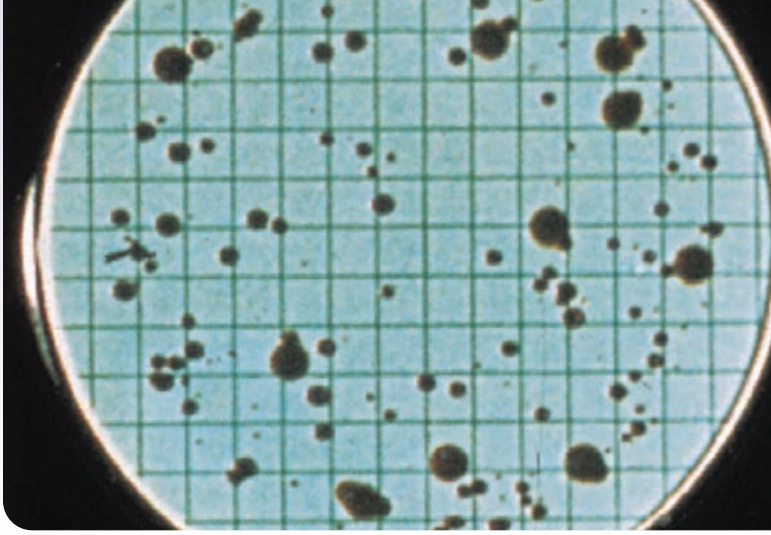
6 Microbial Growth



Membrane filters are used in counting microorganisms. This membrane has been used to obtain a total bacterial count using an indicator to color colonies for easy counting.

Preview

- Most procaryotes reproduce by binary fission. Although simpler than mitosis and meiosis, binary fission and the procaryotic cell cycle are still poorly understood.
- Growth is defined as an increase in cellular constituents and may result in an increase in a microorganism's size, population number, or both.
- When microorganisms are grown in a closed system, population growth remains exponential for only a few generations and then enters a stationary phase due to factors such as nutrient limitation and waste accumulation. In an open system with continual nutrient addition and waste removal, the exponential phase can be maintained for long periods.
- A wide variety of techniques can be used to study microbial growth by following changes in the total cell number, the population of viable microorganisms, or the cell mass.
- Water availability, pH, temperature, oxygen concentration, pressure, radiation, and a number of other environmental factors influence microbial growth. Yet many microorganisms, and particularly procaryotes, have managed to adapt and flourish under environmental extremes that would destroy most higher organisms.
- In the natural environment, growth is often severely limited by available nutrient supplies and many other environmental factors.
- Many microorganisms form biofilms in natural environments. This is an important survival strategy.
- Microbes can communicate with each other and behave cooperatively using population density-dependent signals.

In chapter 5 we emphasize that microorganisms need access to a source of energy and the raw materials essential for the construction of cellular components. All organisms must have carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, and a variety of minerals; many also require one or more special growth factors. The cell takes up these substances by membrane transport processes, the most important of which are facilitated diffusion, active transport, and group translocation. Eucaryotic cells also employ endocytosis.

Chapter 6 concentrates more directly on procaryotic reproduction and growth. First we describe binary fission, the type of cell division most frequently observed among procaryotes, and the procaryotic cell cycle. Cell reproduction leads to an increase in population size, so we consider growth and the ways in which it can be measured next. Then we discuss continuous culture techniques. An account of the influence of environmental factors on microbial growth and microbial growth in natural environments completes the chapter.

Growth may be defined as an increase in cellular constituents. It leads to a rise in cell number when microorganisms reproduce by processes like budding or binary fission. Growth also results when cells simply become longer or larger. If the microorganism is **coenocytic**—that is, a multinucleate organism in which nuclear divisions are not accompanied by cell divisions—growth results in an increase in cell size but not cell number. It is usually not convenient to investigate the growth and reproduction of individual microorganisms because of their small size. Therefore, when studying growth, microbiologists normally follow changes in the total population number.

6.1 The Procaryotic Cell Cycle

The **cell cycle** is the complete sequence of events extending from the formation of a new cell through the next division. Most procaryotes reproduce by **binary fission,** although some procaryotes

The paramount evolutionary accomplishment of bacteria as a group is rapid, efficient cell growth in many environments.

—J. L. Ingraham, O. Maaløe, and F. C. Neidhardt

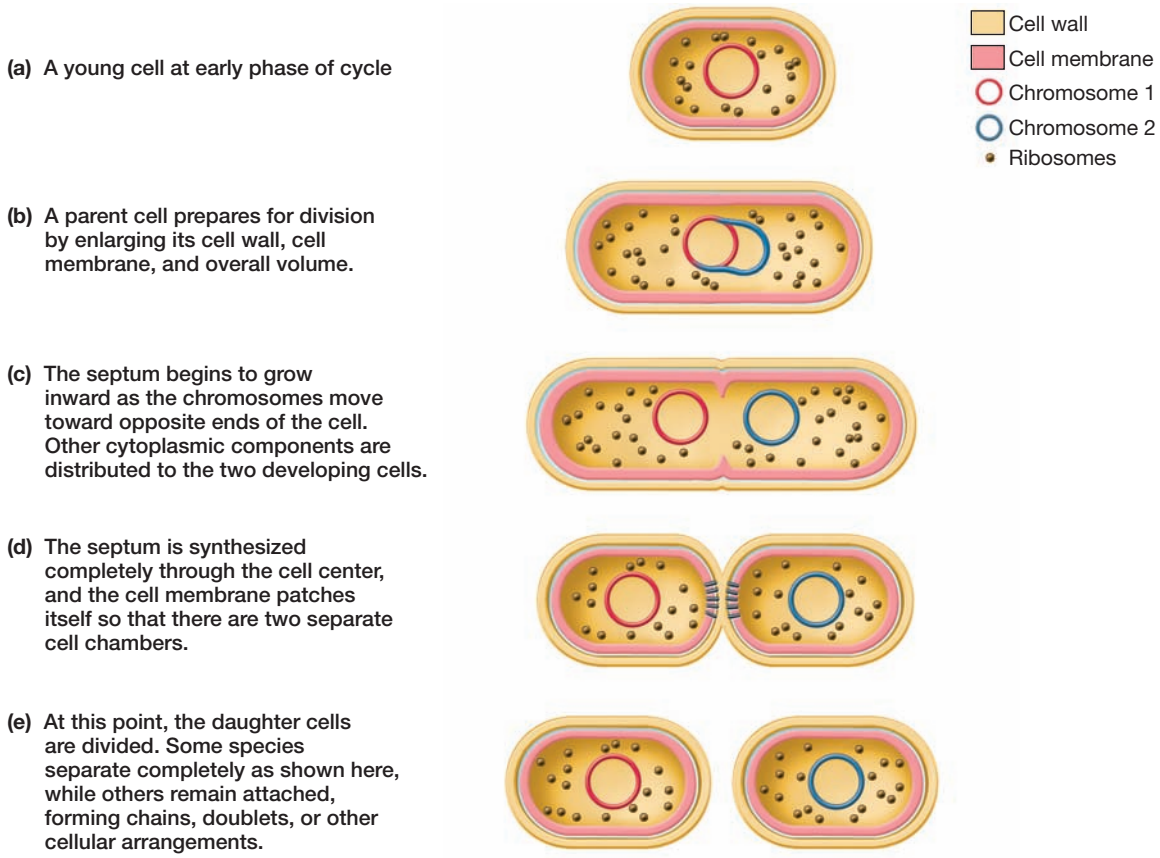


Figure 6.1 Binary Fission.

reproduce by budding, fragmentation, and other means (**figure 6.1**). Binary fission is a relatively simple type of cell division: the cell elongates, replicates its chromosome, and separates the newly formed DNA molecules so there is one chromosome in each half of the cell. Finally, a septum (or cross wall) is formed at midcell, dividing the parent cell into two progeny cells, each having its own chromosome and a complement of other cellular constituents.

Despite the apparent simplicity of the procaryotic cell cycle, it is poorly understood. The cell cycles of *Escherichia coli*, *Bacillus subtilis*, and the aquatic microbe *Caulobacter crescentus* have been examined extensively, and our understanding of the cell cycle is based largely on these studies. Two pathways function during the cell cycle (**figure 6.2**): one pathway replicates and partitions the DNA into the progeny cells, the other carries out cytokinesis (septum formation and formation of progeny cells). Although these pathways overlap, it is easiest to consider them separately.

Chromosome Replication and Partitioning

Recall that most procaryotic chromosomes are circular. Each circular chromosome has a single site at which replication starts called the **origin of replication,** or simply the origin (**figure 6.3**). Replication is completed at the **terminus,** which is located directly opposite the origin. In a newly formed *E. coli* cell, the chromosome is compacted and organized so that the origin and terminus are in opposite halves of the cell. Early in the cell cycle, the origin and terminus move to midcell and a group of proteins needed for DNA synthesis assemble to form the **replisome** at the origin. DNA replication proceeds in both directions from the origin and the parent DNA is thought to spool through the replisome, which remains relatively stationary. As progeny chromosomes are synthesized, the two newly formed origins move toward opposite ends of the cell, and the rest of the chromosome follows in an orderly fashion.

Although the process of DNA synthesis and movement seems rather straightforward, the mechanism by which chromosomes are partitioned to each daughter cell is not well understood. Surprisingly, a picture is emerging in which components of the cytoskeleton are involved. For many years, it was assumed that procaryotes were too small for eucaryotic-like cytoskeletal structures. However, a protein called **MreB,** which is similar to eucaryotic actin, seems to be involved in several processes, including determining cell shape and chromosome movement. MreB polymerizes (that is to say, MreB units are linked together) to form a spiral around the inside periphery of the cell (**figure 6.4*a***). One model suggests that the origin of each newly replicated chromosome associates with

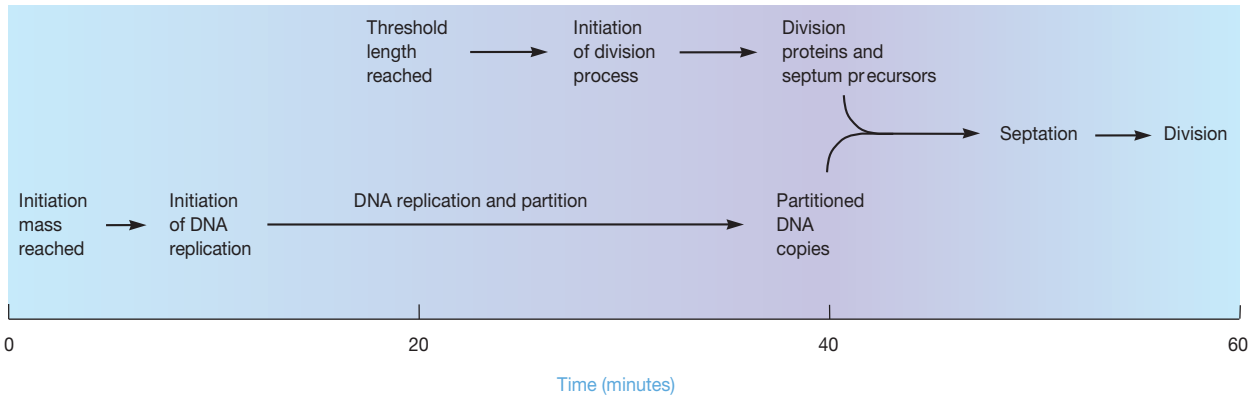


Figure 6.2 The Cell Cycle in *E. coli*. A 60-minute interval between divisions has been assumed for purposes of simplicity (the actual time between cell divisions may be shorter). *E. coli* requires about 40 minutes to replicate its DNA and 20 minutes after termination of replication to prepare for division. The position of events on the time line is approximate and meant to show the general pattern of occurrences.

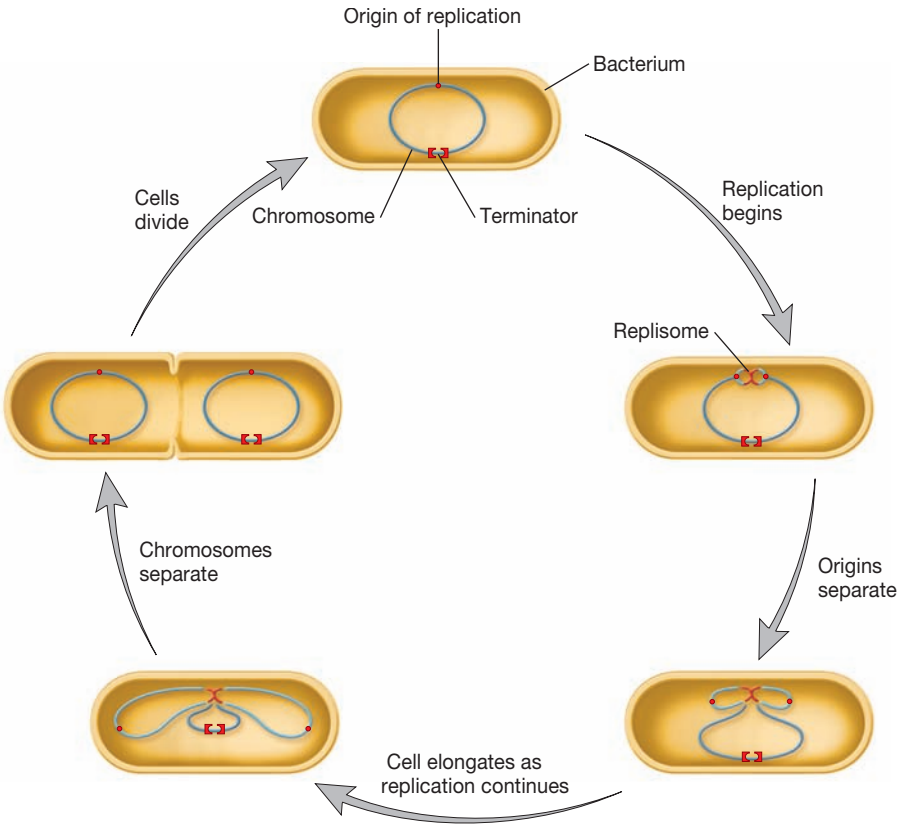


Figure 6.3 Cell Cycle of Slow-Growing *E. coli*. As the cell readies for replication, the origin migrates to the center of the cell and proteins that make up the replisome assemble. As replication proceeds, newly synthesized chromosomes move toward poles so that upon cytokinesis, each daughter cell inherits only one chromosome.

MreB, which then moves them to opposite poles of the cell. The notion that procaryotic chromosomes may be actively moved to the poles is further suggested by the fact that if MreB is mutated so that it can no longer hydrolyze ATP, its source of energy, chromosomes fail to segregate properly.

Cytokinesis

Septation is the process of forming a cross wall between two daughter cells. **Cytokinesis,** a term that has traditionally been used to describe the formation of two eucaryotic daughter cells, is now used to describe this process in procaryotes as well. Septation is

divided into several steps: (1) selection of the site where the septum will be formed; (2) assembly of a specialized structure called the **Z ring,** which divides the cell in two by constriction; (3) linkage of the Z ring to the plasma membrane and perhaps components of the cell wall; (4) assembly of the cell wall-synthesizing machinery; and (5) constriction of the Z ring and septum formation.

The assembly of the Z ring is a critical step in septation, as it must be formed if subsequent steps are to occur. The **FtsZ protein,** a tubulin homologue found in most bacteria and many archaea, forms the Z ring. FtsZ, like tubulin, polymerizes to form filaments, which are thought to create the meshwork that constitutes the Z ring. Numerous studies show that the Z ring is very dynamic, with portions of the meshwork being exchanged constantly with newly formed, short FtsZ polymers from the cytosol. Another protein, called MinCD, is an inhibitor of Z-ring assembly. Like FtsZ, it is very dynamic, oscillating its position from one end of the cell to the other, forcing Z-ring formation only at the center of the cell (figure 6.4*b*). Once the Z-ring forms, the rest of the division machinery is constructed, as illustrated in **figure 6.5**. First one or more anchoring proteins link the Z ring to the cell membrane. Then the cell wall-synthesizing machinery is assembled. The cytoplasmic matrix: The procaryotic cytoskeleton (section 3.3)

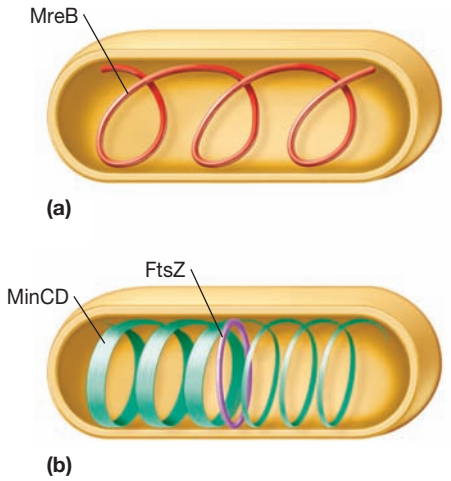


Figure 6.4 Cytoskeletal Proteins Involved in Cytokinesis in Rod-Shaped Bacteria. **(a)** The actin homolog MreB forms spiral filaments around the inside of the cell that help determine cell shape and may serve to move chromosomes to opposite cell poles. **(b)** The tubulin-like protein FtsZ assembles in the center of the cell to form a Z ring, which is essential for septation. MinCD, together with other Min proteins, oscillates from pole to pole, thereby preventing the formation of an off-center Z ring.

The final steps in division involve constriction of the Z ring, accompanied by invagination of the cell membrane and synthesis of the septal wall. Several models for Z-ring constriction have been proposed. One model holds that the FtsZ filaments are shortened by losing FtsZ subunits (i.e., depolymerization) at sites where the Z ring is anchored to the plasma membrane. This model is supported by the observation that Z rings of cells producing an excessive amount of FtsZ subunits fail to constrict.

DNA Replication in Rapidly Growing Cells

The preceding discussion of the cell cycle describes what occurs in slowly growing *E. coli* cells. In these cells, the cell cycle takes approximately 60 minutes to complete: 40 minutes for DNA replication and partitioning and about 20 minutes for septum formation and cytokinesis. However, *E. coli* can reproduce at a much more rapid rate, completing the entire cell cycle in about 20 minutes, despite the fact that DNA replication always requires at least 40 minutes. How can *E. coli* complete an entire cell cycle in 20 minutes when it takes 40 minutes to replicate its chromosome? *E. coli* accomplishes this by beginning a second round of DNA replication (and sometimes even a third or fourth round) before

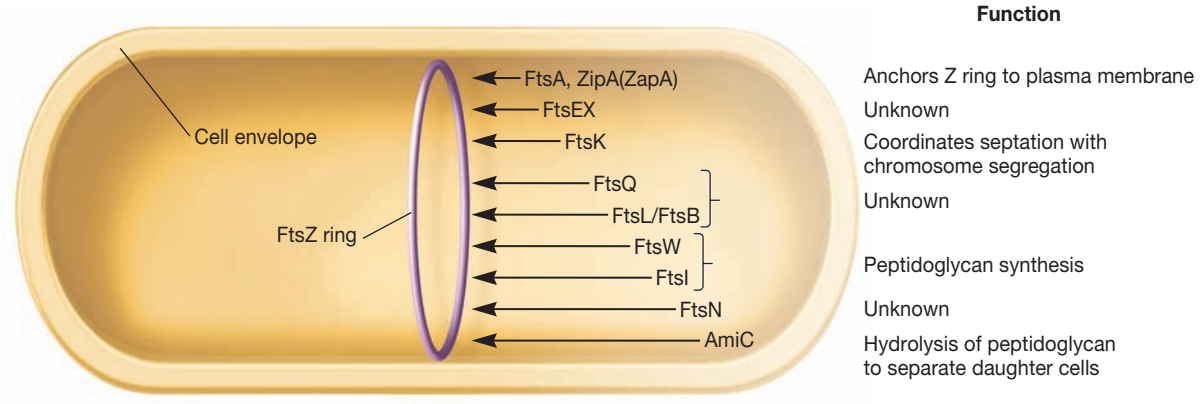


Figure 6.5 Formation of the Cell Division Apparatus in *E. coli*. The cell division apparatus is composed of numerous proteins that are thought to assemble in the order shown. The process begins with the polymerization of FtsZ to form the Z ring. Then FtsA and ZipA (possibly ZapA in *Bacillus subtilis*) proteins anchor the Z ring to the plasma membrane. Although numerous proteins are known to be part of the cell division apparatus, the functions of relatively few are known.

the first round of replication is completed. Thus the progeny cells receive two or more replication forks, and replication is continuous because the cells are always copying their DNA.

1. What two pathways function during the procaryotic cell cycle?
2. How does the procaryotic cell cycle compare with the eucaryotic cell cycle? List two ways they are similar; list two ways they differ.

6.2 The Growth Curve

Binary fission and other cell division processes bring about an increase in the number of cells in a population. Population growth is studied by analyzing the growth curve of a microbial culture. When microorganisms are cultivated in liquid medium, they usually are grown in a **batch culture** or closed system—that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase. The growth of microorganisms reproducing by binary fission can be plotted as the logarithm of the number of viable cells versus the incubation time. The resulting curve has four distinct phases (**figure 6.6**).

Lag Phase

When microorganisms are introduced into fresh culture medium, usually no immediate increase in cell number occurs, so this period is called the **lag phase.** Although cell division does not take place right away and there is no net increase in mass, the cell is synthesizing new components. A lag phase prior to the start of cell division can be necessary for a variety of reasons. The cells may be old and depleted of ATP, essential cofactors, and ribosomes; these must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. Here new enzymes would be needed to use different nutrients. Possibly the microorganisms have been injured and require time to recover. Whatever the causes, eventually the cells retool, replicate their DNA, begin to increase in mass, and finally divide.

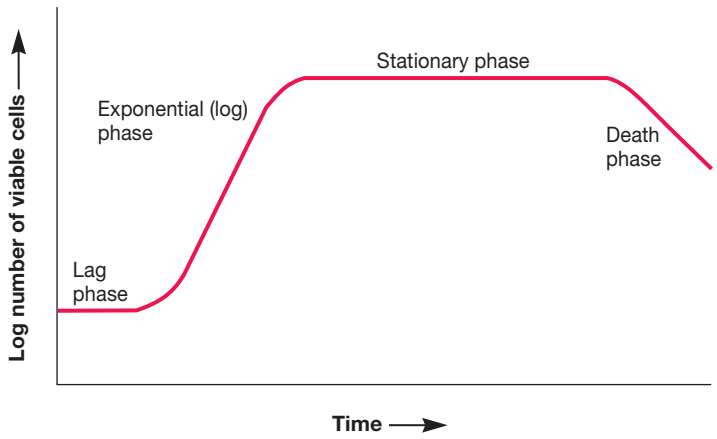


Figure 6.6 Microbial Growth Curve in a Closed System.
The four phases of the growth curve are identified on the curve and discussed in the text.

The lag phase varies considerably in length with the condition of the microorganisms and the nature of the medium. This phase may be quite long if the inoculum is from an old culture or one that has been refrigerated. Inoculation of a culture into a chemically different medium also results in a longer lag phase. On the other hand, when a young, vigorously growing exponential phase culture is transferred to fresh medium of the same composition, the lag phase will be short or absent.

Exponential Phase

During the **exponential** or **log phase,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the conditions under which they are growing. Their rate of growth is constant during the exponential phase; that is, the microorganisms are dividing and doubling in number at regular intervals. Because each individual divides at a slightly different moment, the growth curve rises smoothly rather than in discrete jumps (figure 6.6). The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies.

Exponential growth is **balanced growth.** That is, all cellular constituents are manufactured at constant rates relative to each other. If nutrient levels or other environmental conditions change, **unbalanced growth** results. This is growth during which the rates of synthesis of cell components vary relative to one another until a new balanced state is reached. Unbalanced growth is readily observed in two types of experiments: shift-up, where a culture is transferred from a nutritionally poor medium to a richer one; and shift-down, where a culture is transferred from a rich medium to a poor one. In a shift-up experiment, there is a lag while the cells first construct new ribosomes to enhance their capacity for protein synthesis. This is followed by increases in protein and DNA synthesis. Finally, the expected rise in reproductive rate takes place. In a shift-down experiment, there is a lag in growth because cells need time to make the enzymes required for the biosynthesis of unavailable nutrients. Consequently cell division and DNA replication continue after the shift-down, but net protein and RNA synthesis slow. The cells become smaller and reorganize themselves metabolically until they are able to grow again. Then balanced growth is resumed and the culture enters the exponential phase. These shift-up and shift-down experiments demonstrate that microbial growth is under precise, coordinated control and responds quickly to changes in environmental conditions.

When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present (**figure 6.7*a***). This is the basis of microbiological assays for vitamins and other growth

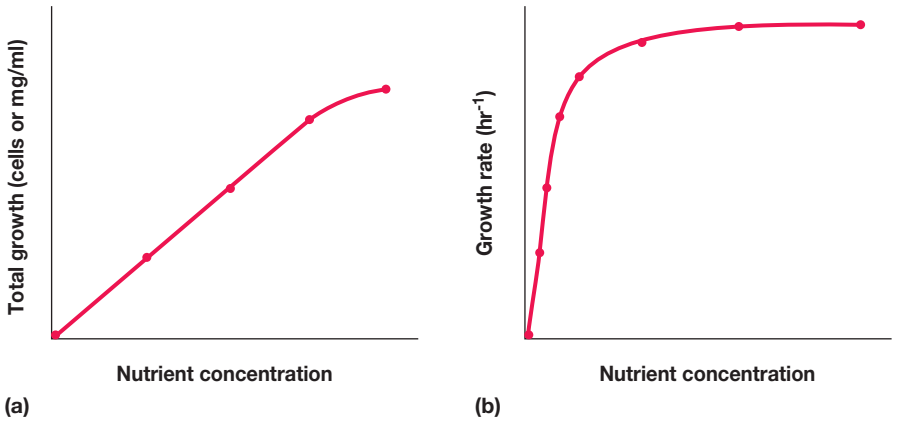


Figure 6.7 Nutrient Concentration and Growth. **(a)** The effect of changes in limiting nutrient concentration on total microbial yield. At sufficiently high concentrations, total growth will plateau. **(b)** The effect on growth rate.

factors. The rate of growth also increases with nutrient concentration (figure 6.7*b*), but in a hyperbolic manner much like that seen with many enzymes (*see figure 8.18*). The shape of the curve seems to reflect the rate of nutrient uptake by microbial transport proteins. At sufficiently high nutrient levels the transport systems are saturated, and the growth rate does not rise further with increasing nutrient concentration. Uptake of nutrients by the cell (section 5.6)

Stationary Phase

Because this is a closed system, eventually population growth ceases and the growth curve becomes horizontal (figure 6.6). This **stationary phase** usually is attained by bacteria at a population level of around 10^9 cells per ml. Other microorganisms normally do not reach such high population densities; protist cultures often have maximum concentrations of about 10^6 cells per ml. Of course final population size depends on nutrient availability and other factors, as well as the type of microorganism being cultured. In the stationary phase the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide but remain metabolically active.

Microbial populations enter the stationary phase for several reasons. One obvious factor is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow. Aerobic organisms often are limited by O_2 availability. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an O_2 concentration adequate for growth. The cells beneath the surface will not be able to grow unless the culture is shaken or aerated in another way. Population growth also may cease due to the accumulation of toxic waste products. This factor seems to limit the growth of many anaerobic cultures (cultures growing in the absence of O_2). For example, streptococci can produce so much lactic acid and other organic acids from sugar fermentation that their medium becomes acidic and growth is inhibited. Streptococcal cultures also can enter the stationary phase due to depletion of their sugar supply. Finally, there is some evidence that growth may cease when a critical population level is reached. Thus entrance into the stationary phase may result from several factors operating in concert.

As we have seen, bacteria in a batch culture may enter stationary phase in response to starvation. This probably often occurs in nature because many environments have low nutrient levels. Procaryotes have evolved a number of strategies to survive starvation. Many do not respond with obvious morphological changes such as endospore formation, but only decrease somewhat in overall size, often accompanied by protoplast shrinkage and nucleoid condensation. The more important changes are in gene expression and physiology. Starving bacteria frequently produce a variety of **starvation proteins,** which make the cell much more resistant to damage in a variety of ways. They increase peptidoglycan crosslinking and cell wall strength. The Dps (*D*NA-binding *p*rotein from *s*tarved cells) protein protects DNA. Chaperone proteins prevent protein denaturation and renature damaged proteins. As a result of these and many other mechanisms, the starved cells become harder to kill and more resistant to starvation itself, damaging temperature changes, oxidative and osmotic damage, and toxic chemicals such as chlorine. These changes are so effective that some bacteria can survive starvation for years. There is even evidence that *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), and some other bacterial pathogens become more virulent when starved. Clearly, these considerations are of great practical importance in medical and industrial microbiology.

Senescence and Death

For many years, the decline in viable cells following stationary cells was described simply as the "death phase." It was assumed that detrimental environmental changes like nutrient deprivation and the buildup of toxic wastes caused irreparable harm resulting in loss of viability. That is, even when bacterial cells were transferred to fresh medium, no cellular growth was observed. Because loss of viability was often not accompanied by a loss in total cell number, it was assumed that cells died but did not lyse.

This view is currently under debate. There are two alternative hypotheses (**figure 6.8**). Some microbiologists believe starving cells that show an exponential decline in density have not irreversibly lost their ability to reproduce. Rather, they suggest that microbes are temporarily unable to grow, at least under the labora-

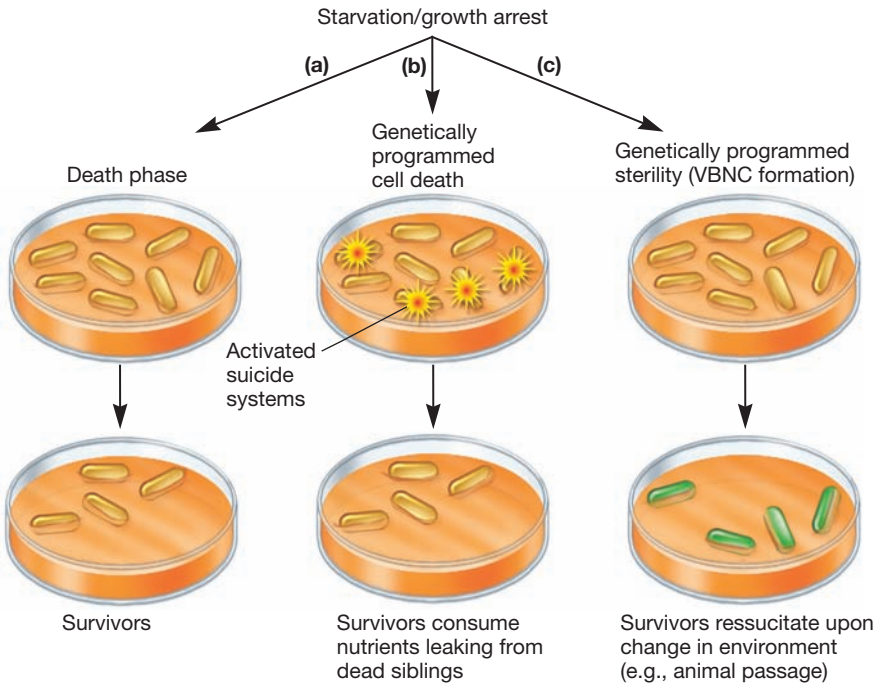


Figure 6.8 Loss of Viability. **(a)** It has long been assumed that as cells leave stationary phase due to starvation or toxic waste accumulation, the exponential decline in culturability is due to cellular death. **(b)** Some believe that a fraction of a microbial population dies due to activation of programmed cell death genes. The nutrients that are released by dying cells supports the growth of other cells. **(c)** The viable but nonculturable (VBNC) hypothesis posits that when cells are starved, they become temporarily nonculturable under laboratory conditions. When exposed to appropriate conditions, some cells will regain the capacity to reproduce.

tory conditions used. This phenomenon, in which the cells are called **viable but nonculturable (VBNC),** is thought to be the result of a genetic response triggered in starving, stationary phase cells. Just as some bacteria form spores as a survival mechanism, it is argued that others are able to become dormant without changes in morphology (figure 6.8*c*). Once the appropriate conditions are available (for instance, a change in temperature or passage through an animal), VBNC microbes resume growth. VBNC microorganisms could pose a public health threat, as many assays that test for food and drinking water safety are culture-based.

The second alternative to a simple death phase is **programmed cell death** (figure 6.8*b*). In contrast to the VBNC hypothesis whereby cells are genetically programmed to survive, programmed cell death predicts that a fraction of the microbial population is genetically programmed to commit suicide. In this case, nonculturable cells are dead (as opposed to nonculturable) and the nutrients that they leak enable the eventual growth of those cells in the population that did not initiate suicide. The dying cells are thus altruistic—that is to say, they sacrifice themselves for the benefit of the larger population.

Phase of Prolonged Decline

Long-term growth experiments reveal that an exponential decline in viability is sometimes replaced by a gradual decline in the number of culturable cells. This decline can last months to years

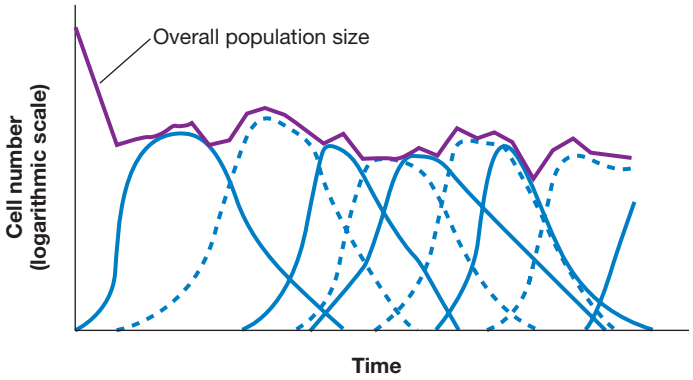


Figure 6.9 Prolonged Decline in Growth. Instead of a distinct death phase, successive waves of genetically distinct subpopulations of microbes better able to use the released nutrients and accumulated toxins survive. Each successive solid or dashed curve represents the growth of a new subpopulation.

(**figure 6.9**). During this time the bacterial population continually evolves so that actively reproducing cells are those best able to use the nutrients released by their dying brethren and best able to tolerate the accumulated toxins. This dynamic process is marked by successive waves of genetically distinct variants. Thus natural selection can be witnessed within a single culture vessel.

The Mathematics of Growth

Knowledge of microbial growth rates during the exponential phase is indispensable to microbiologists. Growth rate studies contribute to basic physiological and ecological research and are applied in industry. The quantitative aspects of exponential phase growth discussed here apply to microorganisms that divide by binary fission.

During the exponential phase each microorganism is dividing at constant intervals. Thus the population will double in number during a specific length of time called the **generation time** or **doubling time.** This situation can be illustrated with a simple example. Suppose that a culture tube is inoculated with one cell that divides every 20 minutes (**table 6.1**). The population will be 2 cells after 20 minutes, 4 cells after 40 minutes, and so forth. Because the population is doubling every generation, the increase in population is always 2^n where n is the number of generations. The resulting population increase is exponential or logarithmic (**figure 6.10**).

These observations can be expressed as equations for the generation time.

Let N_0 = the initial population number

N_t = the population at time t

n = the number of generations in time t

Then inspection of the results in table 6.1 will show that

$$N_t = N_0 \times 2^n.$$

Solving for n, the number of generations, where all logarithms are to the base 10,

$$\log N_t = \log N_0 + n \cdot \log 2, \text{ and}$$

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

The rate of growth during the exponential phase in a batch culture can be expressed in terms of the **mean growth rate constant *(k)*.**

Table 6.1 An Example of Exponential Growth

Time[a]	Division Number	2^n	Population ($N_o \times 2^n$)	$\log_{10} N_t$
0	0	$2^0 = 1$	1	0.000
20	1	$2^1 = 2$	2	0.301
40	2	$2^2 = 4$	4	0.602
60	3	$2^3 = 8$	8	0.903
80	4	$2^4 = 16$	16	1.204
100	5	$2^5 = 32$	32	1.505
120	6	$2^6 = 64$	64	1.806

[a]The hypothetical culture begins with one cell having a 20-minute generation time.

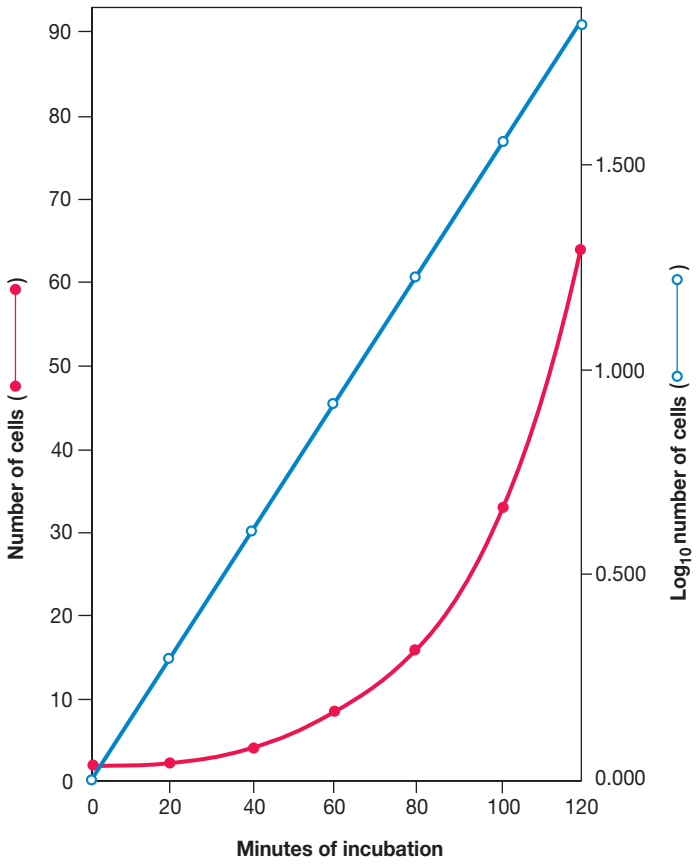


Figure 6.10 Exponential Microbial Growth. The data from table 6.1 for six generations of growth are plotted directly ( ) and in the logarithmic form ( ). The growth curve is exponential as shown by the linearity of the log plot.

This is the number of generations per unit time, often expressed as the generations per hour.

$$k = \frac{n}{t} = \frac{\log N_t - \log N_0}{0.301t}$$

The time it takes a population to double in size—that is, the **mean generation time** or mean doubling time (g)—can now be calculated. If the population doubles ($t = g$), then

$$N_t = 2N_0.$$

Substitute $2N_0$ into the mean growth rate equation and solve for k.

$$k = \frac{\log (2N_0) - \log N_0}{0.301\ g} = \frac{\log 2 + \log N_0 - \log N_0}{0.301\ g}$$

$$k = \frac{1}{g}$$

The mean generation time is the reciprocal of the mean growth rate constant.

$$g = \frac{1}{k}$$

The mean generation time (g) can be determined directly from a semilogarithmic plot of the growth data (**figure 6.11**) and the growth rate constant calculated from the g value. The generation time also may be calculated directly from the previous equations. For example, suppose that a bacterial population increases from 10^3 cells to 10^9 cells in 10 hours.

$$k = \frac{\log 10^9 - \log 10^3}{(0.301)(10\ \text{hr})} = \frac{9 - 3}{3.01\ \text{hr}} = 2.0\ \text{generations/hr}$$

$$g = \frac{1}{2.0\ \text{gen./hr}} = 0.5\ \text{hr/gen. or 30 min/gen.}$$

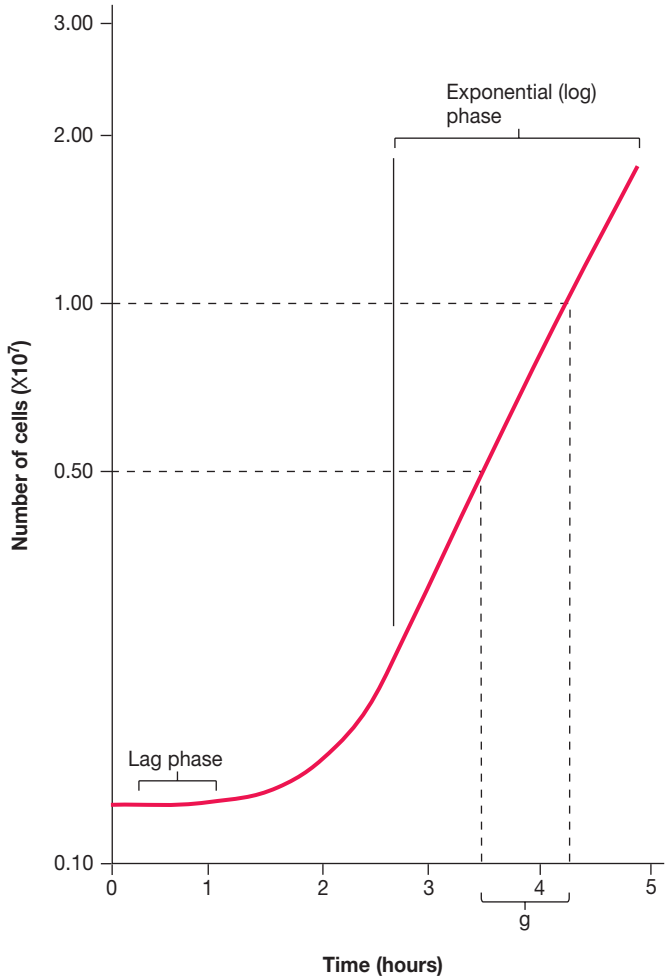


Figure 6.11 Generation Time Determination. The generation time can be determined from a microbial growth curve. The population data are plotted with the logarithmic axis used for the number of cells. The time to double the population number is then read directly from the plot. The log of the population number can also be plotted against time on regular axes.

Table 6.2 Examples of Generation Times[a]

Microorganism	Incubation Temperature (°C)	Generation Time (Hours)
Bacteria		
Beneckea natriegens	37	0.16
Escherichia coli	40	0.35
Bacillus subtilis	40	0.43
Staphylococcus aureus	37	0.47
Pseudomonas aeruginosa	37	0.58
Clostridium botulinum	37	0.58
Rhodospirillum rubrum	25	4.6–5.3
Anabaena cylindrica	25	10.6
Mycobacterium tuberculosis	37	≈12
Treponema pallidum	37	33
Protists		
Tetrahymena geleii	24	2.2–4.2
Scenedesmus quadricauda	25	5.9
Chlorella pyrenoidosa	25	7.75
Asterionella formosa	20	9.6
Leishmania donovani	26	10–12
Paramecium caudatum	26	10.4
Euglena gracilis	25	10.9
Acanthamoeba castellanii	30	11–12
Giardia lamblia	37	18
Ceratium tripos	20	82.8
Fungi		
Saccharomyces cerevisiae	30	2
Monilinia fructicola	25	30

[a] Generation times differ depending on the growth medium and environmental conditions used.

Generation times vary markedly with the species of microorganism and environmental conditions. They range from less than 10 minutes (0.17 hours) for a few bacteria to several days with some eucaryotic microorganisms (**table 6.2**). Generation times in nature are usually much longer than in culture.

1. Define growth. Describe the four phases of the growth curve in a closed system and discuss the causes of each.
2. Why might a culture have a long lag phase after inoculation? Why would cells that are vigorously growing when inoculated into fresh culture medium have a shorter lag phase than those that have been stored in a refrigerator?
3. List two physiological changes that are observed in stationary cells. How do these changes impact the organism's ability to survive?
4. Define balanced growth and unbalanced growth. Why do shift-up and shift-down experiments cause cells to enter unbalanced growth?
5. Define the generation or doubling time and the mean growth rate constant. Calculate the mean growth rate and generation time of a culture that increases in the exponential phase from 5×10^2 to 1×10^8 in 12 hours.

6. Suppose the generation time of a bacterium is 90 minutes and the initial number of cells in a culture is 10^3 cells at the start of the log phase. How many bacteria will there be after 8 hours of exponential growth?
7. What effect does increasing a limiting nutrient have on the yield of cells and the growth rate?
8. Contrast and compare the viable but nonculturable status of microbes with that of programmed cell death as a means of responding to starvation.

6.3 Measurement of Microbial Growth

There are many ways to measure microbial growth to determine growth rates and generation times. Either population number or mass may be followed because growth leads to increases in both. Here the most commonly employed techniques for growth measurement are examined briefly and the advantages and disadvantages of each noted. No single technique is always best; the most appropriate approach will depend on the experimental situation.

Measurement of Cell Numbers

The most obvious way to determine microbial numbers is through direct counting. Using a counting chamber is easy, inexpensive, and relatively quick; it also gives information about the size and morphology of microorganisms. Petroff-Hausser counting chambers can be used for counting procaryotes; hemocytometers can be used for both procaryotes and eucaryotes. These specially designed slides have chambers of known depth with an etched grid on the chamber bottom (**figure 6.12**). Procaryotes are more easily counted in these chambers if they are stained, or when a phase-contrast or a fluorescence microscope is employed. The number of microorganisms in a sample can be calculated by taking into account the chamber's volume and any sample dilutions required. One disadvantage to the technique is that the microbial population must be fairly large for accuracy because such a small volume is sampled.

Larger microorganisms such as protists and yeasts can be directly counted with electronic counters such as the Coulter Counter, although more recently the flow cytometer is increasingly used. The microbial suspension is forced through a small hole or orifice in the Coulter Counter. An electrical current flows through the hole, and electrodes placed on both sides of the orifice measure its electrical resistance. Every time a microbial cell passes through the orifice, electrical resistance increases (or the conductivity drops) and the cell is counted. The Coulter Counter gives accurate results with larger cells and is extensively used in hospital laboratories to count red and white blood cells. It is not as useful in counting bacteria because of interference by small debris particles, the formation of filaments, and other problems. Identification of microorganisms from specimens (section 35.2)

The number of bacteria in aquatic samples is frequently determined from direct counts after the bacteria have been trapped on special membrane filters. In the membrane filter technique, the sample is first filtered through a black polycarbonate membrane filter. Then the bacteria are stained with a fluorescent dye such as acridine orange or the DNA stain DAPI, and observed microscopically. The stained cells are easily observed against the black background of the membrane filter and can be counted when viewed with an epifluorescence microscope. The light microscope: The fluorescence microscope (section 2.2)

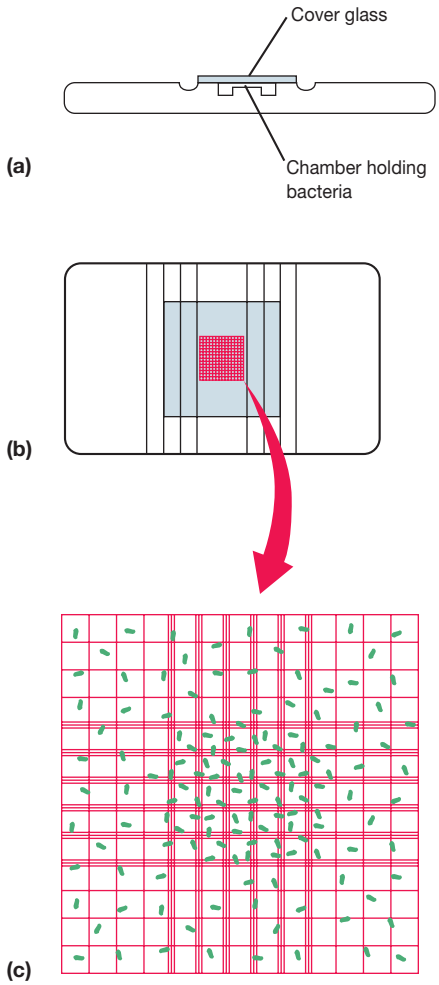


Figure 6.12 The Petroff-Hausser Counting Chamber. **(a)** Side view of the chamber showing the cover glass and the space beneath it that holds a bacterial suspension. **(b)** A top view of the chamber. The grid is located in the center of the slide. **(c)** An enlarged view of the grid. The bacteria in several of the central squares are counted, usually at ×400 to ×500 magnification. The average number of bacteria in these squares is used to calculate the concentration of cells in the original sample. Since there are 25 squares covering an area of 1 mm^2, the total number of bacteria in 1 mm^2 of the chamber is (number/square)(25 squares). The chamber is 0.02 mm deep and therefore,

$$\text{bacteria/mm}^3 = (\text{bacteria/square})(25\ \text{squares})(50).$$

The number of bacteria per cm^3 is 10^3 times this value. For example, suppose the average count per square is 28 bacteria:

$$\text{bacteria/cm}^3 = (28\ \text{bacteria})\ (25\ \text{squares})(50)(10^3) = 3.5 \times 10^7.$$

Traditional methods for directly counting microbes in a sample usually yield cell densities that are much higher than the plat-

ing methods described next because direct counting procedures do not distinguish dead cells from live cells. Newer methods for direct counts avoid this problem. Commercial kits that use fluorescent reagents to stain live and dead cells differently are now available, making it possible to directly count the number of live and dead microorganisms in a sample (*see figures 2.13a and 27.16*).

Several plating methods can be used to determine the number of viable microbes in a sample. These are referred to as viable counting methods because they count only those cells that are alive and able to reproduce. Two commonly used procedures are the spread-plate technique and the pour-plate technique. In both of these methods, a diluted sample of bacteria or other microorganisms is dispersed over a solid agar surface. Each microorganism or group of microorganisms develops into a distinct colony. The original number of viable microorganisms in the sample can be calculated from the number of colonies formed and the sample dilution. For example, if 1.0 ml of a 1×10^{-6} dilution yielded 150 colonies, the original sample contained around 1.5×10^{8} cells per ml. Usually the count is made more accurate by use of a special colony counter. In this way the spread-plate and pour-plate techniques may be used to find the number of microorganisms in a sample. Isolation of pure cultures: The spread plate and streak plate; The pour plate (section 5.8)

Another commonly used plating method first traps bacteria in aquatic samples on a membrane filter. The filter is then placed on an agar medium or on a pad soaked with liquid media (**figure 6.13**) and incubated until each cell forms a separate colony. A colony count gives the number of microorganisms in the filtered sample, and special media can be used to select for specific microorganisms (**figure 6.14**). This technique is especially useful in analyzing water purity. Water purification and sanitary analysis (section 41.1)

Plating techniques are simple, sensitive, and widely used for viable counts of bacteria and other microorganisms in samples of food, water, and soil. Several problems, however, can lead to inaccurate counts. Low counts will result if clumps of cells are not

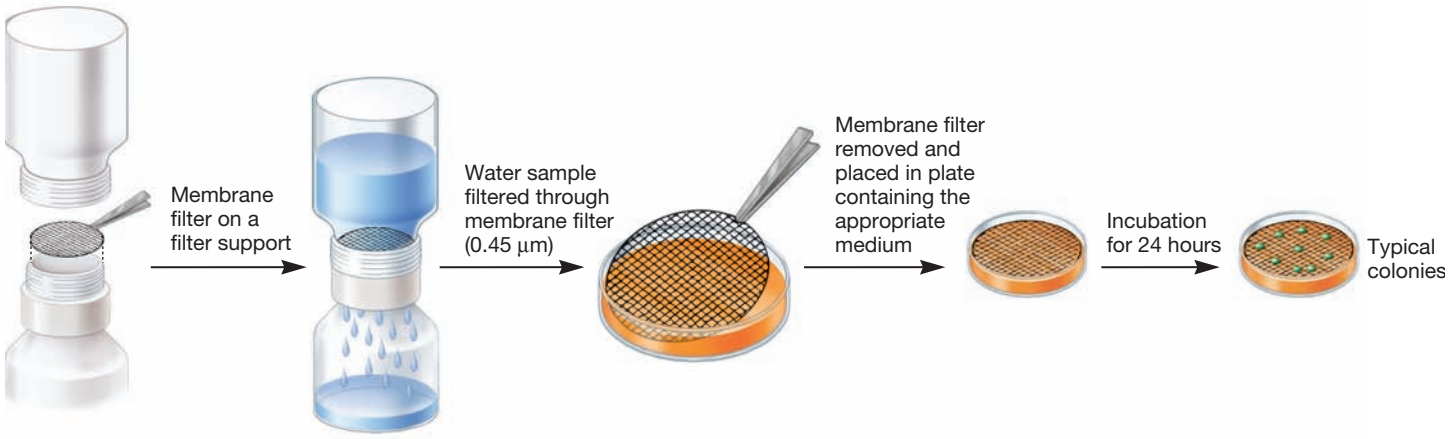


Figure 6.13 The Membrane Filtration Procedure. Membranes with different pore sizes are used to trap different microorganisms. Incubation times for membranes also vary with the medium and microorganism.

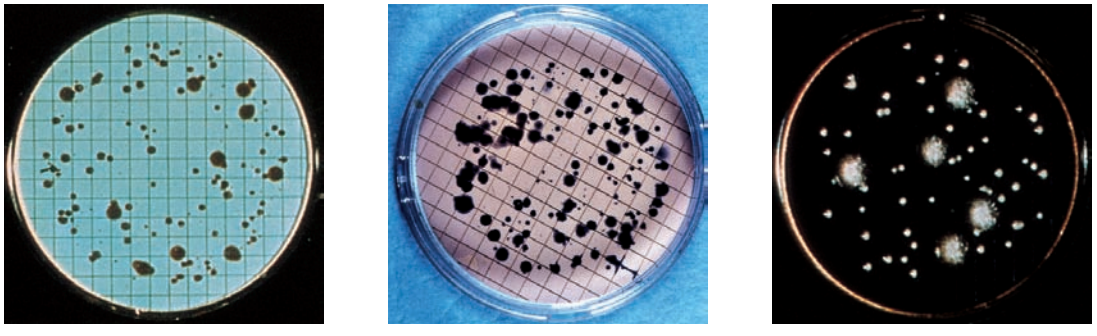


Figure 6.14 Colonies on Membrane Filters. Membrane-filtered samples grown on a variety of media. **(a)** Standard nutrient media for a total bacterial count. An indicator colors colonies red for easy counting. **(b)** Fecal coliform medium for detecting fecal coliforms that form blue colonies. **(c)** Wort agar for the culture of yeasts and molds.

broken up and the microorganisms well dispersed. Because it is not possible to be absolutely certain that each colony arose from an individual cell, the results are often expressed in terms of **colony forming units (CFU)** rather than the number of microorganisms. The samples should yield between 30 and 300 colonies for most accurate counting. Of course the counts will also be low if the agar medium employed cannot support growth of all the viable microorganisms present. The hot agar used in the pour-plate technique may injure or kill sensitive cells; thus spread plates sometimes give higher counts than pour plates.

Measurement of Cell Mass

Increases in the total cell mass, as well as in cell numbers, accompany population growth. Therefore techniques for measuring changes in cell mass can be used in following growth. The most direct approach is the determination of microbial dry weight. Cells growing in liquid medium are collected by centrifugation, washed, dried in an oven, and weighed. This is an especially useful technique for measuring the growth of filamentous fungi. It is time-consuming, however, and not very sensitive. Because bacteria weigh so little, it may be necessary to centrifuge several hundred milliliters of culture to collect a sufficient quantity.

Spectrophotometry can also be used to measure cell mass. These methods are more rapid and sensitive. They depend on the fact that microbial cells scatter light that strikes them. Because microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. When the concentration of bacteria reaches about 10 million cells (10^7) per ml, the medium appears slightly cloudy or turbid. Further increases in concentration result in greater turbidity and less light is transmitted through the medium. The extent of light scattering can be measured by a spectrophotometer and is almost linearly related to cell concentration at low absorbance levels (**figure 6.15**). Thus population growth can be easily measured as long as the population is high enough to give detectable turbidity.

If the amount of a substance in each cell is constant, the total quantity of that cell constituent is directly related to the total microbial cell mass. For example, a sample of washed cells collected from a known volume of medium can be analyzed for total protein or nitrogen. An increase in the microbial population will be reflected in higher total protein levels. Similarly, chlorophyll determinations can be used to measure algal and cyanobacterial populations, and the quantity of ATP can be used to estimate the amount of living microbial mass.

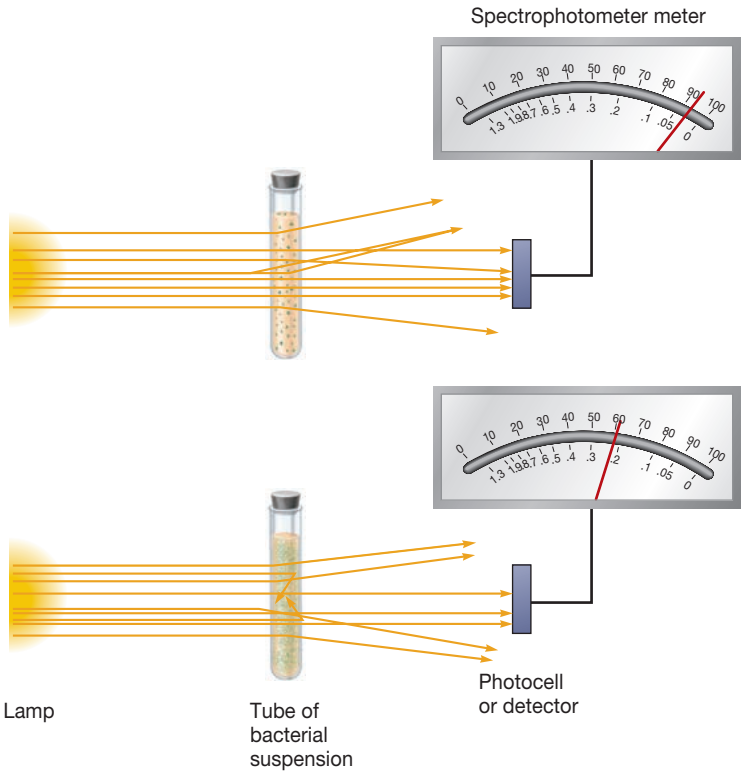


Figure 6.15 Turbidity and Microbial Mass Measurement. Determination of microbial mass by measurement of light absorption. As the population and turbidity increase, more light is scattered and the absorbance reading given by the spectrophotometer increases. The spectrophotometer meter has two scales. The bottom scale displays absorbance and the top scale, percent transmittance. Absorbance increases as percent transmittance decreases.

1. Briefly describe each technique by which microbial population numbers may be determined and give its advantages and disadvantages.
2. When using direct cell counts to follow the growth of a culture, it may be difficult to tell when the culture enters the phase of senescence and death. Why?
3. Why are plate count results expressed as colony forming units?

6.4 The Continuous Culture of Microorganisms

Up to this point the focus has been on closed systems called batch cultures in which nutrient supplies are not renewed nor wastes removed. Exponential growth lasts for only a few generations and soon the stationary phase is reached. However, it is possible to grow microorganisms in an open system, a system with constant environmental conditions maintained through continual provision of nutrients and removal of wastes. These conditions are met in the laboratory by a **continuous culture system.** A microbial population can be maintained in the exponential growth phase and at a constant biomass concentration for extended periods in a continuous culture system.

The Chemostat

Two major types of continuous culture systems commonly are used: (1) chemostats and (2) turbidostats. A **chemostat** is constructed so that sterile medium is fed into the culture vessel at the same rate as the media containing microorganisms is removed (**figure 6.16**). The culture medium for a chemostat possesses an essential nutrient (e.g., an amino acid) in limiting quantities. Because one nutrient is limiting, the growth rate is determined by the rate at which new medium is fed into the growth chamber, and the final cell density depends on the concentration of the limiting nutrient. The rate of nutrient exchange is expressed as the dilution rate (D), the rate at which medium flows through the culture vessel relative to the vessel volume, where f is the flow rate (ml/hr) and V is the vessel volume (ml).

$$D = f/V$$

For example, if f is 30 ml/hr and V is 100 ml, the dilution rate is 0.30 hr^{-1}.

Both the microbial population level and the generation time are related to the dilution rate (**figure 6.17**). The microbial population density remains unchanged over a wide range of dilution rates. The generation time decreases (i.e., the rate of growth increases) as the dilution rate increases. The limiting nutrient will be almost completely depleted under these balanced conditions. If the dilution rate rises too high, the microorganisms can actually be washed out of the culture vessel before reproducing because the dilution rate is greater than the maximum growth rate. This occurs because fewer microorganisms are present to consume the limiting nutrient.

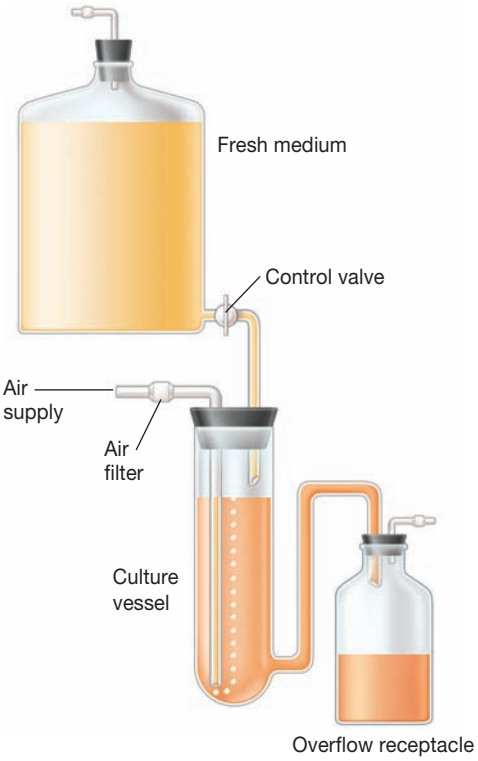


Figure 6.16 A Continuous Culture System: The Chemostat. Schematic diagram of the system. The fresh medium contains a limiting amount of an essential nutrient. Growth rate is determined by the rate of flow of medium through the culture vessel.

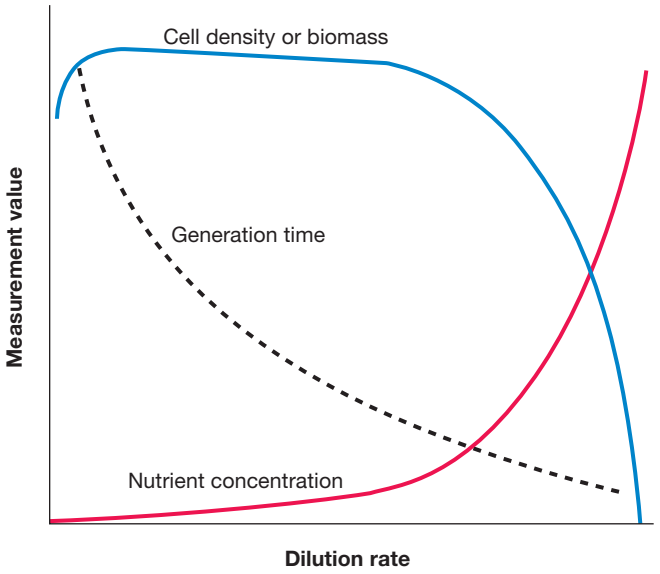


Figure 6.17 Chemostat Dilution Rate and Microbial Growth. The effects of changing the dilution rate in a chemostat.

At very low dilution rates, an increase in *D* causes a rise in both cell density and the growth rate. This is because of the effect of nutrient concentration on the growth rate, sometimes called the Monod relationship (figure 6.7*b*). Only a limited supply of nutrient is available at low dilution rates. Much of the available energy must be used for cell maintenance, not for growth and reproduction. As the dilution rate increases, the amount of nutrients and the resulting cell density rise because energy is available for both maintenance and reproduction. The growth rate increases when the total available energy exceeds the maintenance energy.

The Turbidostat

The second type of continuous culture system, the **turbidostat,** has a photocell that measures the absorbance or turbidity of the culture in the growth vessel. The flow rate of media through the vessel is automatically regulated to maintain a predetermined turbidity or cell density. The turbidostat differs from the chemostat in several ways. The dilution rate in a turbidostat varies rather than remaining constant, and its culture medium contains all nutrients in excess. That is, none of the nutrients is limiting. The turbidostat operates best at high dilution rates; the chemostat is most stable and effective at lower dilution rates.

Continuous culture systems are very useful because they provide a constant supply of cells in exponential phase and growing at a known rate. They make possible the study of microbial growth at very low nutrient levels, concentrations close to those present in natural environments. These systems are essential for research in many areas—for example, in studies on interactions between microbial species under environmental conditions resembling those in a freshwater lake or pond. Continuous systems also are used in food and industrial microbiology (chapters 40 and 41, respectively).

1. How does an open system differ from a closed culture system or batch culture?
2. Describe how the two different kinds of continuous culture systems, the chemostat and turbidostat, operate.
3. What is the dilution rate? What is maintenance energy?
4. How is the rate of growth of a microbial population controlled in a chemostat? In a turbidostat?

6.5 The Influence of Environmental Factors on Growth

As we have seen, microorganisms must be able to respond to variations in nutrient levels, and particularly to nutrient limitation. The growth of microorganisms also is greatly affected by the chemical and physical nature of their surroundings. An understanding of environmental influences aids in the control of microbial growth and the study of the ecological distribution of microorganisms.

The ability of some microorganisms to adapt to extreme and inhospitable environments is truly remarkable. Procaryotes are present anywhere life can exist. Many habitats in which procaryotes thrive would kill most other organisms. Procaryotes such as *Bacillus infernus* are even able to live over 1.5 miles below the Earth's surface, without oxygen and at temperatures above 60°C. Microorganisms that grow in such harsh conditions are often called **extremophiles.**

In this section we shall briefly review how some of the most important environmental factors affect microbial growth. Major emphasis will be given to solutes and water activity, pH, temperature, oxygen level, pressure, and radiation. **Table 6.3** summarizes the way in which microorganisms are categorized in terms of their response to these factors. It is important to note that for most environmental factors, a range of levels supports growth of a microbe. For example, a microbe might exhibit optimum growth at pH 7, but will grow, though not optimally, at pH values down to pH 6 (its pH minimum) and up to pH 8 (its pH maximum). Furthermore, outside this range, the microbe might cease reproducing but will remain viable for some time. Clearly, each microbe must possess adaptations that allow it to adjust its physiology within its preferred range, and it may also have adaptations that protect it in environments outside this range. These adaptations will also be discussed in this section.

Solutes and Water Activity

Because a selectively permeable plasma membrane separates microorganisms from their environment, they can be affected by changes in the osmotic concentration of their surroundings. If a microorganism is placed in a hypotonic solution (one with a lower osmotic concentration), water will enter the cell and cause it to burst unless something is done to prevent the influx. Conversely if it is placed in a hypertonic solution (one with a higher osmotic concentration), water will flow out of the cell. In microbes that have cell walls (i.e., most procaryotes, fungi, and algae), the membrane shrinks away from the cell wall—a process called plasmolysis. Dehydration of the cell in hypertonic environments may damage the cell membrane and cause the cell to become metabolically inactive.

It is important, then, that microbes be able to respond to changes in the osmotic concentrations of their environment. For instance, microbes in hypotonic environments can reduce the osmotic concentration of their cytoplasm. This can be achieved by the use of inclusion bodies. Some bacteria and archaea also have mechanosensitive (MS) channels in their plasma membrane. In a hypotonic environment, the membrane stretches due to an increase in hydrostatic pressure and cellular swelling. MS channels then open and allow solutes to leave. Thus they can act as escape valves to protect cells from bursting. Since many protists do not have a cell wall, they must use contractile vacuoles (*see figures 25.5 and 25.17b*) to expel excess water. Many microorganisms, whether in hypotonic or hypertonic environments, keep the osmotic concentration of their protoplasm somewhat above that of the habitat by the use of compatible solutes, so that the plasma membrane is always pressed firmly against their cell wall. **Compatible solutes** are solutes that do not interfere with metabolism and growth when at high intracellular concentrations. Most procaryotes increase their