

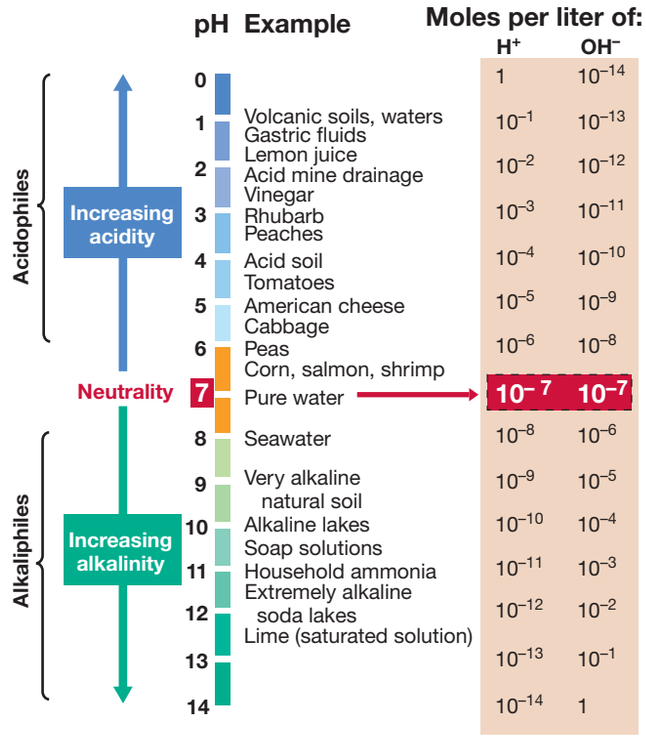


Figure 5.24 **The pH scale.** Although some microorganisms can live at very low or very high pH, the cell's internal pH remains near neutrality.

Table 5.2 *Relationships of microorganisms to pH*

Physiological class (optima range)	*Appoximate 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*.

extremely rare. Most acidophiles cannot grow at pH 7 and many cannot grow at greater than two pH 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 hydrogen ions are actually *required* for membrane stability. For example, the most acidophilic prokaryote known is *Picrophilus oshimae*, a species of *Archaea* that grows optimally at pH 0.7 and 60°C (the organism is also a thermophile). Above pH 4, cells of *P. oshimae* spontaneously lyse. As one would expect, *P. oshimae* inhabits extremely acidic thermal soils associated with volcanic activity.

Alkaliphiles

A few extremophiles have very high pH optima for growth, sometimes as high as pH 10, and some of these can still grow at even higher pH. Microorganisms showing growth 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 prokaryotes are certain *Bacillus* species, such as *Bacillus firmus*. This organism is alkaliphilic, but has an unusually broad pH range for growth, from 7.5 to 11. Some extremely alkaliphilic bacteria are also halophilic (salt-loving), and most of these are *Archaea* (Section 19.2). Some phototrophic purple bacteria (Section 17.2) are strongly alkaliphilic. Certain alkaliphiles have industrial uses because they produce hydrolytic enzymes, such as proteases and lipases, which are excreted from the cell and thus function well at alkaline pH. These enzymes are produced on a large scale and added as supplements to laundry detergents.

Alkaliphiles are of basic interest for several reasons but particularly because of the bioenergetic problems they face living at such high pH. For example, imagine trying to generate a proton motive force (Section 4.10) when the external surface of your cytoplasmic membrane is so alkaline. Some strategies for this are known. In *B. firmus* a sodium (Na^+) motive force rather than a proton motive force drives transport reactions and motility. Remarkably, however, a proton motive force drives ATP synthesis in cells of *B. firmus*, even though the external membrane surface is awash in hydroxyl ions (OH^-). It is thought that H^+ are in some way kept very near the outer surface of the cytoplasmic membrane such that they cannot combine with OH^- to form water.

Internal Cell pH

The optimal pH for growth of any organism is a measure of the pH of the *extracellular* environment only. The *intracellular* pH must remain relatively close to neutrality to prevent destruction of macromolecules in the cell. For the majority of microorganisms whose pH optimum for growth is between pH 6 and 8, organisms called *neutrophiles*, the cytoplasm remains neutral or very nearly so. However, in acidophiles and alkaliphiles the internal pH can vary from neutrality. For example, in the previously mentioned acidophile *P. oshimae*, the internal pH has been measured at pH 4.6, and in extreme alkaliphiles an intracellular pH as high as 9.5 has been measured. If these are not the lower and upper limits of cytoplasmic pH, respectively, they are extremely close to the limits. This is because DNA is acid-labile and RNA is alkaline-labile; if a cell cannot maintain these key macromolecules in a stable state, it obviously cannot survive.

Buffers

In batch cultures, the pH can change during growth as the result of metabolic reactions of microorganisms that consume or produce acidic or basic substances. Thus, *buffers* are frequently added to microbial culture media to keep the pH relatively constant. However, a given buffer works over only a narrow pH range. Hence, different buffers must be used at different pH values.

For near neutral pH ranges, potassium phosphate (KH_2PO_4) and calcium carbonate ($CaCO_3$) are good buffers. Many other buffers for use in microbial growth media or for the assay of enzymes extracted from microbial cells are available, and the best buffering system for one organism or enzyme may be considerably different from that for another. Thus, the optimal buffer for use in a particular situation must usually be determined empirically. For assaying enzymes *in vitro*, though, a buffer that works well in an assay of the enzyme from one organism will usually work well for assaying the same enzyme from other organisms.

MiniQuiz

- What is the increase in concentration of H^+ when going from pH 7 to pH 3?
- What terms are used to describe organisms whose growth pH optimum is either very high or very low?

5.16 Osmotic Effects

Water is the solvent of life, and water availability is an important factor affecting the growth of microorganisms. Water availability not only depends on the absolute water content of an environment, that is, how moist or dry it is, but it is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in the water. Dissolved substances have an affinity for water, which makes the water associated with solutes less available to organisms.

Table 5.3 *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	*Saccharomyces bailii, 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 prokaryotes or fungi capable of growth in culture media adjusted to the stated water activity.

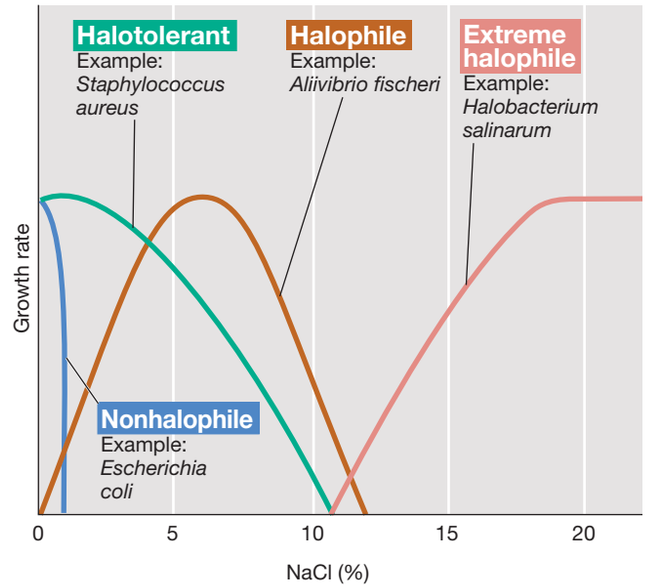


Figure 5.25 **Effect of sodium chloride (NaCl) concentration on growth of microorganisms of different salt tolerances or requirements.** The optimum NaCl concentration for marine microorganisms such as *Aliivibrio fischeri* is about 3%; for extreme halophiles, it is between 15 and 30%, depending on the organism.

Water Activity and Osmosis

Water availability is expressed in physical terms as **water activity**. Water activity, abbreviated a_w, is defined as the ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure of pure water. Thus, values of a_w vary between 0 and 1; some representative values are given in Table 5.3. Water activities in agricultural soils generally range between 0.90 and 1.

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 water tends to diffuse into the cell. Under such conditions, the cell is said to be in *positive water balance*. However, when a cell finds itself in an environment where the solute concentration exceeds that of the cytoplasm, water will flow out of the cell. This can cause serious problems if a cell has no way to counteract it because a dehydrated cell cannot 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. Marine microorganisms usually have a specific requirement for NaCl and grow optimally at the water activity of seawater (Figure 5.25). Such organisms are called **halophiles**. By definition, halophiles require at least some NaCl for growth, but the optimum varies with the organism and its habitat. For example, marine organisms typically grow best with 1–4% NaCl, organisms from hypersaline environments (environments that are more salty than seawater), 3–12%, and organisms from extremely hypersaline environments require even higher levels of NaCl. And the growth requirement for NaCl cannot be replaced by KCl, meaning that halophiles have an absolute requirement for Na^+.

Most microorganisms are unable to cope with environments of very low water activity and either die or become dehydrated and dormant under such conditions. **Halotolerant** organisms can tolerate some reduction in the a_w of their environment, but grow best in the absence of the added solute (Figure 5.25). By contrast, some organisms thrive and indeed require low water activity for growth. These organisms are of interest not only from the standpoint of their adaptation to life under these conditions, but also from an applied standpoint, for example, in the food industry, where solutes such as salt and sucrose are commonly used as preservatives to inhibit microbial growth.

Organisms capable of growth in very salty environments are called **extreme halophiles** (Figure 5.25). These organisms require 15–30% NaCl, depending on the species, for optimum growth. Organisms able to live in environments high in sugar as a solute are called **osmophiles**, and those able to grow in very dry environments (made dry by lack of water rather than from dissolved solutes) are called **xerophiles**. Examples of these various classes of organisms are given in Table 5.4.

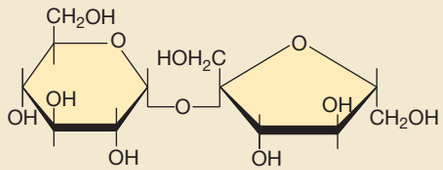
Compatible Solutes

When an organism grows in a medium with a low water activity, it can obtain water from its environment only by increasing its internal solute concentration and driving water in by osmosis. The internal solute concentration can be raised by either pumping solutes into the cell from the environment or by synthesizing a solute. Many organisms are known that employ one or the other of these strategies, and several examples are given in Table 5.4.

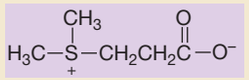
The solute used inside the cell for adjustment of cytoplasmic water activity must be noninhibitory to macromolecules within the cell. Such compounds are called **compatible solutes**, and

Table 5.4 *Compatible solutes of microorganisms*

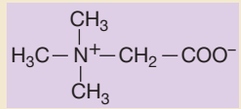
Organism	*Major cytoplasmic solute(s)*	*Minimum a_w for growth*
Nonphototrophic *Bacteria*/freshwater cyanobacteria	Amino acids (mainly glutamate or proline[a])/sucrose, trehalose[b]	0.98–0.90
Marine cyanobacteria	α-Glucosylglycerol[b]	0.92
Marine algae	Mannitol,[b] various glycosides, dimethylsulfoniopropionate	0.92
Salt lake cyanobacteria	Glycine betaine	0.90–0.75
Halophilic anoxygenic phototrophic purple *Bacteria*	Glycine betaine, ectoine, trehalose[b]	0.90–0.75
Extremely halophilic *Archaea* and some *Bacteria*	KCl	0.75
Dunaliella (halophilic green alga)	Glycerol	0.75
Xerophilic and osmophilic yeasts	Glycerol	0.83–0.62
Xerophilic filamentous fungi	Glycerol	0.72–0.61



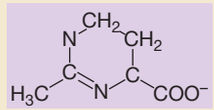
Sucrose



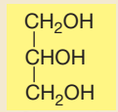
Dimethylsulfoniopropionate



Glycine betaine



Ectoine



Glycerol

[a] See Figure 6.29 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.

several such solutes are known. These substances are typically highly water-soluble molecules, such as sugars, alcohols, or amino acid derivatives (Table 5.4). The compatible solute of extremely halophilic *Archaea*, such as *Halobacterium*, and a very few extremely halophilic *Bacteria*, is KCl (↻ Section 19.2).

The concentration of compatible solute in a cell is a function of the level of solutes present in its environment; however, in any given organism the maximal amount of compatible solute is a genetically directed characteristic. As a result, different organisms can tolerate different ranges of water potential (Tables 5.3 and 5.4). Nonhalotolerant, halotolerant, halophilic, and extremely halophilic microorganisms (Figure 5.25) are to a major extent defined by their genetic capacity to produce or accumulate compatible solutes.

Gram-positive cocci of the genus *Staphylococcus* are notoriously halotolerant (in fact, a common isolation procedure for them is to use media containing 7.5–10% NaCl), and these organisms use the amino acid proline as a compatible solute. Glycine betaine is an analog of the amino acid glycine in which the hydrogen atoms on the amino group are replaced by methyl groups. This places a positive charge on the N atom and greatly increases solubility. Glycine betaine is widely distributed as a compatible solute among halophilic phototrophic bacteria, as is ectoine, a cyclic derivative of the amino acid aspartate (Table 5.4). Other common compatible solutes include various sugars and dimethylsulfoniopropionate produced by marine algae, and glycerol produced by several organisms including xerophilic fungi that grow at the lowest water potential of all known organisms (Table 5.4).

MiniQuiz

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

5.17 Oxygen and Microorganisms

Because animals require molecular oxygen (O_2), it is easy to assume that all organisms require O_2. However, this is not true; many microorganisms can, and some must, live in the total absence of oxygen.

Table 5.5 *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*). Representative of either domain of prokaryotes 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.

Oxygen is poorly soluble in water, and because of the constant respiratory activities of microorganisms in aquatic habitats, O_2 can quickly become exhausted. Thus, *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. In these anoxic habitats, microorganisms, particularly prokaryotes, thrive.

Oxygen Classes of Microorganisms

Microorganisms vary in their need for, or tolerance of, O_2. In fact, microorganisms can be grouped according to their relationship with O_2, as outlined in **Table 5.5**. **Aerobes** can grow at full oxygen tensions (air is 21% O_2) and respire O_2 in their metabolism. Many aerobes can even tolerate elevated concentrations of oxygen (hyperbaric oxygen). **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 their limited capacity to respire or because they contain some O_2-sensitive molecule such as an O_2-labile enzyme. Many aerobes are **facultative**, meaning that under the appropriate nutrient and culture conditions they can grow under either oxic or anoxic conditions.

Some organisms cannot respire oxygen; such organisms 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 use it, and **obligate anaerobes**, which are inhibited or even killed by O_2 (Table 5.5). The reason obligate anaerobes are killed by O_2 is unknown, but it is likely because they are unable to detoxify some of the products of O_2 metabolism (Section 5.18).

So far as is known, obligate anaerobiosis is found in only three groups of microorganisms: a wide variety of *Bacteria* and *Archaea*, a few fungi, and a few protozoa. The best-known group of obligately anaerobic *Bacteria* belongs to the genus *Clostridium*, a group of gram-positive endospore-forming rods. Clostridia are widespread in soil, lake sediments, and the intestinal tracts of warm-blooded animals, and are often responsible for spoilage of canned foods. Other obligately anaerobic organisms are the methanogens and many other *Archaea*, the sulfate-reducing and acetogenic bacteria, and many of the bacteria that inhabit the animal gut and oral cavity. Among obligate anaerobes, however, the sensitivity to O_2 varies greatly. Some species can tolerate traces of O_2 or even full exposure to O_2, whereas others cannot.

Culture Techniques for Aerobes and Anaerobes

For the growth of many 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 simple 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. Aerobes typically grow better with forced aeration than with O_2 supplied only by diffusion.

For the culture of anaerobes, the problem is not to provide O_2, but to exclude it. Obligate anaerobes vary in their sensitivity to O_2, and procedures are available for reducing the O_2 content of cultures. Some of these techniques are simple and suitable mainly for less O_2-sensitive organisms; others are more complex, but necessary for growth of obligate anaerobes. Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stoppers provide suitably anoxic conditions for organisms that are not overly sensitive to small amounts of O_2. A chemical called a *reducing agent* may be added to culture media; the reducing agent reacts with oxygen and reduces it to water (H_2O). An example is thioglycolate, which is added to thioglycolate broth, a medium commonly used to test an organism's requirements for O_2 (**Figure 5.26**).

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

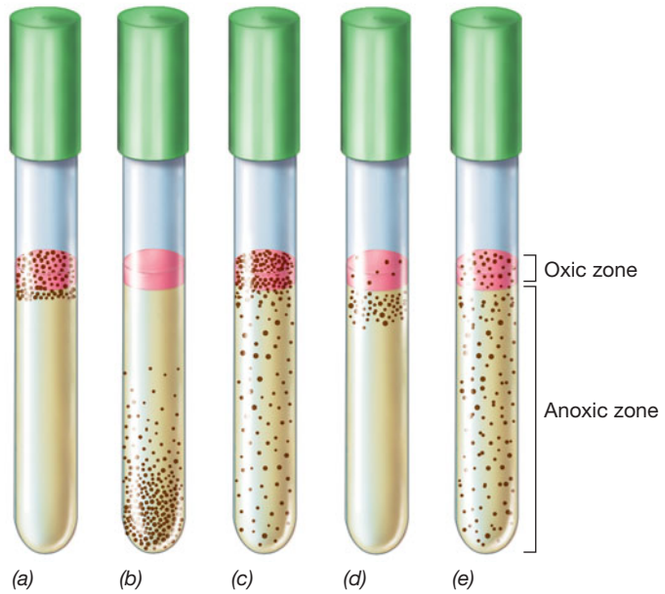


Figure 5.26 **Growth versus oxygen (O_2) concentration.** From left to right, aerobic, anaerobic, facultative, microaerophilic, and aerotolerant anaerobe growth, as revealed by the position of microbial colonies (depicted here as black dots) within tubes of thioglycolate broth culture medium. A small 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.

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 added to the medium to differentiate oxic from anoxic 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 5.26).

To remove all traces of O_2 for the culture of strict anaerobes, one can place an oxygen-consuming system in a jar holding the tubes or plates. One of the simplest devices for this is an anoxic jar, a glass or gas-impermeable plastic jar fitted with a gastight seal within which tubes, plates, or other containers are placed for incubation (**Figure 5.27*a***). The air in the jar is replaced with a mixture of H_2 and CO_2, and in the presence of a palladium catalyst, the traces of O_2 left in the jar and culture medium are consumed in the formation of water ($H_2 + O_2 \rightarrow H_2O$), eventually leading to anoxic conditions.

For obligate anaerobes it is usually necessary to not only remove all traces of O_2, but also to carry out all manipulations of cultures in a completely anoxic atmosphere. Strict anaerobes can be killed by even a brief exposure to O_2. In these cases, a culture medium is first boiled to render it O_2-free, then a reducing agent such as H_2S is added, and the mixture is sealed under an O_2-free gas. All manipulations are carried out under a jet of sterile O_2-free H_2 or N_2 that is directed into the culture vessel when it is open, thus driving out any O_2 that might enter. For extensive research on anaerobes, special devices called *anoxic glove bags* permit work with open cultures in completely anoxic atmospheres (Figure 5.27*b*).

Deborah O. Jung and M. T. Madigan

(a)

Coy Laboratory Products

(b)

Figure 5.27 **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.

MiniQuiz

- How does an obligate aerobe differ from a facultative aerobe?
- How does a reducing agent work? Give an example of a reducing agent.

5.18 Toxic Forms of Oxygen

O_2 is a powerful oxidant and the best electron acceptor for respiration. But O_2 can also be a poison to obligate anaerobes. Why? It turns out that O_2 itself is not poisonous, but instead it is toxic derivatives of oxygen that can damage cells that are not prepared to deal with them. We consider this topic here.

Oxygen Chemistry

Oxygen in its ground state is called *triplet* oxygen ($^3O_2$). However, other electronic configurations of oxygen are possible, and most are toxic to cells. One major form of toxic oxygen is *singlet* oxygen ($^1O_2$), a higher-energy form of oxygen in which outer shell electrons surrounding the nucleus become highly reactive and can carry out spontaneous and undesirable oxidations within the cell. Singlet oxygen is produced both photochemically and biochemically, the latter through the activity of various peroxidase enzymes. Organisms that frequently encounter singlet oxygen, such as airborne bacteria and phototrophic microorganisms, often contain colored pigments called *carotenoids*, which function to convert singlet oxygen to nontoxic forms.

Superoxide and Other Toxic Oxygen Species

Besides singlet oxygen, many other toxic forms of oxygen exist, including *superoxide anion* (O_2^-), *hydrogen peroxide* (H_2O_2), and *hydroxyl radical* (OH•). All of these are produced as by-products of the reduction of O_2 to H_2O in respiration (**Figure 5.28**). Flavoproteins, quinones, and iron–sulfur proteins (↻ Section 4.9), found in virtually all cells, can also catalyze the reduction of O_2 to O_2^-. Thus, whether or not it can respire O_2 (Table 5.5), a cell can be exposed to toxic oxygen species from time to time.

Superoxide anion and OH• are strong oxidizing agents and can oxidize virtually any organic compound in the cell, including macromolecules. Peroxides such as H_2O_2 can also damage cell components but are not as toxic as O_2^- or OH•. The latter is the most reactive of all toxic oxygen species but is transient and quickly removed in other reactions. Later we will see that certain cells of the immune system make toxic oxygen species for the specific purpose of killing microbial invaders (↻ Section 29.1).

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

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

Figure 5.28 **Four-electron reduction of O_2 to H_2O by stepwise addition of electrons.** All the intermediates formed are reactive and toxic to cells, except for water, of course.

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

(a) **Catalase**

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

(b) **Peroxidase**

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

(c) **Superoxide dismutase**

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

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

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

(e) **Superoxide reductase**

Figure 5.29 **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.

Superoxide Dismutase and Other Enzymes That Destroy Toxic Oxygen

With so many toxic oxygen derivatives to deal with, it is not surprising that organisms have evolved enzymes that destroy these compounds (**Figure 5.29**). Superoxide and H_2O_2 are the most common toxic oxygen species, so enzymes that destroy these compounds are widely distributed. The enzyme *catalase* attacks H_2O_2, forming O_2 and H_2O; its activity is illustrated in Figure 5.29*a* and **Figure 5.30**. Another enzyme that destroys H_2O_2 is *peroxidase* (Figure 5.29*b*), which differs from catalase in that it requires a reductant, usually NADH, for activity and produces only H_2O as a product. Superoxide is destroyed by the enzyme *superoxide dismutase*, an enzyme that generates H_2O_2 and O_2 from two molecules of O_2^- (Figure 5.29*c*). Superoxide dismutase and catalase thus work in concert to bring about the conversion of O_2^- to O_2 plus H_2O (Figure 5.29*d*).

Cells of aerobes and facultative aerobes typically contain both superoxide dismutase and catalase. Superoxide dismutase is an essential enzyme for aerobes, and the absence of this enzyme in obligate anaerobes was originally thought to explain why O_2 is toxic to them (but see next paragraph). Some aerotolerant anaerobes, such as the lactic acid bacteria, also lack superoxide dismutase, but

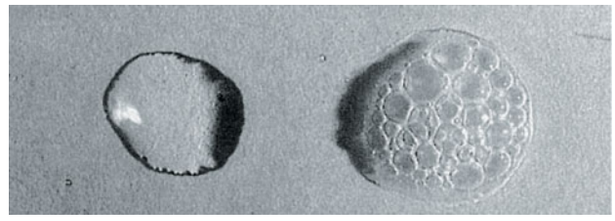


Figure 5.30 **Method for testing a microbial culture for the presence of catalase.** A heavy loopful of cells from an agar culture was mixed on a slide (right) with a drop of 30% hydrogen peroxide. The immediate appearance of bubbles is indicative of the presence of catalase. The bubbles are O_2 produced by the reaction $H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$.

they use protein-free manganese (Mn^{2+}) complexes 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 may have functioned as a primitive form of this enzyme in ancient anaerobic organisms faced with O_2 for the first time when cyanobacteria first appeared on Earth.

Superoxide Reductase

Another means of superoxide disposal is present in certain obligately anaerobic *Archaea*. In the hyperthermophile *Pyrococcus furiosus*, for example, superoxide dismutase is absent, but a unique enzyme, *superoxide reductase*, is present and functions to remove O_2^-. However, unlike superoxide dismutase, superoxide reductase reduces O_2^- to H_2O_2 without the production of O_2 (Figure 5.29*e*), thus avoiding exposure of the organism to O_2. The electron donor for superoxide reductase activity is rubredoxin, an iron–sulfur protein with low reduction potential. *P. furiosus* also lacks catalase, an enzyme that, like superoxide dismutase, also generates O_2 (Figure 5.29*a*). Instead, the H_2O_2 produced by superoxide reductase is removed by the activity of peroxidase-like enzymes that yield H_2O as a final product (Figure 5.29*b*).

Superoxide reductases are present in many other obligate anaerobes as well, such as sulfate-reducing bacteria (*Bacteria*) and methanogens (*Archaea*), as well as in certain microaerophilic species of *Bacteria*, such as *Treponema*. Thus these organisms, previously thought to be O_2-sensitive because they lacked superoxide dismutase, can indeed consume superoxide. The sensitivity of these organisms to O_2 may therefore be for entirely different and as yet unknown reasons.

Many obligately anaerobic hyperthermophiles such as *Pyrococcus* inhabit deep-sea hydrothermal vents (Section 23.12) but are quite tolerant of cold, oxic conditions. Although they do not grow under these conditions, superoxide reductase presumably prevents their killing when they are exposed to O_2. It is thought that O_2 tolerance may be an important factor enabling transport of these organisms in fully oxic ocean water from one deep-sea hydrothermal system to another.

MiniQuiz

- How does superoxide dismutase protect a cell from toxic oxygen?
- How does the activity of superoxide dismutase differ from that of superoxide reductase?

Big Ideas

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 the actual division event that yields two daughter cells. Most microorganisms grow by binary fission.

5.2
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.

5.3
MreB protein helps define cell shape, and in rod-shaped cells, MreB forms a cytoskeletal coil that directs 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. Shape and cell division proteins in eukaryotes have prokaryotic counterparts.

5.4
During bacterial growth new cell wall material is synthesized by the insertion of new glycan tetrapeptide units into preexisting wall material. 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.

5.5
Microbial populations show a characteristic type of growth pattern called exponential growth. A plot of the logarithm of cell numbers versus time is called a semilogarithmic plot and can be used to derive the doubling time of the exponentially growing population.

5.6
From knowledge of the initial and final cell numbers and the time of exponential growth, the generation time and growth rate constant of a cell population can be calculated directly. Key parameters here are n, the number of generations; t, time; and g, generation time. The generation time is expressed as $g = t/n$.

5.7
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 or toxic products build up, growth ceases and the population enters the stationary phase. If incubation continues, cells may begin to die.

5.8
The chemostat is an open system used to maintain cell populations in exponential growth for extended periods. In a chemostat, the rate at which a culture is diluted with fresh growth medium controls the 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.

5.9
Cell counts can be done under the microscope using special counting chambers. Microscopic counts measure the total number of cells in the sample and are very useful for assessing a microbial habitat for total cell numbers. Certain stains can be used to target specific cell populations in a sample.

5.10
Viable cell counts (plate 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 how they are used, plate counts can be fairly accurate assessments or highly unreliable.

5.11
Turbidity measurements are an indirect but very rapid and useful method of measuring microbial growth. However, in order to relate a turbidity value to a direct cell number, a standard curve plotting these two parameters against one another must first be established.

5.12
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 and can differ dramatically from one organism to the next. Microorganisms can be grouped by their cardinal temperature as psychrophiles, mesophiles, thermophiles, and hyperthermophiles.

5.13
Organisms with cold temperature optima are called psychrophiles, and the most extreme representatives inhabit constantly cold environments. Psychrophiles have evolved macromolecules that function best at cold temperatures, but that can be unusually sensitive to warm temperatures.

5.14
Organisms with growth temperature optima between 45 and 80°C are called thermophiles and 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.

5.15
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 nucleic acid destruction.

5.16
The water activity of an aqueous environment is controlled by the dissolved solute concentration. To survive in high-solute environments, organisms produce or accumulate compatible solutes to maintain the cell in positive water balance. Some microorganisms grow best at reduced water potential and some even require high levels of salts for growth.

5.17
Aerobes require O_2 to live, whereas 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.

5.18
Several toxic forms of oxygen can form in the cell, but enzymes are present that neutralize most of them. Superoxide in particular seems to be a common toxic oxygen species.

Review of Key Terms

Acidophile an organism that grows best at low pH; typically below pH 5.5
Aerobe an organism that can use oxygen (O_2) in respiration; some require O_2
Aerotolerant anaerobe a microorganism unable to respire O_2 but whose growth is unaffected by oxygen
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
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
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
Divisome a complex of proteins that directs cell division processes in prokaryotes
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 salt (NaCl), usually greater than 10% and in some cases near to saturation, for growth
Extremophile an organism that grows optimally under one or more chemical or physical extremes, such as high or low temperature or pH
Facultative with respect to O_2, an organism that can grow in either its presence or absence
FtsZ a protein that forms a ring along the midcell division plane to initiate cell division
Generation time the time required for a population of microbial cells to double
Growth 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 prokaryote that has a growth temperature optimum of 80 °C or greater
Mesophile an organism that grows best at temperatures between 20 and 45 °C
Microaerophile an aerobic organism that can grow only when O_2 tensions are reduced from that present in air
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
pH the negative logarithm of the hydrogen ion (H^+) concentration of a solution
Psychrophile an organism with a growth temperature optimum of 15 °C or lower and a maximum growth temperature below 20 °C
Plate count a viable counting method where the number of colonies on a plate is used as a measure of cell numbers
Psychrotolerant capable of growing at low temperatures but having an optimum above 20 °C
Thermophile an organism whose growth temperature optimum lies between 45 and 80 °C
Transpeptidation formation of peptide cross-links between muramic acid residues in peptidoglycan synthesis
Viable capable of reproducing
Water activity the ratio of the vapor pressure of air in equilibrium with a solution to that of pure water
Xerophile an organism that is able to live, or that lives best, in very dry environments

Review Questions

1. Describe the key molecular processes that occur when a cell grows and divides (Section 5.1).
2. Describe the role of proteins present at the divisome (Section 5.2).
3. In what way do derivatives of the rod-shaped bacterium *Escherichia coli* carrying mutations that inactivate the protein MreB look different microscopically from wild-type (unmutated) cells? What is the reason for this (Section 5.3)?
4. Describe how new peptidoglycan subunits are inserted into the growing cell wall. How does the antibiotic penicillin kill bacterial cells, and why does it kill only growing cells (Section 5.4)?
5. What is the difference between the specific growth rate (k) of an organism and its generation time (g) (Sections 5.5 and 5.6)?
6. Describe the growth cycle of a population of bacterial cells from the time this population is first inoculated into fresh medium (Section 5.7).
7. How can a chemostat regulate growth rate and cell numbers independently (Section 5.8)?
8. What is the difference between a total cell count and a viable cell count (Sections 5.9 and 5.10)?
9. How can turbidity be used as a measure of cell numbers (Section 5.11)?
10. Examine the graph describing the relationship between growth rate and temperature (Figure 5.18). Give an explanation, in biochemical terms, of why the optimum temperature for an organism is usually closer to its maximum than its minimum (Section 5.12).
11. Describe a habitat where you would find a psychrophile; a hyperthermophile (Sections 5.13 and 5.14).
12. Concerning the pH of the environment and of the cell, in what ways are acidophiles and alkaliphiles different? In what ways are they similar (Section 5.15)?
13. Write an explanation in molecular terms for how a halophile is able to make water flow into the cell while growing in a solution high in NaCl (Section 5.16).
14. Contrast an aerotolerant and an obligate anaerobe in terms of sensitivity to O_2 and ability to grow in the presence of O_2. How does an aerotolerant anaerobe differ from a microaerophile (Section 5.17)?
15. Compare and contrast the enzymes catalase, superoxide dismutase, and superoxide reductase from the following points of view: substrates, oxygen products, organisms containing them, and role in oxygen tolerance of the cell (Section 5.18).

Application Questions

1. Calculate g and k in a growth experiment in which a medium was inoculated with 5×10^6 cells/ml of *Escherichia coli* cells and, following a 1-h lag, grew exponentially for 5 h, after which the population was 5.4×10^9 cells/ml.
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?

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