PART A

FUNDAMENTALS OF MICROBIOLOGY

1

THE MICROBIAL WORLD

Wastewater Microbiology, Fourth Edition, by Gabriel Bitton

1.1 INTRODUCTION

The three domains of life are *bacteria*, *archaea*, and *eukarya* (Fig. 1.1; Rising and Reysenbach, 2002; Woese, 1987). Bacteria, along with actinomycetes and cyanobacteria (blue-green algae), belong to the *prokaryotes*, while fungi, protozoa, algae, plant, and animal cells belong to the *eukaryotes* or eukarya.

Viruses are obligate intracellular parasites that belong to neither of these two groups.

The main characteristics that distinguish prokaryotes from eukaryotes are the following (Fig. 1.2):

- Eukaryotic cells are generally more complex than prokaryotic cells.
- DNA is enclosed in a nuclear membrane and is associated with histones and other proteins only in eukaryotes.
- Organelles are membrane-bound in eukaryotes.
- Prokaryotes divide by binary fission, whereas eukaryotes divide by mitosis.
- Some structures are absent in prokaryotes, for example, Golgi complex, endoplasmic reticulum, mitochondria, and chloroplasts.

Other differences between prokaryotes and eukaryotes are shown in Table 1.1.

We will now review the main characteristics of prokaryotes, archaea, and eukaryotes. Later on, we will focus on their importance in process microbiology and public health. We will also introduce the reader to environmental virology and parasitology, the study of the fate of viruses, and protozoan and helminth parasites that are of public health significance in wastewater and other fecally contaminated environments.

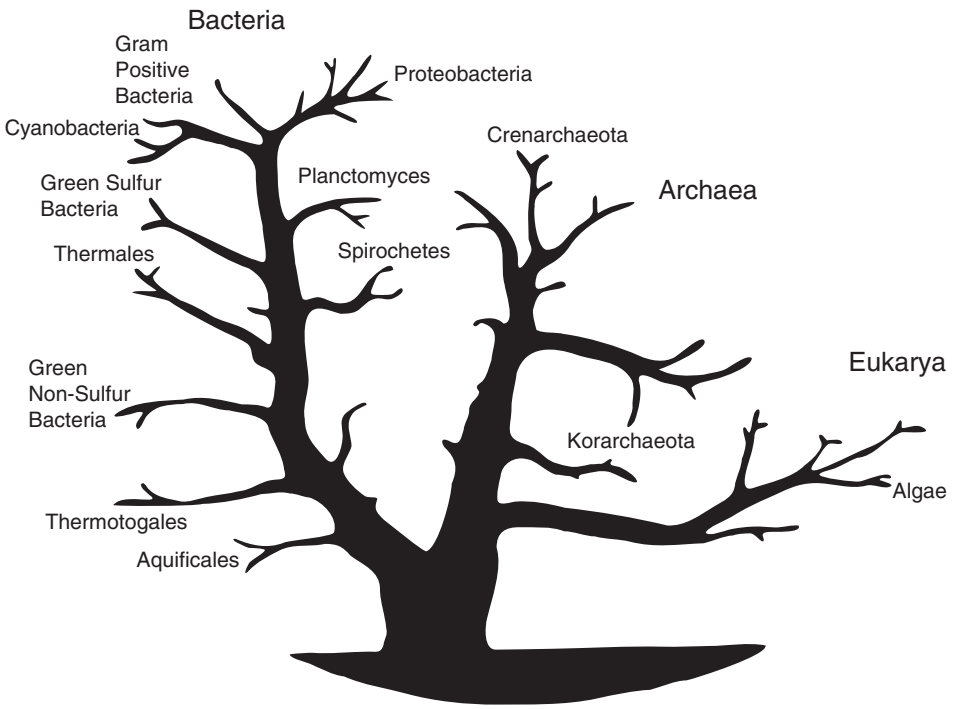

Figure 1.1 The Tree of Life. From Rising and Reysenbach (2002) and Woese (1987).

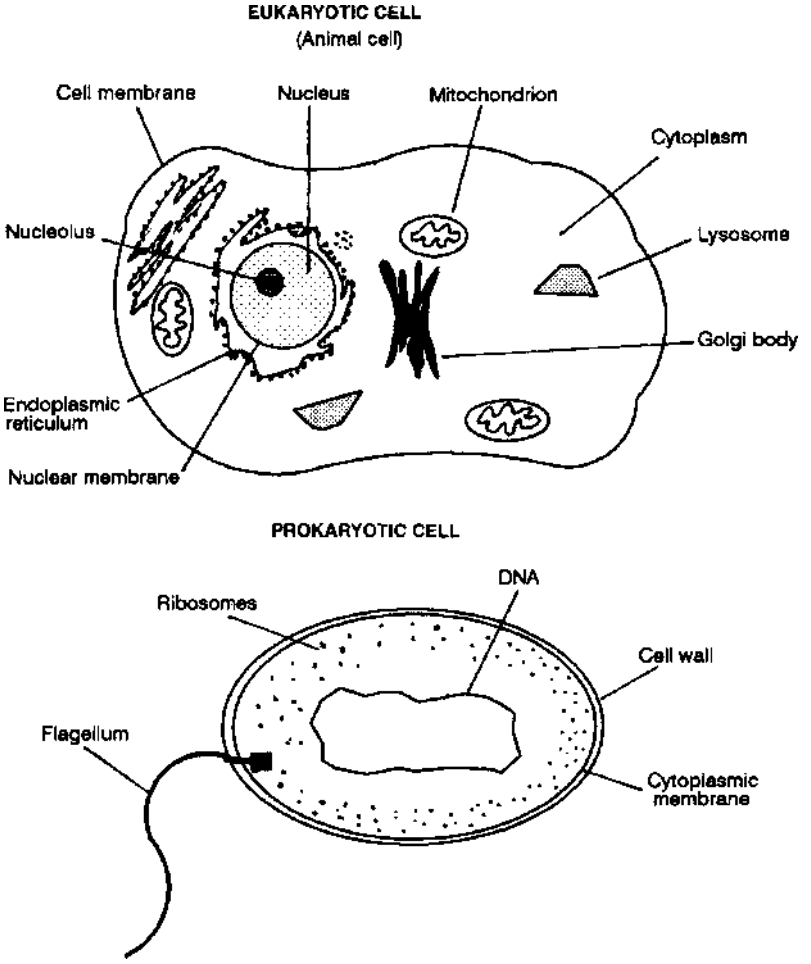

Figure 1.2 Prokaryotic and eukaryotic cells.

1.2 CELL STRUCTURE

1.2.1 Cell Size

Except for filamentous bacteria, prokaryotic cells are generally smaller than eukaryotic cells. Small cells have a higher growth rate than larger cells. This may be explained by the fact that small cells have a higher surface-to-volume ratio than larger cells. Thus, the higher metabolic activity of small cells is due to additional membrane surface available for transport of nutrients into, and waste products out of, the cell.

1.2.2 Cytoplasmic Membrane (Plasma Membrane)

The cytoplasmic membrane is a 40- to 80-Å-thick semipermeable membrane that contains a phospholipid bilayer with proteins embedded within the bilayer (fluid mosaic model; Fig. 1.3). The phospholipid bilayer is made of hydrophobic fatty acids oriented toward the inside of the bilayer, and hydrophilic glycerol moieties oriented toward the outside of the

TABLE 1.1. Comparison of Prokaryotes and Eukaryotes

Feature	Prokaryotes (Bacteria)	Eukaryotes (1 ungi, Protozoa, Algae, Plants, Animals)
Cell wall	Present in most prokaryotes (absent in mycoplasma); made of peptidoglycan	Absent in animal; present in plants, algae, and fungi
Cell membrane	Phospholipid bilayer	Phospholipid bilayer + sterols
Ribosomes	70S in size	80S in size
Chloroplasts	Absent	Present
Mitochondria	Absent; respiration associated with plasma membrane	Present
Golgi complex	Absent	Present
Endoplasmic reticulum	Absent	Present
Gas vacuoles	Present in some species	Absent
Endospores	Present in some species	Absent
Locomotion	Flagella composed of one fiber	Flagella or cilia composed of microtubules; amoeboid movement
Nuclear membrane	Absent	Present
DNA	One single molecule	Several chromosomes where DNA is associated with histones
Cell division	Binary fission	Mitosis

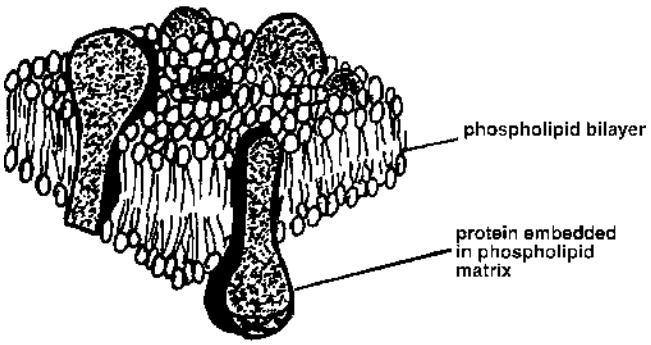

Figure 1.3 Structure of cytoplasmic membrane. Adapted from Alberts et al. (1989).

bilayer. Cations such as Ca^{2+} and Mg^{2+} help stabilize the membrane structure. Sterols are other lipids that enter into the composition of plasma membranes of eukaryotic cells as well as some prokaryotes, such as mycoplasma (these bacteria lack a cell wall).

Chemicals cross biological membranes by diffusion, active transport, and endocytosis.

Diffusion. Because of the hydrophobic nature of the plasma membrane, lipophilic compounds diffuse better through the membrane than ionized compounds. The rate of diffusion across cell membranes depends on their lipid solubility and concentration gradient across the membrane.

Active transport. Hydrophilic compounds (i.e., lipid insoluble) may be transferred through the membrane by active transport. This transport involves highly specific *carrier proteins*, requires energy in the form of adenosine triphosphate (ATP) or phosphoenol-pyruvate (PEP), and allows cells to accumulate chemicals against a concentration gradient. There are specific active transport systems for sugars, amino acids, and ions.

Toxic chemicals gain entry into cells mainly by diffusion, and some may use active transport systems similar to those used for nutrients.

Endocytosis. In eukaryotes cells, substances can cross the cytoplasmic membranes by endocytosis, in addition to diffusion and active transport. Endocytosis includes *phagocytosis* (uptake of particles) and *pynocytosis* (uptake of dissolved substances).

1.2.3 Cell Wall

All bacteria, except *Mycoplasma,* have a cell wall. This structure confers rigidity to cells and maintains their characteristic shape, and it protects them from high osmotic pressures. Cell walls are composed of a mucopolysaccharide called peptidoglycan or murein (glycan strands cross-linked by peptide chains). Peptidoglycan is composed of N-acetylglucosamine and N-acetylmuramic acid and amino acids. A cell wall stain, called the Gram stain differentiates between *gram-negative* and *gram-positive* bacteria on the basis of the cell wall chemical composition. Peptidoglycan layers are thicker in gram-positive bacteria than in gram-negative bacteria. In addition to peptidoglycan, gram-positive bacteria contain teichoic acids made of alcohol and phosphate groups.

Animal cells do not have cell walls, but in other eukaryotic cells, the cell walls may be composed of cellulose (e.g., plant cells, algae), chitin (e.g., fungi), silica (e.g., diatoms), or polysaccharides such as glucan and mannan (e.g., yeasts).

1.2.4 Outer Membrane

The outermost layer of gram-negative bacteria contains phospholipids, lipopolysaccharides (LPS), and proteins (Fig. 1.4). LPS constitute about 20% of the outer membrane by weight and consist of a hydrophobic region bound to an oligosaccharide core. The LPS molecules are held together with divalent cations. Proteins constitute about 60% of the outer membrane weight and are partially exposed to the outside. Some of the proteins form water-filled pores, called *porins*, for the passage of hydrophilic compounds. Other proteins have a structural role, as they help anchor the outer membrane to the cell wall. The outer membrane of gram-negative bacteria is an efficient barrier against hydrophobic chemicals, namely some antibiotics and xenobiotics, but is permeable to hydrophilic compounds, some of which are essential nutrients.

Chemicals (e.g., ethylenediamine tetraacetic acid [EDTA], polycations) and physical (e.g., heating, freeze-thawing, drying, and freeze-drying) treatments, as well as genetic alterations, can increase the permeability of outer membranes to hydrophobic compounds.

1.2.5 Glycocalyx

The *glycocalyx* is made of extracellular polymeric substances (EPS), which surround some microbial cells and are composed mainly of polysaccharides. In some cells, the glycocalyx

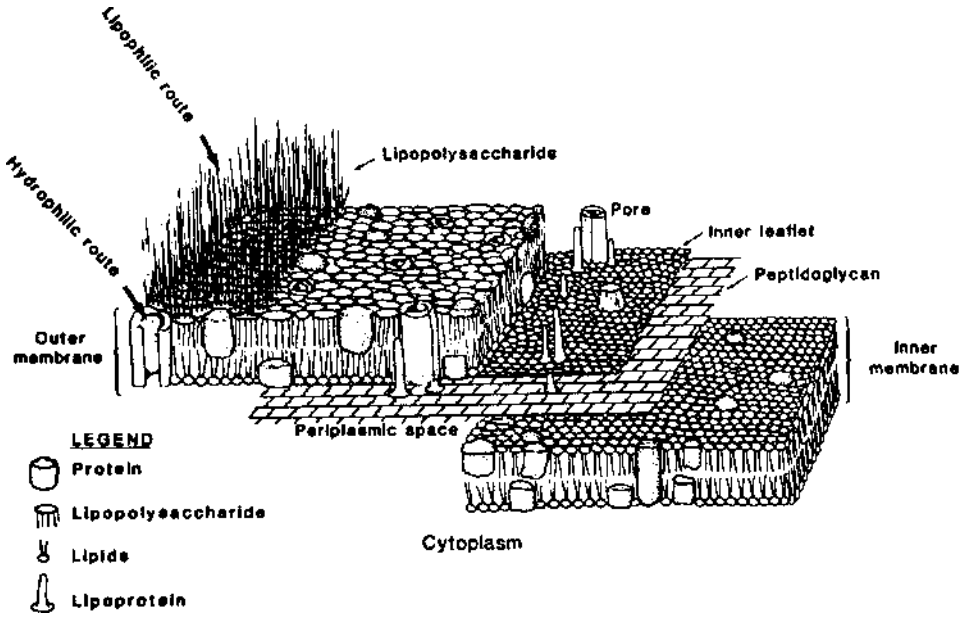

Figure 1.4 Outer membrane of gram-negative bacteria. From Godfrey and Bryan (1984). Courtesy of Academic Press.

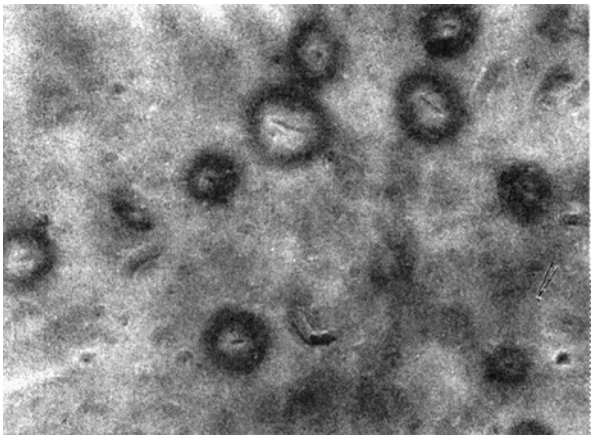

Figure 1.5 Capsules of *Klebsiella aerogenes*. From Bitton.

is organized as a capsule (Fig. 1.5). Other cells produce loose polymeric materials, which are dispersed in the growth medium.

EPS are important from medical and environmental viewpoints: (1) capsules contribute to pathogen virulence; (2) encapsulated cells are protected from phagocytosis in the body and in the environment; (3) EPS help bacteria adsorb to surfaces such as teeth, mucosal surfaces, and environmentally important surfaces such as soils or water distribution pipes; (4) capsules protect cells against desiccation; (5) capsules play a role in metal complexation, particularly in wastewater treatment plants; and (6) capsules play a role in microbial flocculation in the activated sludge process (see Chapter 9).

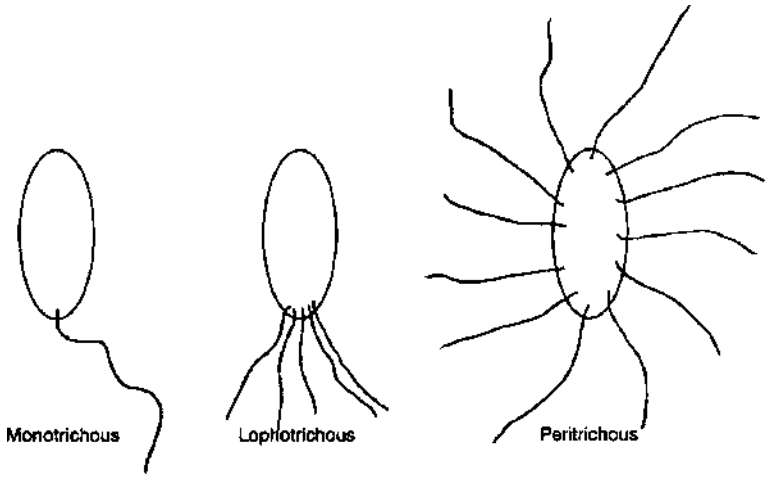

Figure 1.6 Flagellar arrangements in bacteria.

1.2.6 Cell Motility

Microbial cells can move by means of flagella, cilia, or *pseudopods*. Bacteria display various flagellar arrangements ranging from *monotrichous* (polar flagellum, e.g., *Vibrio coma*), *lophotrichous* (bundle of flagella at one end of the cell, e.g., *Spirillum volutans*) to *peritrichous* (several flagella distributed around the cell, e.g., *Escherichia coli*; Fig. 1.6). The flagellum is composed of a protein called *flagellin* and is anchored by a hook to a basal body located in the cell envelope. Flagella enable bacteria to attain speeds of 50–100 μm/s. They enable cells to move toward food (chemotaxis), light (phototaxis), or oxygen (aerotaxis). *Chemotaxis* is the movement of a microorganism toward a chemical, generally a nutrient. It also enables the movement away from a harmful chemical (negative chemotaxis). Chemotaxis can be demonstrated by placing a capillary containing a chemical attractant into a bacterial suspension. Bacteria, attracted to the chemical, swarm around the tip and move inside the capillary. Two sets of proteins, chemoreceptors and transducers, are involved in triggering flagellar rotation and subsequent cell movement. From an ecological viewpoint, chemotaxis provides a selective advantage to bacteria, allowing them to detect carbon and energy sources. Toxicants (e.g., hydrocarbons, heavy metals) inhibit chemotaxis by blocking chemoreceptors, thus affecting food detection by motile bacteria as well as predator–prey relationships in aquatic environments.

Eukaryotic cells move by means of flagella, cilia, or cytoplasmic streaming (i.e., amoeboid movement). Flagella have a more complex structure than that of prokaryotic flagella. Cilia are shorter and thinner than flagella. Ciliated protozoa use cilia for locomotion and for pushing food inside the *cytostome*, a mouthlike structure. Some eukaryotes (e.g., amoeba, slime molds) move by amoeboid movement by means of pseudopods (i.e., false feet), which are temporary projections of the cytoplasm.

1.2.7 Pili

Pili are structures that appear as short and thin flagella, attached to cells in a manner similar to that of flagella. They play a role in cell attachment to surfaces, are involved in conjugation (involvement of a sex pilus), and act as specific receptors for certain types of phages.

1.2.8 Storage Products

Cells may contain inclusions that contain storage products serving as a source of energy or building blocks. These inclusions may be observed under a microscope using special stains.

- Carbon storage in the form of glycogen, starch, and poly-β-hydroxybutyric acid (PHB), which stains with Sudan black, a fat-soluble stain. PHB occurs exclusively in prokaryotic microorganisms.
- *Volutin granules*, which contain polyphosphate reserves. These granules, also called *metachromatic granules*, appear red when specifically stained with basic dyes such as toluidine blue or methylene blue.
- *Sulfur granules*, which are found in sulfur filamentous bacteria (e.g., *Beggiatoa*, *Thiothrix*) and purple photosynthetic bacteria. They use H_2S as an energy source and electron donor. H_2S is oxidized to S^0, which accumulates inside sulfur granules, readily visible under a light microscope. Upon depletion of the H_2S source, the elemental sulfur is further oxidized to sulfate.

1.2.9 Gas Vacuoles

Gas vacuoles are found in cyanobacteria, halobacteria (i.e., salt-loving bacteria), and photosynthetic bacteria. Electron microscopic studies have shown that gas vacuoles are made of gas vesicles, which are filled with gases and surrounded by a protein membrane. Their role is to regulate cell buoyancy in the water column. Owing to this flotation device, cyanobacteria and photosynthetic bacteria sometimes form massive blooms at the surface of lakes or ponds.

1.2.10 Endospores

Endospores are formed inside bacterial cells and are released when cells are exposed to adverse environmental conditions. The location of the spore may vary. There are central, subterminal, and terminal spores. Physical and chemical agents trigger spore germination to form vegetative cells. Bacterial endospores are very resistant to heat, and this is probably due to the presence of a dipicolinic acid–Ca complex in endospores. Endospores are also quite resistant to desiccation, radiation, and harmful chemicals. This is significant from a public health viewpoint because endospores are much more resistant to chemical disinfectants than vegetative bacteria in water and wastewater treatment plants (see Chapters 5 and 6).

1.2.11 Eukaryotic Organelles

Specialized structures, called organelles, are located in the cytoplasm of eukaryotic cells and carry out several important cell functions. We will now briefly review some of these organelles.

1.2.11.1 Mitochondria. Mitochondria (singular: mitochondrion) are oval or spherical structures surrounded by a double membrane. The outer membrane is very permeable to the passage of chemicals, and the inner membrane is folded and form shelves called *cristae* (singular: crista). The innermost compartment is the *matrix* (Fig. 1.7). Mitochondria are the powerhouses of the cell as they are the *site of cell respiration and adenosine*

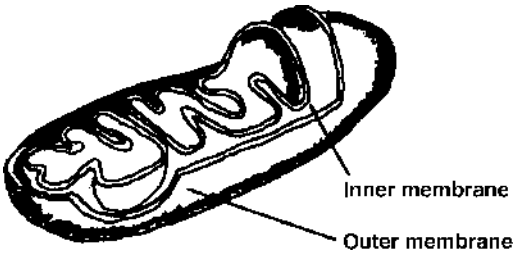

Figure 1.7 Mitochondrion structure.

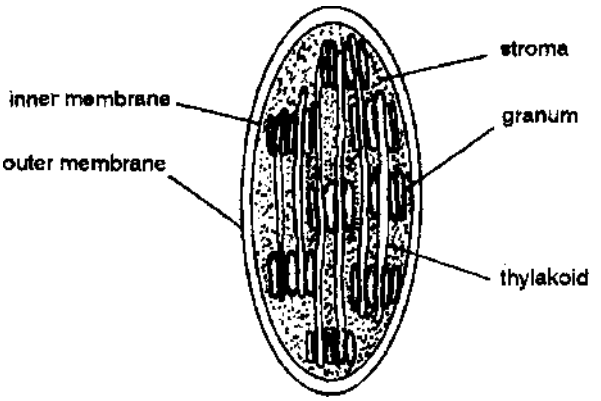

Figure 1.8 Chloroplast structure.

triphosphate (ATP) production in eukaryotic cells (see Chapter 2). They contain their own DNA as well as ribosomes and enzymes involved in protein synthesis. The number of mitochondria per cell varies with the type and the metabolic level of cells.

1.2.11.2 Chloroplasts and Other Plastids. Chloroplasts are relatively large chlorophyll-containing structures found in plant and algal cells and are also surrounded by a double membrane. They are made of units called *grana*, interconnected by lamellae. Each granum consists of a stack of disks called thylakoids bathing into a matrix called stroma (Fig. 1.8). As shown for mitochondria, chloroplasts contain their own DNA, ribosomes, and enzymes required for protein synthesis. Chloroplasts are the sites for photosynthesis in plant and algal cells. The light and dark reactions of photosynthesis occur in the thylakoids and stroma, respectively (see Chapter 2).

Other plastids found in plant cells are the *leucoplasts* (storage of proteins, lipids, and starch) and the *chromoplasts* (storage of plant pigments).

1.2.11.3 Other Organelles. Other important organelles that are found in eukaryotic cells but not in prokaryotic cells are the following:

- The *Golgi complex* consists of a stack of flattened membranous sacs, called *saccules*, which form vesicles that collect proteins, carbohydrates, and enzymes.
- The e*ndoplasmic reticulum* is a system of folded membranes attached to both the cell membrane and the nuclear membrane. The rough endoplasmic reticulum is associated

with ribosomes and is involved in protein synthesis. The smooth endoplasmic reticulum is found in cells that make and store hormones, carbohydrates, and lipids.

- *Lysosomes* are sacs that contain hydrolytic (digestive) enzymes and help in the digestion of phagocytized cells and various biological molecules by eukaryotic cells. They are particularly important in animal cells.
- *Microbodies*, generally known as peroxisomes, are structures involved in oxidative reactions in the cells.
- *Microtubules* and *microfilaments* are implicated in eukaryotic cell movements (e.g., flagella, cytoplasmic streaming).

1.3 CELL GENETIC MATERIAL

1.3.1 DNA in Prokaryotes and Eukaryotes

In prokaryotes, DNA occurs as a single circular molecule, which is tightly packed to fit inside the cell and is not enclosed in a nuclear membrane. Prokaryotic cells may also contain small circular DNA molecules called *plasmids*.

Eukaryotes have a distinct nucleus surrounded by a nuclear membrane with very small pores that allow exchanges between the nucleus and the cytoplasm. Deoxyribonucleic acid (DNA) is present as chromosomes consisting of DNA associated with histone proteins. Cell divide by mitosis, which leads to a doubling of the chromosome numbers. Each daughter cell has a full set of chromosomes.

1.3.2 Nucleic Acids

1.3.2.1 DNA and RNA. Deoxyribonucleic acid (DNA) is a double-stranded molecule that is made of several millions of units (e.g., approximately 4 million base pairs (bp) in *E. coli* chromosome) called *nucleotides*. The double-stranded DNA is organized into a *double helix* (Fig. 1.9). Each nucleotide is made of a five-carbon sugar (deoxyribose), a phosphate group and a nitrogen-containing base linked to the C-5 and C-1 of the deoxyribose molecule, respectively. The nucleotides on a strand are linked together via a phosphodiester bridge. The hydroxyl group of a C-3 of a pentose (3′ carbon) is linked to the phosphate group on the C-5 (5′ carbon) of the next pentose. There are four different bases in DNA: two purines (adenine and guanine) and two pyrimidines (cytosine and thymine). A base on one strand pairs through hydrogen bonding with another base on the complementary strand. Guanine always pairs with cytosine, while adenine always pairs with thymine (Fig. 1.10). One strand runs in the $5' \rightarrow 3'$ direction, while the complementary strand runs in the $3' \rightarrow 5'$ direction. Physical and chemical agents cause DNA to unwind, leading to the separation of the two strands.

Ribonucleic acid (RNA) is generally single-stranded (some viruses have double-stranded RNA), contains ribose in lieu of deoxyribose, and uracil in lieu of thymine. In RNA, guanine binds to cytosine, while adenine binds to uracil. There are three forms of RNA: messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA).

1.3.2.2 DNA Replication and Protein Synthesis

- *Replication*

The DNA molecule can make an exact copy of itself. The two strands separate and new complementary strands are formed. The double helix unwinds and each of the DNA strands

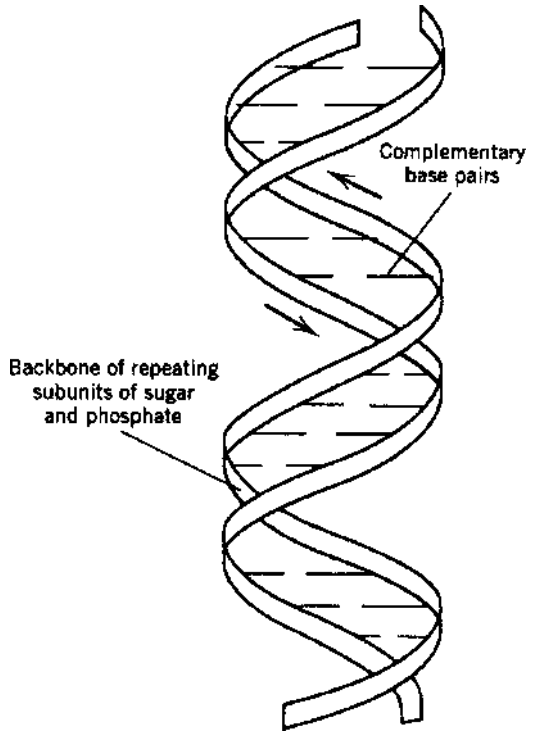

Figure 1.9 DNA structure.

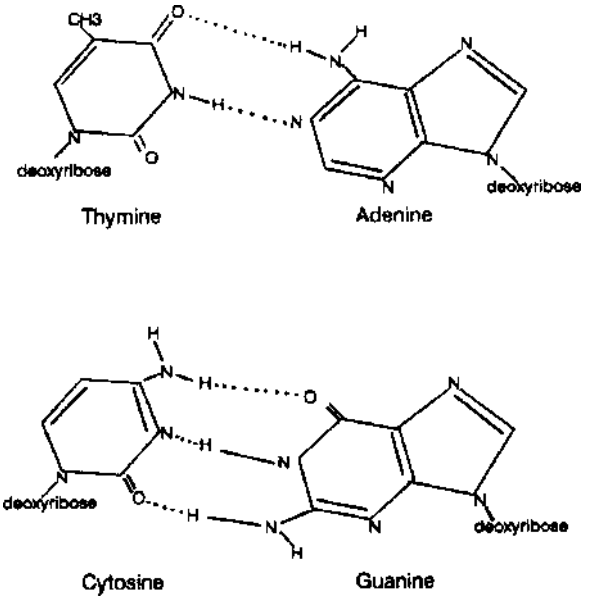

Figure 1.10 Base-pairing in DNA.

act as a template for a new complementary strand. Nucleotides move into the *replication fork* and align themselves against the complementary bases on the template. The addition of nucleotides is catalyzed by an enzyme called *DNA polymerase*.

- *Transcription*

Transcription is the process of transfer of information from DNA to RNA. The complementary single-stranded RNA (ssRNA) molecule is called *messenger RNA* (*mRNA*). mRNA carries the information from the DNA to the ribosomes, where it controls protein synthesis. Transcription is catalyzed by an enzyme called *RNA polymerase*. Enzyme regulation (repression or induction) occurs at the level of transcription. Sometimes, the product formed through the action of an enzyme represses the synthesis of that enzyme. The enzyme product acts as a co-repressor, which, along with a *repressor*, combines with the operator gene to block transcription and, therefore, enzyme synthesis. The synthesis of other enzymes, called *inducible enzymes*, occurs only when the substrate is present in the medium. Enzyme synthesis is induced because the substrate, the inducer, combines with the repressor to form a complex that has no affinity for the operator gene.

- *Translation*

mRNA controls protein synthesis in the cytoplasm. This process is called *translation*. Another type of RNA is the *transfer RNA* (*tRNA*), which has attachment sites for both mRNA and amino acids and brings specific amino acids to the ribosome.

Each combination of three nucleotides on the mRNA is called a *codon* or *triplet*. Each of these triplets codes for a specific amino acid. The sequence of codons determines that of amino acids in a protein. Some triplets code for the initiation and termination of amino acid sequences. There are 64 possible codons.

The sequence of events involved in protein synthesis is illustrated in Figure 1.11 (MacGregor, 2002).

1.3.3 Plasmids

A *plasmid* is a circular extrachromosomal circular DNA containing 1000–200,000 bp, and reproducing independent from the chromosomal DNA. Plasmids are inherited by daughter cells after cell division. Plasmid replication can be inhibited by *curing* the cells with compounds such as ethidium bromide. Some of the plasmids may exist in a limited number (1-3) of copies (stringent plasmids) or relatively large number (10–220) of copies (relaxed plasmids). Relaxed plasmids are most useful as cloning vectors. Some plasmids cannot coexist, making them incompatible with other plasmids in the same cell.

There are several categories of plasmids:

Conjugative plasmids. These plasmids carry genes that code for their own transfer to other cells. *F factors* or *sex factors* are conjugative plasmids that can become integrated into the chromosomes. *E. coli* strains that possess the chromosome-integrated F factors are called *Hfr* (high frequency of recombination).

Resistance transfer factors (R factors). These plasmids confer upon the host cell resistance to antibiotics (e.g., tetracycline, chloramphenicol, streptomycin) and heavy metals (e.g., mercury, nickel, cadmium). There is a great concern over these plasmids by the medical profession. The widespread use of antibiotics in medicine and agriculture results in the selection of multiple drug-resistant bacteria with R factors (see Chapter 4).

- *Col factors* are plasmids that code for production of colicins, which are proteinaceous bacteria-inhibiting substances.

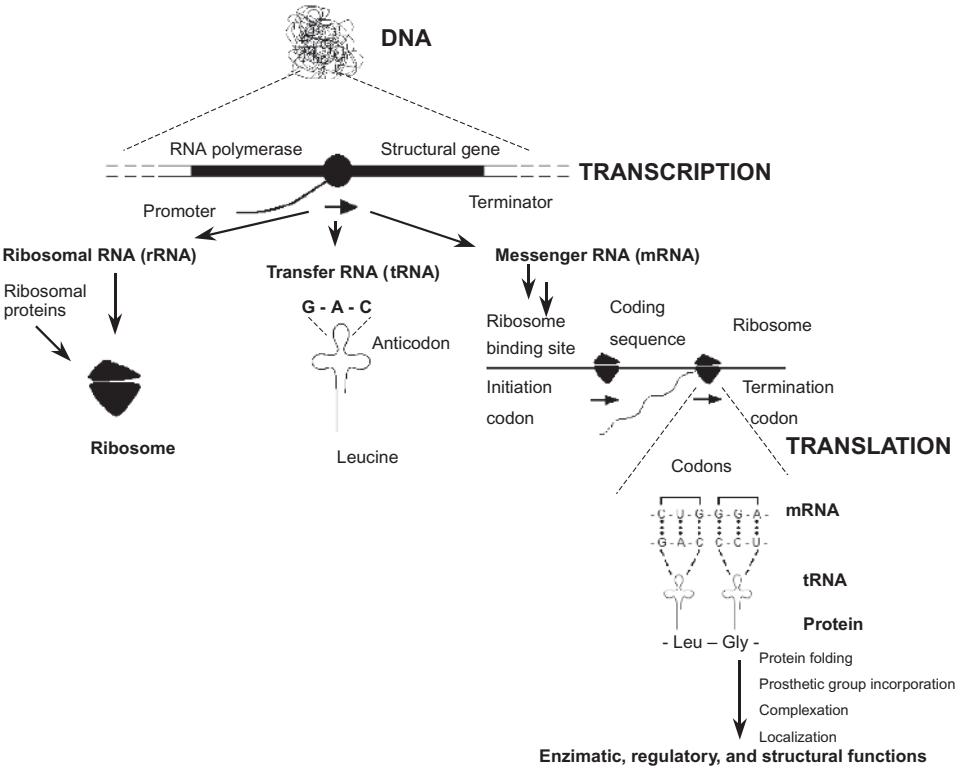

Figure 1.11 Protein synthesis: Transcription and translation. From MacGregor (2002).

- *Catabolic plasmids* code for enzymes that drive the degradation of unusual molecules such as camphor, naphthalene, and other xenobiotics found in environmental samples. They are important in the field of pollution control. Plasmids can be engineered to contain desired genes and can be replicated by introduction into an appropriate host (see Section 1.3.6).

1.3.4 Mutations

Mutations, caused by physical and chemical agents, change the DNA code and impart new characteristics to the cell, allowing it, for example, to degrade a given xenobiotic or survive under high temperatures. Spontaneous mutations occur in one out of 10^6 cells. However, the DNA molecule is capable of self-repair.

Conventional methods are used to obtain desired mutations in a cell. The general approach consists of exposing cells to a mutagen (e.g., ultraviolet [UV] light, chemical) and then exposing them to desired environmental conditions. These conditions select for cells having the desired traits.

1.3.5 Genetic Recombinations

Recombination is the transfer of genetic material (plasmid or chromosomal DNA) from a donor cell to a recipient cell. There are four means by which DNA is transferred to recipient cells (Brock and Madigan, 1991; Fig. 1.12).

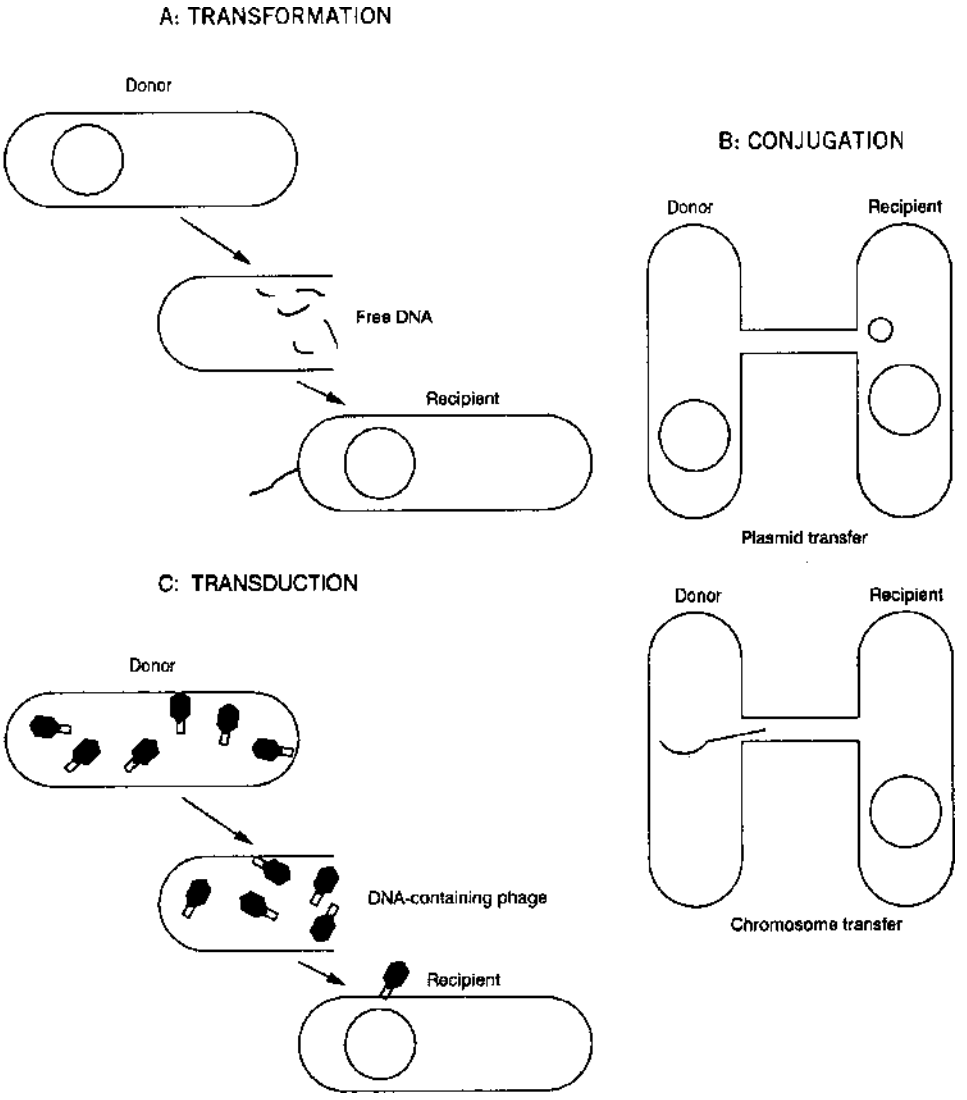

Figure 1.12 Genetic recombinations among bacteria. Adapted from Brock and Madigan (1988).

1.3.5.1 Transformation. Exogenous DNA enters a cell and becomes an integral part of a chromosome or plasmid. A cell capable of transformation by exogenous DNA is called a *competent* cell. Cell competence is affected by the growth phase of the cells (i.e., physiological state of bacteria), as well as the composition of the growth medium. During transformation, the transforming DNA fragment attaches to the competent cell, is incorporated into the cell, becomes single-stranded, and one strand is integrated into the recipient cell DNA, while the other strand is broken down. Transformation efficiency is increased by treating cells with high concentrations of calcium under cold conditions. The widespread occurrence of DNAses in the environment, particularly in wastewater, affects the transformation frequency.

If the transforming DNA is extracted from a virus, the process is called *transfection*. DNA can be introduced into eukaryotic cells by electroporation (the use of an electric field

to produce pores in the cell membrane) or through the use of a particle gun to shoot DNA inside the recipient cell.

1.3.5.2 Conjugation. This type of genetic transfer necessitates cell-to-cell contact. The genetic material (plasmid or a fragment of a chromosome mobilized by a plasmid) is transferred upon direct contact between a donor cell (F^+ or male cells) and a recipient cell (F^- or female cells). A special surface structure, called *sex pilus*, of the donor cell triggers the formation of a conjugation bridge that allows the transfer of the genetic material from the donor to the recipient cell. The conjugative pili are encoded by the *tra* genes. Certain bacteria (e.g., enterococci) have a specialized conjugation system induced by signal peptides secreted by the recipient cells. The signal peptides induce the synthesis by the donor cells of proteins involved in cell clumping.

Gene transfer, through conjugation, has been demonstrated in natural environments and engineered systems, including wastewater, freshwater, seawater, sediments, leaf surfaces, soils, and in the intestinal tract. The transfer rates vary between 10^{-2} and 10^{-8} transconjugants per recipient cell. Plasmids coding for antibiotic resistance can be transferred from environmental isolates to laboratory strains. Biotic and abiotic factors (e.g., cell type and density, temperature, oxygen, pH, surfaces) affect gene transfer by conjugation, but their impact under environmental conditions is not well known.

1.3.5.3 Transduction. It is the transfer of genetic material from a donor to a recipient cell using a bacterial phage as a carrier. A small fragment of the DNA from a donor cell is incorporated into the phage particle. Upon infection of a donor cell, the DNA of the transducing phage particle may be integrated into the recipient cell DNA. Contrary to conjugation, transduction is specific, due to the limited host range of phage. Transducing phages are involved in the horizontal transmission of the Shiga toxins among enterohemorrhagic strains of *E. coli* in food and aquatic environments (Imamovic et al., 2009; Muniesa et al., 2006). There are reports on the occurrence of transduction in freshwater and wastewater treatment plants.

1.3.5.4 Transposition. Another recombination process is *transposition* that consists of the movement (i.e., "jumping") of small pieces of plasmid or chromosomal DNA, called *transposons* ("jumping genes"), from one location to another on the genome. Transposons, which can move from one chromosome to another or from one plasmid to another, carry genes that code for the enzyme transposase, which catalyzes their transposition.

1.3.6 Recombinant DNA Technology: Construction of a Genetically Engineered Microorganism

Recombinant DNA technology, commonly known as genetic engineering or gene cloning, is the deliberate manipulation of genes to produce useful gene products (e.g., proteins, toxins, hormones). There are two categories of recombination experiments: (1) *in vitro recombination,* which consists of using purified enzymes to break and rejoin isolated DNA fragments in test tubes; and (2) *in vivo recombination*, which consists of encouraging DNA rearrangements that occur in living cells.

A typical gene cloning protocol consists of the following steps (Fig. 1.13):

1. *Isolation of the source DNA.* Several methods are used for the isolation of DNA from a wide range of cells.

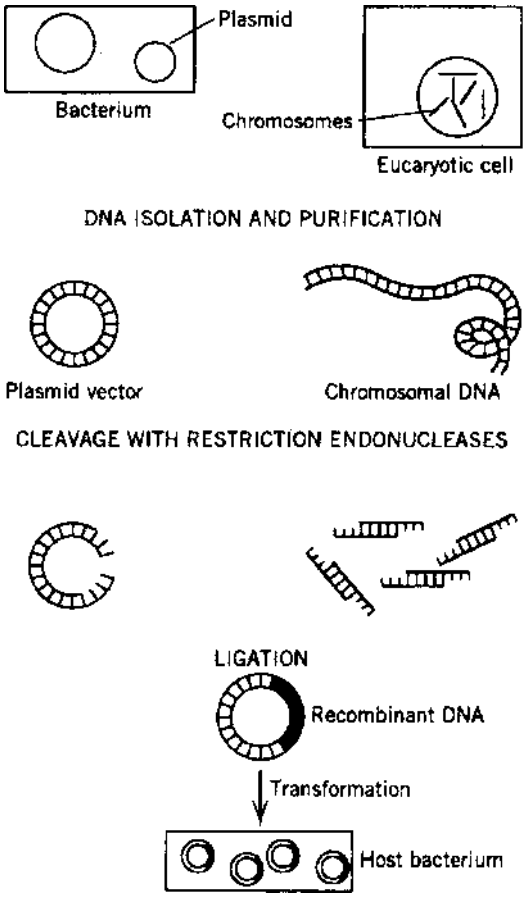

Figure 1.13 Steps involved in gene cloning.

2. *DNA fragmentation or splicing.* Restriction endonucleases are used to cleave the double-stranded DNA at specific sites. These enzymes normally help cells cope with foreign DNA and protect bacterial cells against phage infection. They are named after the microorganism from which they were initially isolated. For example, the restriction enzyme *EcoRI* was isolated from *E. coli*, whereas *HindII* enzyme was derived from *Haemophilus influenza*. *EcoRI* recognizes the following sequence on the double-stranded DNA:

-G-A-A-T-T-C-

-C-T-T-A-A-G-

and produces the following fragments:

-G- A-A-T-T-C-

-C-T-T-A-A -G-

The DNA fragments can be separated according to their size by electrophoresis.

3. *DNA ligation.* DNA fragments are joined to a cloning vector using another enzyme called *DNA ligase*. Ligation is possible because both the source DNA and the cloning vector DNA were cut with the same restriction enzyme. Commonly used cloning vectors are plasmids (e.g., pBR322) or phages (e.g., phage λ).
4. *Incorporation of the recombinant DNA into a host.* The recombinant DNA is introduced into a cell for replication and expression. The recombinant DNA may be introduced in the host cell by transformation, for example. The most popular hosts are prokaryotes such as *E. coli*, or eukaryotes such as *Saccharomyces cerevisiae*. The host microorganism now containing the recombinant DNA will divide and make clones.
5. *Selection of the desirable clones.* Clones that have the desired recombinant DNA can be screened using markers like antibiotic resistance. However, the selection of clones having the desired gene can be accomplished by using nucleic acid probes (see Section 1.3.7) or by screening for the gene product. If the gene product is an enzyme (e.g., β-galactosidase), clones are selected by looking for colonies that have the enzyme of interest (hosts cells are grown in the presence of the enzyme substrate).

Biotechnological applications of genetically engineered microorganisms (GEMs) have been realized in various fields, including the pharmaceuticals industry, agriculture, medicine, food industry, energy, and pollution control (see Chapter 16). Notorious applications are the production of human insulin and viral vaccines. In agriculture, research is focusing on the production of *transgenic plants* (i.e., genetically altered whole plants) that are resistant to insects, herbicides, or diseases.

The potential use of GEMs in pollution control is becoming increasingly attractive. It has been proposed to use GEMs to clean up hazardous waste sites and wastewaters by constructing microbial strains capable of degrading recalcitrant molecules. However, there are potential problems associated with the deliberate release of GEMs into the environment. This is because, unlike chemicals, GEMs have the potential ability to grow and reproduce under *in situ* environmental conditions.

1.3.7 Review of Selected Molecular Techniques

Several molecular tools are now available to investigate the structure and function of microbial communities and understand the survival of pathogens and parasites in environmental samples.

1.3.7.1 DNA Probes. DNA isolated from an environmental sample may be hybridized with a labeled probe, cloned into a plasmid, or amplified by polymerase chain reaction (PCR; MacGregor, 2002). Nucleic acid probes help detect specific microorganisms in an environmental mixture of cells. They are based on nucleic acid hybridization. The two strands of DNA are said to be complementary. Single complementary strands are produced via DNA denaturation, using heat or alkali. Under appropriate conditions, the complementary strands *hybridize* (i.e., bind to each other). A DNA probe is a small piece of DNA (oligonucleotide) that contains specific sequences that, when combined with single-stranded target DNA, will hybridize with the complementary sequence in the target DNA and form a double-stranded structure (Sayler and Blackburn, 1989). For easy detection, the probe is labeled with a radioisotope (e.g., ^{32}P), enzymes (e.g., β-galactosidase, peroxidase, or alkaline phosphatase), or fluorescent compounds (e.g., fluorescein isothiocyanate). Gene probes can be used to detect gene sequences in bacterial colonies growing on a solid

medium. This technique is called *colony hybridization*. *Dot hybridization* consists of spotting nucleic acid on a filter and then probing to show the presence or absence of a given sequence. Gene databases are available for checking the gene sequences of microorganisms.

PCR greatly enhances the sensitivity of DNA probes. Some of these probes that can be combined with PCR technology are available for detecting bacterial, viral, and protozoan pathogens and parasites (Sayler and Layton, 1990). However, DNA probes cannot be relied upon to evaluate the safety of disinfected water since they cannot distinguish between infectious and noninfectious pathogens.

The following are some of the applications of nucleic acid probes:

- *Detection of pathogens in clinical samples.* Probes have been developed for clinically important microorganisms such as *Legionella* spp., *Salmonella* species, enteropathogenic *E. coli*, *Neisseria gonorrhoea*, human immunodeficiency virus (HIV), herpes viruses, or protozoan cysts of *Cryptosporidium* and *Giardia*. Probe sensitivity can be increased by using PCR technology (see Section 1.3.7.5 for details on this technology).
- *Detection of metal resistance genes in environmental isolates.* For example, a probe was constructed for detecting the *mer* operon, which controls mercury detoxification.
- *Tracking of numerous microorganisms in the environment.* Probes are useful in detecting and following the fate of specific environmental isolates (e.g., nitrogen-fixing bacteria, bacteria capable of degrading a specific pollutant, protozoan parasites) and genetically engineered microbes in water, sediments, wastewater, biosolids, and soils.

1.3.7.2 RNA-Based Methods. rRNA is a good target to probe because of the large number of ribosomes in living cells. RNA-based methods provide information about the activity of microbial communities. These methods aim at detecting rRNA or mRNA.

rRNA probes are hybridized with extracted target RNA that has been blotted onto positively charged membranes (membrane hybridization) or with fixed target cells (fluorescent *in situ* hybridization [FISH]). rRNA probes give information on community activity and are used for the identification and classification of indigenous microorganisms in environmental samples. These probes can be designed to target specific groups of microorganisms ranging from the subspecies to the domain level (DeLong, 1993; Head et al., 1998; Hofle, 1990; MacGregor, 2002). Their sensitivity is much greater than that of DNA probes. One major advantage of rRNA probes is the availability of thousands of RNA sequences in public databases, such as GenBank or the Ribosomal Database Project (RBD), which are available online (Wilderer et al., 2002). However, because indigenous bacteria have a lower number of copies of ribosomes than cultured bacteria, signal (e.g., fluorescence) amplification is sometimes needed. For example, up to 20-fold amplification of the signal was observed when using thyramide (Lebaron et al., 1997). *r*RNA probes are quite helpful to microbial ecologists in their efforts to identify nonculturable bacteria and to gain knowledge on the biodiversity of complex microbial communities (Amann and Ludwig, 2000). The application of rRNA probes to the identification of filamentous bacteria in activated sludge was discussed in Chapter 10.

mRNA carries out information from the DNA to the ribosome. The detection of mRNA, present at much lower number of copies than rRNA, gives information on gene expression and function.

1.3.7.3 FISH. We have seen that rRNA is a convenient target molecule occurring as thousands of copies inside a target cell. Gene probes, labeled with a fluorescent compound (called reporter), hybridize with whole permeabilized cells *in situ*. A universal probe targeting the Bacteria domain (EUB338) is used in combination with more specific probes. Intact fluorescent cells with the desirable sequence are counted under a fluorescence microscope (Dorigo et al., 2005; Wagner et al., 2003; Wilderer et al., 2002). FISH can also provide information about cell activity in environmental samples. It can provide information about the level of metabolic activity by measuring the fluorescence intensity inside the cell (Talbot et al., 2008). This approach is useful in the identification of filamentous microorganisms in bulking sludge (Kanagawa et al., 2000; Wagner et al., 1994), nitrifying activated sludge (Juretschko et al., 1998), or sulfate-reducing biofilms (Ramsing et al., 1993). FISH also provides information on the interactions between different microorganisms in an environmental sample.

In oligotrophic environments, there are slow-growing or starving microorganisms with a low rRNA content. Their detection by FISH is sometimes difficult due to low fluorescent signal. To address this problem, a number of modified amplification methods have been proposed. The catalyzed reporter deposition (CARD)–FISH method consists of using horseradish peroxidase-labeled oligonucleotide probes along with tyramide to amplify the signal in permeabilized cells (the cells are embedded in low-gelling agarose prior to permeabilization with lysozyme to avoid cell death; Pernthaler et al., 2002). A recent study showed that the sensitivity of CARD–FISH was 26- to 41-fold higher than the conventional FISH (Hoshino et al., 2008). The higher sensitivity of CARD–FISH was confirmed regarding the detection of bacteria in acid mine drainage sites (Kock and Schippers, 2008).

Another modified version of FISH is the tyramide signal amplification (TSA)–FISH method, where the fluorescence inside the cells is amplified 20 to 40 times following treatment with tyramide (Dorigo et al., 2005). FISH can also be combined with microautoradiography (FISH–MAR) to obtain information about the uptake of labeled substrates by the probed microorganisms.

1.3.7.4 Nucleic Acid Fingerprinting. This approach involves the use of PCR (see Section 1.3.7.5) to amplify specific fragments of DNA to be analyzed by denaturing gradient gel electrophoresis (DGGE), which allows the separation of fragments, based on length but with different sequences. The gel contains an increasing concentration of denaturant (formamide and urea). Thus, this approach gives information concerning the microbial diversity in a microbial community.

- *Length heterogeneity PCR (LH-PCR):* The distinction between microorganisms is based on natural length variations within the PCR-amplified rRNA genes variable regions (Talbot et al., 2008).
- *Terminal restriction fragment length polymorphism (T-RFLP):* This is another rapid fingerprinting technique to determine biodiversity in microbial communities. The DNA extracted from the microbial community is subjected to PCR. One primer used in PCR is fluorescently labeled at the 5′ terminus and is used to amplify a selected region of genes encoding for 16S rRNA. The resulting amplicons are fluorescently labeled at the end. After purification, the amplicons are digested with a restriction enzyme, generating fragments, which are separated by gel or capillary electrophoresis. The resulting profile has different peaks, which are detected by a laser (Dorigo et al., 2005; Liu et al., 1997; Nocker et al., 2007b). The T-RFLP technique is illustrated in Figure 1.14 (Grüntzig et al., 2002).

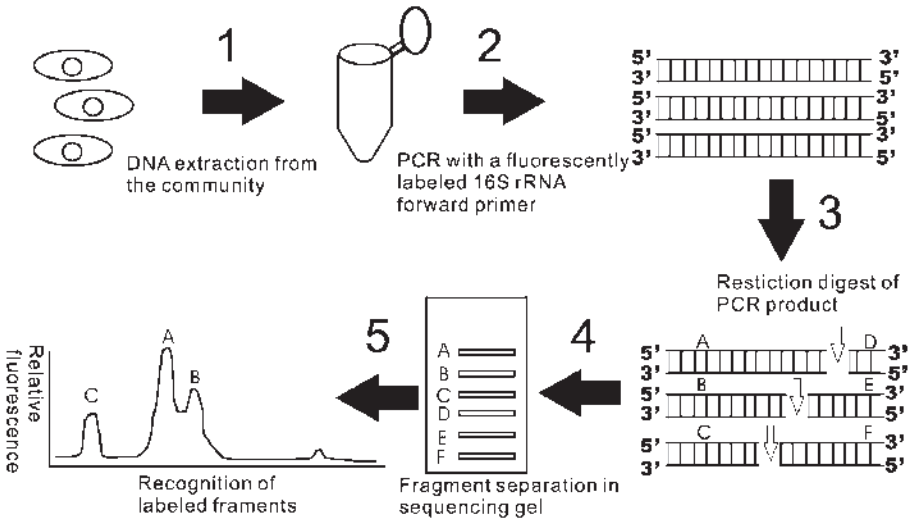

Figure 1.14 Steps involved in T-RFLP. From Grüntzig et al. (2002).

- *Ribosomal intergenic spacer analysis (RISA):* Conceived by Borneman and Triplett (1997), RISA consists of the PCR amplification of the intergenic region between the small16S and large 23S rRNA subunits. This region is called the *intergenic spacer region*, which varies in size (150–1500 bp) and nucleotide sequence. The PCR products of variable sizes are separated via polyacrylamide gel electrophoresis (Dorigo et al., 2005). An automated RISA (ARISA) consists of amplifying the intergenic region using a fluorescently labeled forward primer. The amplicons are detected by capillary electrophoresis.
- *DGGE:* This technique is used for rapid assessment of microbial biodiversity. The genes encoding 16S rRNA are amplified and separated via polyacrylamide gel electrophoresis using a gradient of increasing concentrations of denaturants (urea and formamide; Dorigo et al., 2005).

1.3.7.5 PCR. The PCR technique was developed in 1986 at Cetus Corporation by Mullis and collaborators (Mullis and Fallona, 1987). This technique essentially simulates *in vitro* the DNA replication process occurring *in vivo*; it consists of amplifying discrete fragments of DNA by generating millions of copies of the target DNA (Atlas, 1991; Oste, 1988).

During cell division, two new copies of DNA are made and one set of genes is passed on to each daughter cell. Copies of genes increase exponentially as the number of generations increase. PCR simulates *in vitro* the DNA duplication process and can create millions of copies of the target DNA sequence. PCR consists of three steps that constitute one cycle in DNA replication (Fig. 1.15):

1. *DNA denaturation (strand separation).* When incubated at high temperature, the target double-stranded DNA fragment is denatured and dissociates into two strands.
2. *Annealing of primers.* When the temperature is lowered, the target DNA fragment anneals to synthetic nucleotides primers made of 18–28 nucleotides and which flank the target DNA fragment. These primers are complementary to the section of the

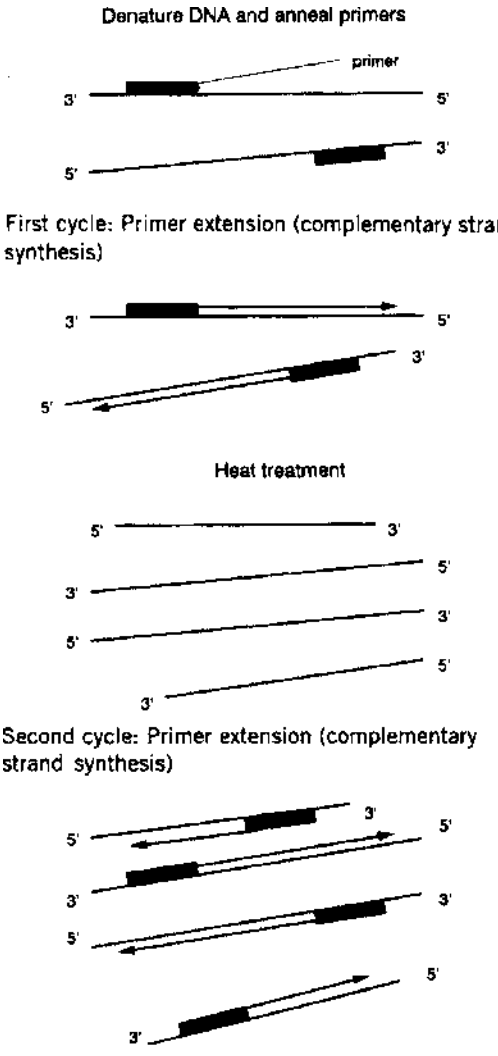

Figure 1.15 Polymerase chain reaction (PCR).

DNA to be replicated. The primers target phylogenetic groups ranging from universal to subspecies level.

3. *Primer extension or amplification step.* The primers are extended with the help of thermostable DNA polymerase, the enzyme responsible for DNA replication in cells. This thermostable enzyme (*Taq* DNA polymerase) is extracted from *Thermus aquaticus*, a bacterium that is found in hot springs.

After approximately 30 cycles lasting approximately 3 h, the target DNA fragment is amplified and accumulates exponentially. The PCR technique can be automated by using a DNA thermal cycler that automatically controls the temperatures necessary for the denaturation and annealing steps.

This procedure is very useful in cloning, DNA sequencing, tracking genetic disorders, and forensic analysis. It is a powerful tool in diagnostic microbiology, virology, and para-

sitology. The tests presently used to detect individuals who have been exposed to HIV detect only antibodies to the virus and not the virus itself. However, HIV was identified directly, using PCR, in the blood of acquired immunodeficiency syndrome (AIDS) patients from which the virus was also isolated by more traditional tissue culture techniques. Virus identification via PCR is relatively rapid as compared with traditional culture techniques (Ou et al., 1988). The following are some environmental applications of PCR technology (Atlas, 1991):

- *Detection of specific bacteria.* Specific bacteria in environmental samples, including wastewater, effluents, and sludges can be detected by PCR.
- *Environmental monitoring of GEMs.* Genetically engineered bacteria that perform certain useful functions (e.g., pesticide or hydrocarbon degradation) can be tracked using PCR technology. The target DNA sequence would be amplified *in vitro* and then hybridized to a constructed DNA probe.
- *Detection of indicator and pathogenic microorganisms.* Methodology for the detection of pathogens has shifted from cell cultures to molecular-based techniques because some enteric viruses grow poorly or fail to grow on tissue cultures (Metcalf et al., 1995). Hence, PCR technology has been considered for the detection of foodborne and waterborne pathogens and parasites in water, wastewater, and food. Examples of pathogens and parasites detected by PCR are invasive *Shigella flexneri*, enterotoxigenic *E. coli, Legionella pneumophila, Salmonella*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* (Bej et al., 1991a; 1992; Kapperud et al., 1993; Koide et al., 1993; Lampel et al., 1990; Olive, 1989; Tsai et al., 1993), hepatitis A virus (HAV; Altmar et al., 1995; Divizia et al., 1993; Le Guyader et al., 1994; Prevot et al., 1993; Morace et al., 2002), Norwalk virus (Altmar et al., 1995; DeLeon et al., 1992), rotaviruses (Gajardo et al., 1995; Le Guyader et al., 1994), adenoviruses (Girones et al., 1993), astroviruses (Abad et al., 1997), enteroviruses (Abbaszadegan et al., 1993), HIV (Ansari et al., 1992), *Giardia* (Mahbubani et al., 1991), *Cryptosporidium* (Johnson et al., 1993), and indigenous and nonindigenous microorganisms in pristine environments (Baker et al., 2003). Multiplex PCR using several sets of primers allows the simultaneous detection of gene sequences of several pathogens or different genes within the same organism. For example, a triplex reverse transcriptase (RT)-PCR method was proposed for the simultaneous detection of poliovirus, HAV, and rotavirus in wastewater (Tsai et al., 1994; Way et al., 1993). Functional genes can also be amplified to study subpopulations with certain physiological characteristics. Examples of genetic markers include the ones coding for ammonium monooxygenase (*amo*A), mercuric reductase (*mer*A), nitrogenase (*nif*H), methane monooxygenase (*pmo*A), dissimilatory sulfate reductase (*dsr*AB), β-galactosidase (*lac*Z), or β-glucuronidase (*uid*A).

Some advantages of PCR are rapid detection of pathogens and parasites, specificity, sensitivity, and use in the detection of nonculturable (e.g., noroviruses) or noncytopathic (HAV) viruses (Rodríguez et al., 2009).

Some disadvantages of PCR are the following:

- *PCR assay is affected by inhibitory compounds.* Environmental samples as well as chemicals used for virus concentration contain substances (e.g., humic and fulvic acids, heavy metals, beef extract used in virus concentration, and other unknown substances) that interfere with pathogen or parasite detection via PCR. DNA or rRNA purification by gel filtration (using Sephadex) followed by treatment by an ion

exchange resin (using Chelex) helps remove the interference due to humic substances and heavy metals, respectively (Abbaszadegan et al., 1993; Straub et al., 1995; Tsai and Olson, 1992; Tsai et al., 1993). Inhibitors found in shellfish extracts can be removed by treatment with cetyltrimethylammonium bromide prior to PCR (Atmar et al., 1993; Moran et al., 1993) or granular cellulose (Le Guyader et al., 1994). The interference can also be removed by using a magnetic-antibody capture method, as shown for the detection by PCR of *Giardia* and *Cryptosporidium* in environmental samples (Bifulco and Schaefer, 1993; Johnson et al., 1995a, b) or by using an electropositive membrane (ZP60S; AMF Cuno Division, Meriden, CT; Queiroz et al., 2001).

- *Indication of cell viability or viral infectivity.* Concerns are raised over the failure of PCR to give an indication of the viability of the pathogens and parasites detected in environmental samples, although some investigators report a relationship between detection of viral RNA by PCR and the presence of infectious viruses (Le Guyader et al., 1994; Limsawat and Ohgaki, 1997).

To overcome these disadvantages, PCR can be combined with cell culture. Viruses are allowed to replicate in a suitable cell culture and then amplified via PCR. This approach has been used for detecting infectious viruses (HAV, enteroviruses, adenoviruses, astroviruses, and reoviruses) in the environment (Rodríguez et al., 2009). The inclusion of an enzymatic treatment (treatment with proteinase K and RNAse) prior to DNA extraction and PCR can help track viral infectivity by reducing the number of false-positive signals (Nuanualsuwan and Cliver, 2003; Pecson et al., 2009).

As regards bacterial cells, the incorporation of propidium azide in the PCR protocol was found to be useful in the distinction between live and dead bacterial cells by quantifying gene copies in dead cells and extracellular DNA (Bae and Wuertz, 2009; Nocker et al., 2007a; 2009). Similarly, the combination of ethidium monoazide bromide, a nucleic acid-binding dye, with quantitative PCR, selectively detects viable cells. The dye penetrates cells with damaged membranes and covalently binds to DNA after photoactivation. This prevents DNA amplification in dead cells and results in signal reduction during quantitative PCR (Delgado-Viscogliosi et al., 2009).

Other PCR protocols are the following:

- *Quantitative real-time PCR (qRT-PCR).* This emerging technique is more sensitive, reproducible, and quicker than the conventional PCR. In conventional PCR, the final concentration of the fluorescent PCR product is determined, while in qRT-PCR the amount of PCR product fluorescence is monitored in real time during the amplification cycles, using dsDNA-binding dyes such as SYBR Green I (Zhang and Fang, 2006). This gives an estimation of the initial DNA/RNA concentration. The results of qRT-PCR are expressed as the number of gene copies per mL of sample (Talbot et al., 2008). As an illustration, Figure 1.16 (Zhang and Fang, 2006) shows the quantification of a cyanobacterium, *Microcystis aeruginosa*, by qRT-PCR. In Figure 1.16a, fluorescence intensity is plotted against the number of cycles for different concentrations of *M. aeruginosa* suspensions. The threshold C_t is the cycle above which there is a logarithmic increase in fluorescence and is inversely proportional to the log of the starting number of target cells (see Fig. 1.16b). The higher the number, the sooner the detection of a fluorescent signal significantly higher than the background. This standard curve should have a slope between −3.0 and −3.9 and an R^2 greater than 0.95 (Zhang and Fang, 2006).
- *Competitive PCR (cPCR).* It involves the co-amplification of the target DNA and an internal standard, or competitor DNA, which is similar to but distinguishable from

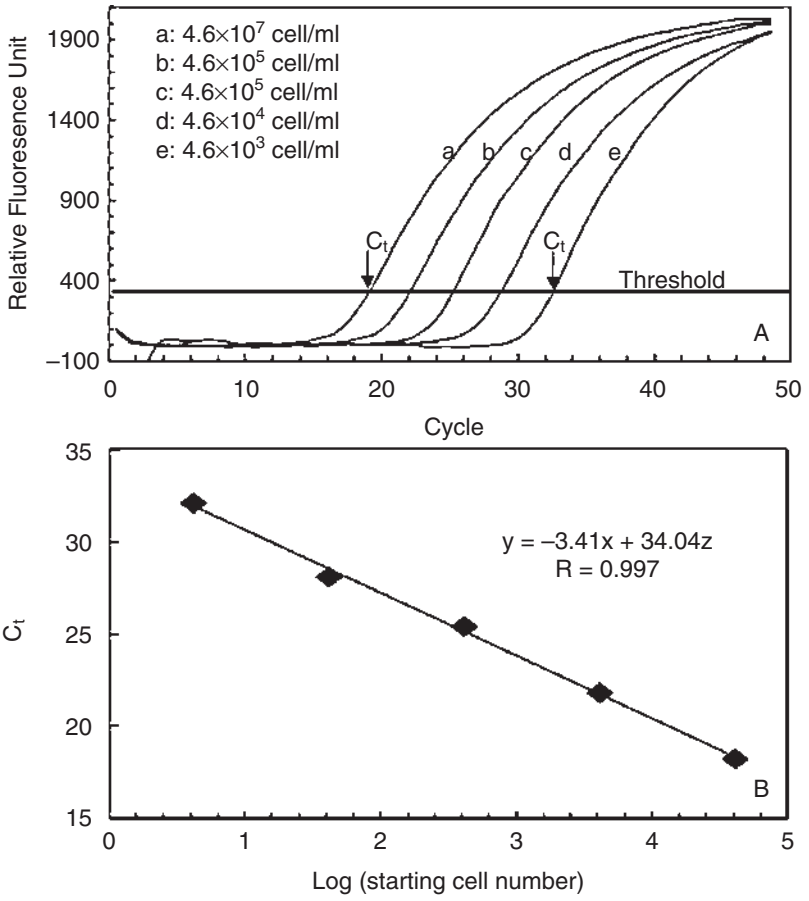

Figure 1.16 Quantification of *Microcystis aeruginosa* PCC 7820 using qRT-PCR. From Zhang and Fang (2006).

the target DNA. Estimation of the number of target sequences is achieved by comparison of ratios between target and competitor sequences with those of a standard curve generated by the amplification of competitor DNA with a range of target DNA concentrations. This standard curve is used to calculate the concentration of the target DNA in the sample (Phillipsa et al., 2000; Zimmermann and Mannhalter, 1996).

The proper decontamination of equipment and surfaces is necessary to avoid false positives by PCR. Among several disinfectants tested, chlorine appears to be the most efficient for degrading nucleic acid sequences contaminants (Ma et al., 1994).

1.3.7.6 Microarrays. Microarrays, also called gene chips, consist of large sets of DNA sequences (probes) or oligonucleotides attached to a nonporous solid support and are hybridized with target fluorescently labeled sequences that have been isolated from environmental samples. Cy-3 and Cy-5 fluorescent dyes are generally used as labels, but alternative fluorescent dyes have been utilized to detect targets in microarrays. The probes, made of PCR products or oligonucleotides, are attached to the solid support by three main printing technologies such as photolithography, ink-jet ejection, or mechanical

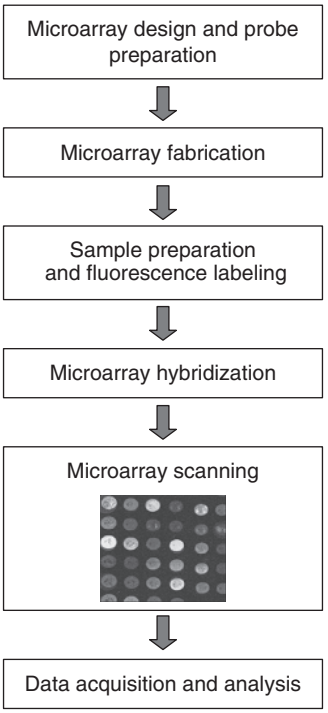

Figure 1.17 Steps involved in microarray preparation and utilization. Adapted from Zhou and Thompson (2002). (See color insert.)

microspotting. Following hybridization of the probes with the targets, the microarrays are scanned with a high-resolution scanner and the digital images are analyzed using commercially available software (Call et al., 2003; Zhou and Thompson, 2002). Figure 1.17 (Zhou and Thompson, 2002) shows the various steps involved in microarray preparation and utilization. In *isotope microarrays*, the environmental sample is pre-incubated with a radioactively labeled substrate. Fluorescent and radioactive spots provide information about both the identity and metabolism of the substrate (Wagner et al., 2006).

Microarrays offer a powerful tool for monitoring gene expression and function as well as the detection and characterization of pathogens in environmental samples.

They offer several advantages that include the ability to attach thousands of probes over a very small surface area, high sensitivity, ability to detect several target sequences labeled with different fluorescent tags, low background fluorescence, amenability to automation, and potential for use in field-based studies (Bavykin et al., 2001; Wu et al., 2001; Zhou and Thompson, 2002).

1.3.7.7 Metaproteomics. Metagenomics is a culture-independent way of characterizing the collective genome of the microorganisms (archaea, bacteria, eukarya, viruses) in an ecosystem and provides information of the structure and diversity in microbial communities (Hugenholtz and Tyson, 2008). However, it does not provide information on the function of the detected genes. The function of microorganisms in an ecosystem can be known only though analysis of RNA transcripts (metatranscriptomics) or expressed proteins (metaproteomics) (Fig. 1.18; Maron et al., 2007). Following extraction, the proteins

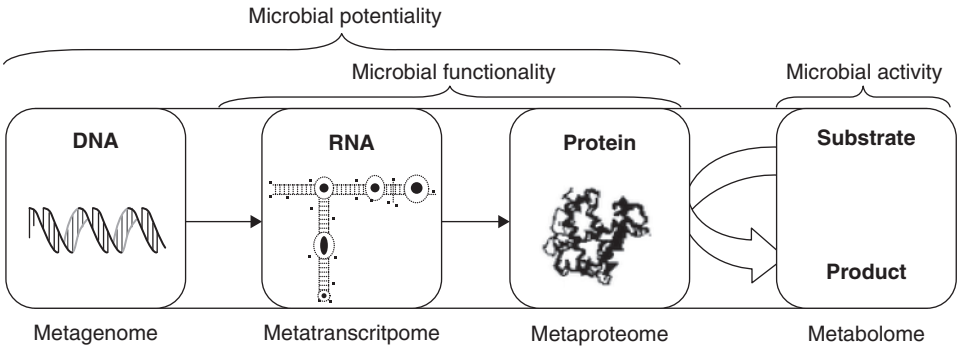

Figure 1.18 Metaproteomics. From Maron et al. (2007).

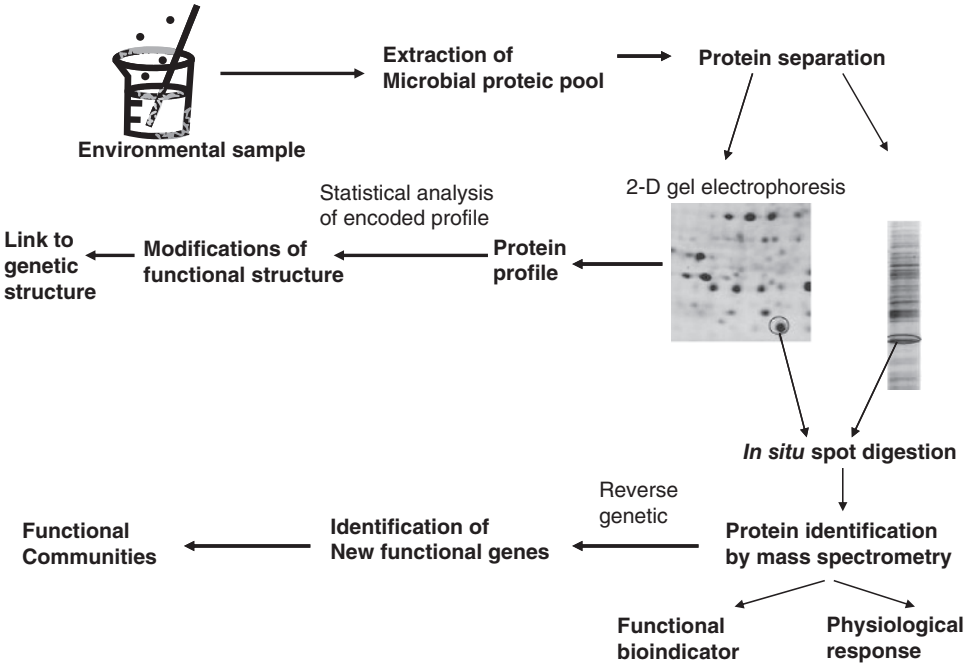

Figure 1.19 Metaproteome characterization. From Maron et al. (2007).

are separated by 2D gel electrophoresis and identified by mass spectrometry. The end result is a protein profile (i.e., fingerprint) that will shed light on the link between phylogenetic analysis and the function of microorganisms (Fig. 1.19; Maron et al., 2007). Metaproteomics can be applied to track new functional genes and to monitor the response of microbial communities to various stresses. Metaproteomics helped distinguish between an enhanced biological phosphorus removal (EBPR)-activated sludge from a non-EBPR sludge. The two sludges had a distinct protein profile (metaproteome; Wilmes and Bond, 2006).

1.4 BRIEF SURVEY OF MICROBIAL GROUPS

We will now discuss the various microbial groups encountered in environmental samples. They include the bacteria (prokaryotes), the archaea, the eukaryotes (algae, protozoa, fungi), and viruses.

1.4.1 Bacteria

1.4.1.1 Bacterial Size and Shape. Except for filamentous bacteria (size may be greater than 100 μm) or cyanobacteria (size range approximately 5–50 μm), bacterial cell size generally ranges between 0.3 μm (e.g., *Bdellovibrio bacteriovorus*; *Mycoplasma*) and 1–2 μm (e.g., *E. coli*; *Pseudomonas*). Australian researchers have discovered an unusually large gram-positive bacterium, *Epulopiscium fishelsoni*, that can reach several hundred micrometers in length. It lives in a symbiotic relationship in the gut of surgeonfish (Clements and Bullivant, 1991).

Bacteria occur in three basic shapes: cocci (spherical shape, e.g., *Streptococcus*), bacilli (rods, e.g., *Bacillus subtilis*), and spiral forms (e.g., *Vibrio cholera; S. volutans*; Fig. 1.20). Because of their relatively small size, bacteria have a high surface-to-volume ratio, a critical factor in substrate uptake.

1.4.1.2 "Unusual" Types of Bacteria (Fig. 1.21)

- *Sheathed Bacteria*

These bacteria are filamentous microorganisms surrounded by a tube-like structure called sheath. The bacterial cells inside the sheath are gram-negative rods that become flagellated (swarmer cells) when they leave the sheath. The swarmer cells produce a new sheath at a relatively rapid rate. They are often found in polluted streams and in wastewater treatment plants. This group includes three genera: *Sphaerotilus, Leptothrix,* and *Crenothrix*. These bacteria have the ability to oxidize reduced iron to ferric hydroxide (e.g., *Sphaerotilus natans, Crenothrix*) or manganese to manganese oxide (e.g., *Leptothrix*). In Chapter 10, we will discuss the role of *S. natans* in activated sludge bulking.

- *Stalked Bacteria*

They are aerobic, flagellated (polar flagellum) gram-negative rods that possess a stalk, a structure that contains cytoplasm and is surrounded by a membrane and a wall. At the end of the stalk is a holdfast that allows the cells to adsorb to surfaces. Cells may adhere to one another and form rosettes. *Caulobacter* is a typical stalked bacterium that is found

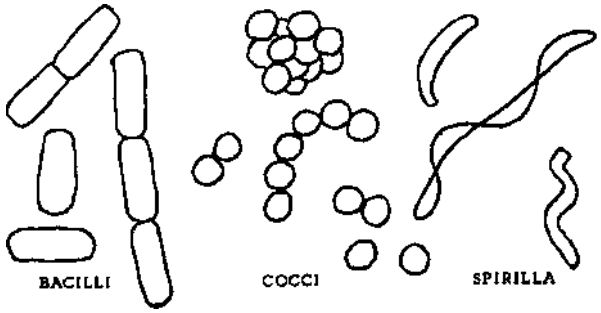

Figure 1.20 Bacterial cell shapes. Adapted from Edmonds (1978).

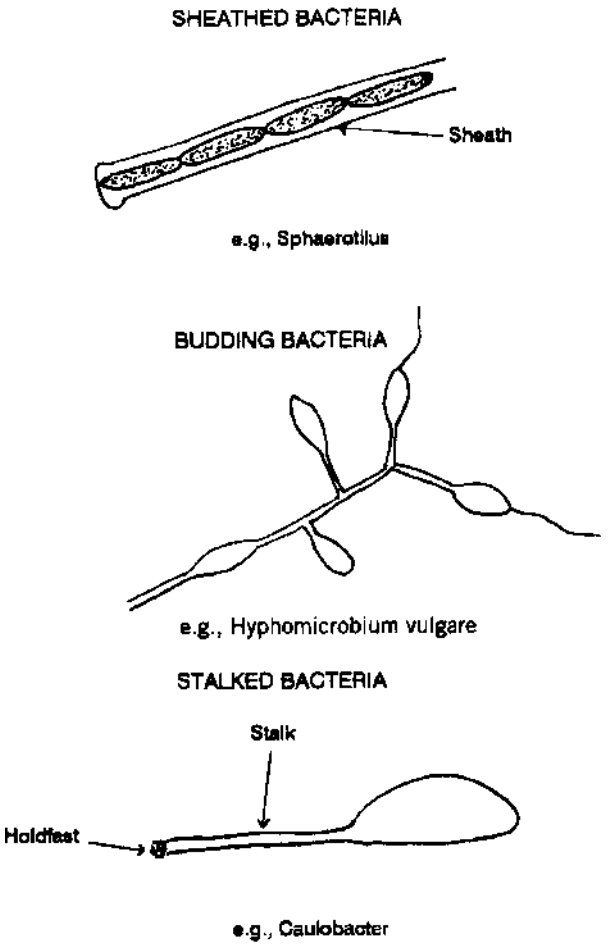

Figure 1.21 Some "unusual" types of bacteria. Adapted from LeChevalier and Pramer (1971).

in aquatic environments with low organic content. *Gallionella* (e.g., *G. ferruginea*) is another stalked bacterium that makes a twisted stalk, sometimes called "ribbon," consisting of an organic matrix surrounded by ferric hydroxide. These bacteria are present in iron-rich waters and oxidize Fe^{2+} to Fe^{3+}. They are found in metal pipes in water distribution systems.

- *Budding Bacteria*

After attachment to a surface, budding bacteria multiply by budding. They make filaments or hyphae, at the end of which a bud is formed. The bud acquires a flagellum (the cell is now called a swarmer), settles on a surface, and forms a new hypha with a bud at the tip. *Hyphomicrobium* is widely distributed in soils and aquatic environments and requires one-carbon (e.g., methanol) compounds for growth. A phototrophic bacterium, *Rhodomicrobium*, is another example of budding bacteria.

- *Gliding Bacteria*

These filamentous gram-negative bacteria move by gliding, a slow motion on a solid surface. They resemble certain cyanobacteria, except that they are colorless. *Beggiatoa*

and *Thiothrix* are gliding bacteria that oxidize H_2S to S^0, which accumulates as sulfur granules inside the cells. *Thiothrix* filaments are characterized by their ability to form rosettes (more details are given in Chapters 3 and 10). Myxobacteria are another group of gliding microorganisms. They feed by lysing bacterial, fungal, or algal cells. Vegetative cells aggregate to make "fruiting bodies," which lead to the formation of resting structures called myxospores. Under favorable conditions, myxospores germinate into vegetative cells.

- *Bdellovibrio (B. bacteriovorus)*

These small (0.2–0.3 μm), flagellated (polar flagellum) bacteria are predatory on gram-negative bacteria. After attaching to the bacterial prey, *Bdellovibrio* penetrates the cells and multiplies in the periplasmic space (i.e., the space between the cell wall and the plasma membrane). Because *Bdellovibrio* lyse their prey, they are able to form plaques on a lawn of the host bacterium. Some *Bdellovibrio* can grow independently on complex organic media.

- *Actinomycetes*

Actinomycetes are gram-positive filamentous bacteria characterized by mycelial growth (i.e., branching filaments), which is analogous to fungal growth. However, the diameter of the filaments is similar in size to bacteria (approximately 1 μm). Most actinomycetes are strict aerobes, but a few of them require anaerobic conditions. Most of these microorganisms produce spores, and their taxonomy is based on these reproductive structures (e.g., single spores in *Micromonospora* or chains of spores in *Streptomyces*). They are commonly found in water, wastewater treatment plants, and soils (preference for neutral and alkaline soils). Some of them (e.g., *Streptomyces*) produce a characteristic "earthy" odor due to the production of volatile compounds called *geosmins.* They degrade polysaccharides (e.g., starch, cellulose), hydrocarbons, and lignin. Many of them produce clinically important antibiotics (e.g., streptomycin, erythromycin, tetracycline, chloramphenicol), and marine actinomycetes may be the source of novel antibiotics in the future (Baltz, 2007). Two well-known genera of actinomycetes are *Streptomyces* and *Nocardia* (now called *Gordonia*; Fig. 1.22). *Streptomyces* forms a mycelium with conidial spores at the tip of the hyphae. These actinomycetes are important industrial microorganisms that produce hundreds of antibiotic substances. *Gordonia* is commonly found in water and wastewater and degrades hydrocarbons and other recalcitrant (i.e., hard to degrade) compounds. *Gordonia* is a significant constituent of foams in activated sludge units (see Chapter 10).

- *Cyanobacteria*

Often referred to as blue-green algae, cyanobacteria are prokaryotic organisms that differ from photosynthetic bacteria in that they carry out oxygenic photosynthesis (see Chapter 2; Fig. 1.23). They contain chlorophyll *a* and accessory pigments such as *phycocyanin* (blue pigment) and *phycoerythrin* (red pigment). The characteristic blue-green color exhibited by these organisms is due to the combination of chlorophyll *a* and phycocyanin. Cyanobacteria occur as unicellular, colonial, or filamentous organisms. They propagate by binary fission or fragmentation, and some may form resting structures, called akinetes, which, under favorable conditions, germinate into a vegetative form. Many contain gas vacuoles, which increase cell buoyancy and helps the cells float to the top of the water column, where light is most available for photosynthesis. Some cyanobacteria (e.g., *Anabaena*) are able to fix nitrogen; the site of nitrogen fixation is a structure called heterocyst.

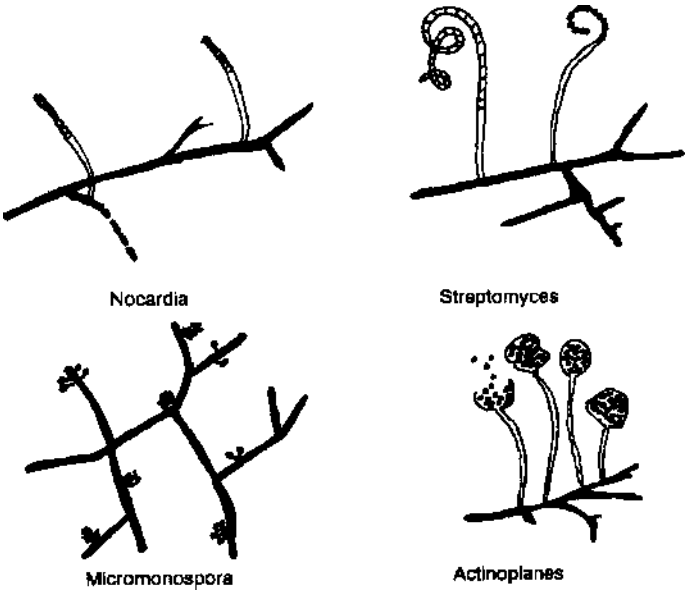

Figure 1.22 Some common actinomycetes. Adapted from Cross and Goodfellow (1973).

Cyanobacteria are ubiquitous and, owing to their resistance to extreme environmental conditions (e.g., high temperatures, desiccation), found in desert soils and hot springs. They are responsible for algal blooms in lakes and other aquatic environments, and some are quite toxic.

1.4.2 Archaea

In the late 1970s, Carl Woese and his collaborators at the University of Illinois proposed the *archaea* as the third domain of living organisms. This domain is subdivided into three kingdoms: Crenarchaeotes, Euryarchaeotes, and Korarchaeotes. The Euryarchaeota kingdom includes the methanogens, which will be discussed in Chapter 14.

Although the archaea have been considered in the past as prokaryotic cells, they possess certain characteristics that are different from those of bacteria:

- Their membranes are made of branched hydrocarbon chains attached to glycerol by ether linkages.
- Their cell walls do not contain peptidoglycan.
- Their rRNA is different from eukaryotic and prokaryotic rRNA.

It appears that archaea are more closely related to eukaryotes than to bacteria. As regards their metabolism, archaea may range from organotrophs (use of organic compounds as source of carbon and energy) to chemoautotrophs (use of CO_2 as carbon source).

Most of the archaea live in extreme environments and are called *extremophiles*. They include the thermophiles, hyperthermophiles, psychrophiles, acidophiles, alkaliphiles, and halophiles. Thus, their unique products are of great interest to biotechnologists. Archaeal enzymes display attractive properties such as tolerance to high and low temperatures, high salt concentrations, high hydrostatic pressures, and organic solvents. The enzymes of

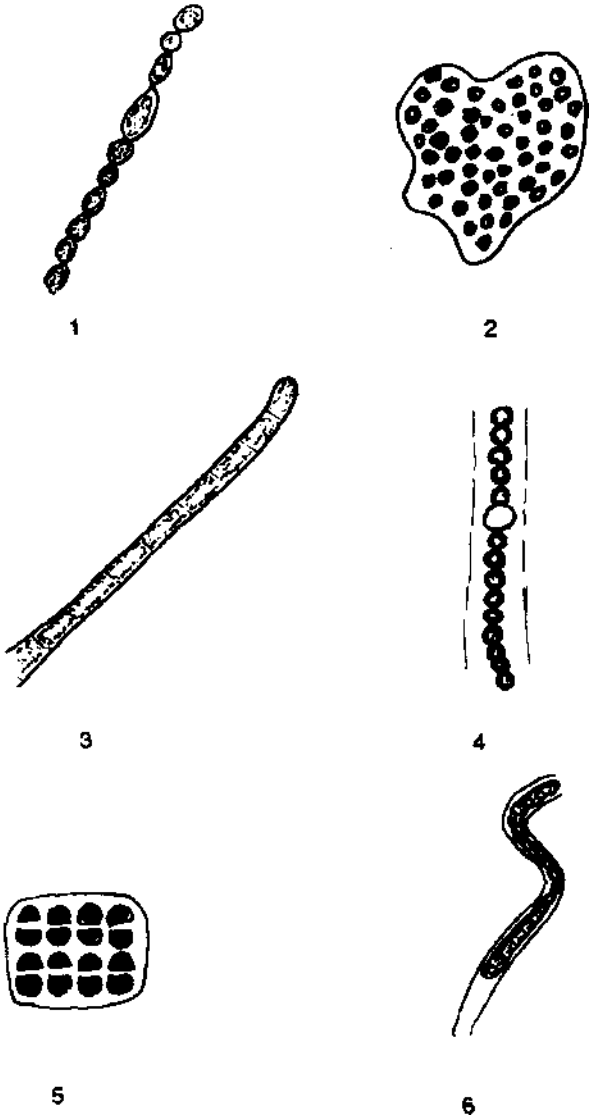

Figure 1.23 Some common cyanobacteria (blue-green algae). (1) *Anabaena*, (2) *Anacystis*, (3) *Oscillatoria*, (4) *Nostoc*, (5) *Agmenellum*, (6) *Lyngbya*. Adapted from Benson (1973).

interest to biotechnologists include the glycosyl hydrolases (e,g., cellulases, amylases, xylanases), proteases, DNA polymerases, and restriction endonucleases (Cowan and Burton, 2002).

1.4.3 Eukaryotes

1.4.3.1 Fungi. Fungi are eukaryotic organisms that produce long filaments called hyphae, which form a mass called mycellium. Chitin is a characteristic component of the cell wall of hyphae. In most fungi, the hyphae are septate and contain cross-walls that

divide the filament into separate cells containing one nucleus each. In some others, the hyphae are non-septate and contain several nuclei. They are called coenocytic hyphae.

Fungi are heterotrophic organisms that include both macroscopic and microscopic forms. They use organic compounds as carbon source and energy and thus play an important role in nutrient recycling in aquatic and soil environments. Some fungi form traps that capture protozoa and nematodes. They grow well under acidic conditions (pH = 5) in foods, water, or wastewater. Most fungi are aerobic, although some (e.g., yeast) can grow under facultatively anaerobic conditions. Fungi are significant components of the soil microflora, and a great number of fungal species are pathogenic to plants, causing significant damages to agricultural crops. A limited number of species are pathogenic to humans and cause fungal diseases called mycoses. Airborne fungal spores are responsible for allergies in humans. Fungi are implicated in several industrial applications, such as fermentation processes and antibiotic production (e.g., penicillin). In Chapter 13, we will discuss their role in composting.

Identification of fungi is mainly based on the type of reproductive structure. Most fungi produce spores (sexual or asexual spores) for reproduction, dispersal, and resistance to extreme environmental conditions. Asexual spores are formed from the mycelium and germinate, producing organisms identical to the parent. The nuclei of two mating strains fuse to give a diploid zygote, which gives haploid sexual spores following meiosis.

There are four major groups of fungi (Fig. 1.24):

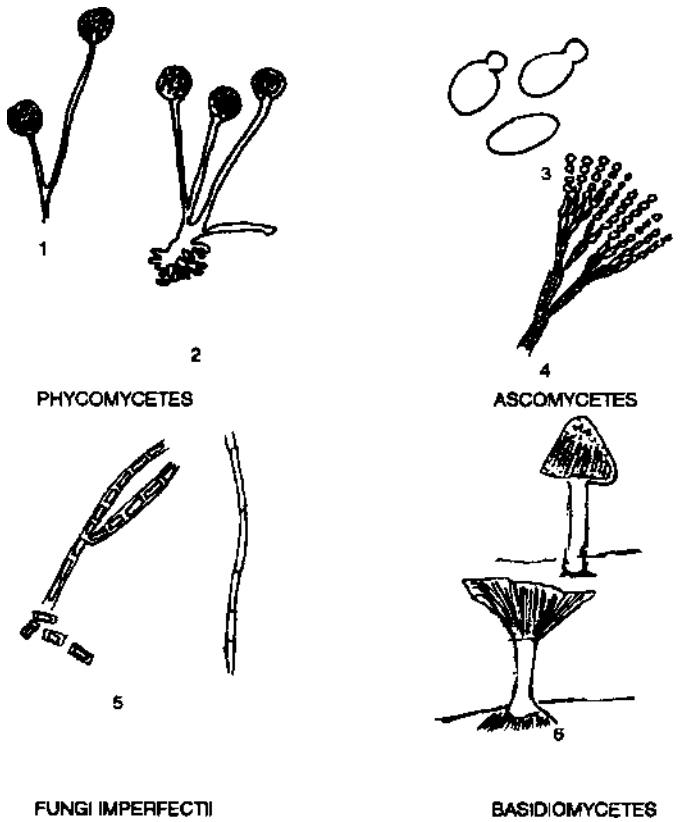

Figure 1.24 Classes of fungi. (1) *Mucor*, (2) *Rhizopus*, (3) *Saccharomyces*, (4) *Penicillium*, (5) *Geotrichum*, (6) fruiting bodies of mushrooms (*Basidiomycetes*).

• *Phycomycetes*

These fungi are known as the *water molds* and occur on the surface of plants and animals in aquatic environments. They have non-septate hyphae and reproduce by forming a sac called *sporangium,* which eventually ruptures to liberate *zoospores*, which settle and form a new organism. Some phycomycetes produce sexual spores. There are also terrestrial phycomycetes, such as the common bread mold (*Rhizopus*), which reproduces asexually as well as sexually.

• *Ascomycetes*

Ascomycetes have septate hyphae. Their reproduction is carried out by sexual spores (*ascospores*) contained in a sac called *ascus* (eight or more ascopores in an ascus), or asexual spores called *conidia,* which are often pigmented. *Neurospora crassa* is a typical ascomycete. Most of the yeasts (e.g., baker's yeast *Saccharomyces cerevisiae*) are classified as ascomycetes. They form relatively large cells that reproduce asexually via budding or fission, and sexually by conjugation and sporulation. Some of these organisms (e.g., *Candida albicans*) are pathogenic to humans. Yeasts, especially the genus *Saccharomyces*, are important industrial microorganisms involved in the manufacture of bread, wine, and beer.

• *Basidiomycetes*

Basidiomycetes also have a septate mycelium. They produce sexual spores called *basidiospores* on the surface of a structure called *basidium*. Four basidiospores are formed on the surface of each basidium. Certain basidiomycetes, the *wood-rotting fungi*, play a significant role in the decomposition of cellulose and lignin. Common edible mushrooms (e.g., *Agaricus*) belong to the basidiomycete group. Unfortunately, some of them (e.g., *Amanita*) are quite poisonous.

• *Fungi imperfectii*

F. imperfectii have septate hyphae but no known sexual stage. Some of them (e.g., *Penicillium*) are used for the commercial production of important antibiotics. These fungi cause plant diseases and are responsible for mycoses in animals and humans (e.g., athlete's foot).

1.4.3.2 Algae. Most of algae are floating unicellular microorganisms and are called phytoplankton. Many of them are unicellular, some are filamentous (e.g., *Ulothrix*), and others are colonial (e.g., *Volvox*). Most of them are free-living organisms, but some form symbiotic associations with fungi (lichens), animals (corals), protozoa, and plants.

Algae play the role of primary producers in aquatic environments, including oxidation ponds for wastewater treatment. Most algae are phototrophic microorganisms (see Chapter 2). They all contain chlorophyll *a*; some contain chlorophyll *b* and *c* as well as other pigments such as xanthophyll and carotenoids. They carry out oxygenic photosynthesis (i.e., they use light as a source of energy and H_2O as electron donor) and grow in mineral media with vitamin supplements and with CO_2 as the carbon source (see Chapter 2). Under environmental conditions, vitamins are generally provided by bacteria. Some algae (e.g., euglenophyta) are heterotrophic and use organic compounds (simple sugars and organic acids) as a source of carbon and energy. Algae have either asexual or sexual reproduction.

The classification of algae is based mainly on the type of chlorophyll, cell wall structure, and nature of carbon reserve material produced by algae cells (Fig. 1.25).

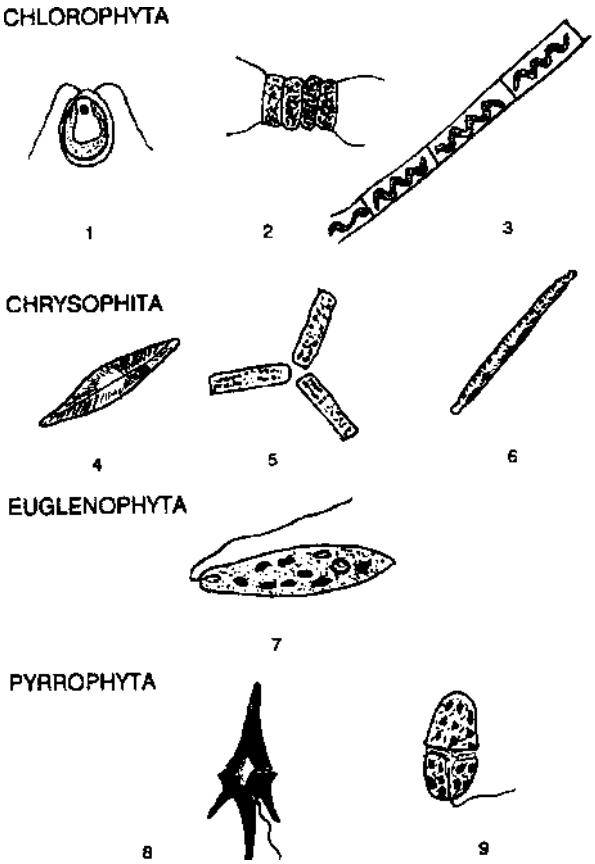

Figure 1.25 Some algae found in water and wastewater. (1) *Chlamydomonas*, (2) *Scenedesmus*, (3) *Spyrogira*, (4) *Navicula*, (5) *Tabellaria*, (6) *Synedra*, (7) *Euglena*, (8) *Ceratium*, (9) *Gymnodynium*. Adapted from Benson (1973).

- *Phylum Chlorophyta* (green algae). These algae contain chlorophylls *a* and *b*, have a cellulosic cell wall, and produce starch as a reserve material.
- *Phylum Chrysophyta* (golden-brown algae). This phylum contains an important group, the diatoms. They are ubiquitous, found in marine and freshwater environments, sediments, and soils. They contain chlorophylls *a* and *c* and their cell wall typically contains silica (they are responsible for geological formations of diatomaceous earth); some produce lipids as reserve materials.
- *Phylum Euglenophyta*. The euglenophytes contain chlorophylls *a* and *b*, have no cell wall, and store reserves of paramylon, a glucose polymer. *Euglena* is a typical euglenophyte.
- *Phylum Pyrrophyta* (dinoflagellates). The pyrrophyta contain chlorophylls *a* and *c*, have a cellulosic cell wall, and store starch. They are responsible for harmful algal blooms by producing potent toxins that harm humans and marine animals. The Gulf Coast of Florida "red tide" is caused by *Karenia brevis*, a marine dinoflagellate that produces neurotoxins called brevetoxins, which are associated with shellfish poisoning and respiratory problems in humans (Fleming et al., 2005; 2009; Hoagland et al., 2009).

- *Phylum Rhodophyta* (red algae). They are found exclusively in the marine environment. They contain chlorophylls *a* and *d* and other pigments such as phycoerythrin; they store starch, and their cell wall is made of cellulose.
- *Phylum Phaeophyta* (brown algae). The cells of these exclusively marine algae contain chlorophylls *a* and *c* and xanthophylls; they store laminarin (β 1,3-glucan) as reserve materials, and their cell walls are made of cellulose.

1.4.3.3 Protozoa. Protozoa are unicellular organisms that are important from public health and process microbiology standpoints in water and wastewater treatment plants. Cells are surrounded by a cytoplasmic membrane covered by a protective structure called pellicle. They form *cysts* under adverse environmental conditions. These cysts are quite resistant to desiccation, starvation, high temperatures, lack of oxygen, and chemical insult, namely disinfection in water and wastewater treatment plants (see Chapter 7). Protozoa are found in soils and aquatic environments, including wastewater. Some are parasitic to animals, including humans.

Protozoa are heterotrophic organisms that can absorb soluble food that is transported across the cytoplasmic membrane. Others, the holozoic protozoa, are capable of engulfing particles such as bacteria. Ciliated protozoa use their cilia to move particles toward a mouthlike structure called *cytostome*. They reproduce by binary fission, although sexual reproduction occurs in some species of protozoa (e.g., *Paramecium*).

The type of locomotion is a basis for classification of protozoa (Fig. 1.26). The medically important protozoa that may be transmitted through water and wastewater will be discussed in more detail in Chapter 4.

- *Sarcodina (amoeba)*

These protozoa move by means of *pseudopods* (i.e., false feet). Movement by pseudopods is achieved by changes in the viscosity of the cytoplasm. Many of the amoeba are free-living, but some are parasitic (e.g., *Entamoeba histolytica*). Amoeba feed by absorbing soluble food or by phagocytosis of the prey. The foraminifera are sarcodina, found in the marine environment, that have shells called tests. They are found as fossils in geological formations.

- *Mastigophora (flagellates)*

The mastigophora move by means of flagella. Protozoologists include in this group the phytomastigophora, which are photosynthetic (e.g., *Euglena*; this alga is also heterotrophic). Another example of flagellate is the human parasite, *Giardia lamblia* (see Chapter 4). Still another flagellate, *Trypanosoma gambiense*, transmitted to humans by the Tsetse fly, causes the African sleeping sickness, which is characterized by neurological disorders.

- *Ciliophora (ciliates)*

These organisms use cilia for locomotion, but they also help in feeding. A well-known large ciliate with a shoe-like shape is *Paramecium*. Some are parasitic to animals and humans. For example, *Balantidium coli* causes dysentery when cysts are ingested.

- *Sporozoa*

The sporozoa have no means of locomotion and are exclusively parasitic. They feed by absorbing food and produce infective spores. A well-known sporozoan is *Plasmodium vivax,* which causes malaria.

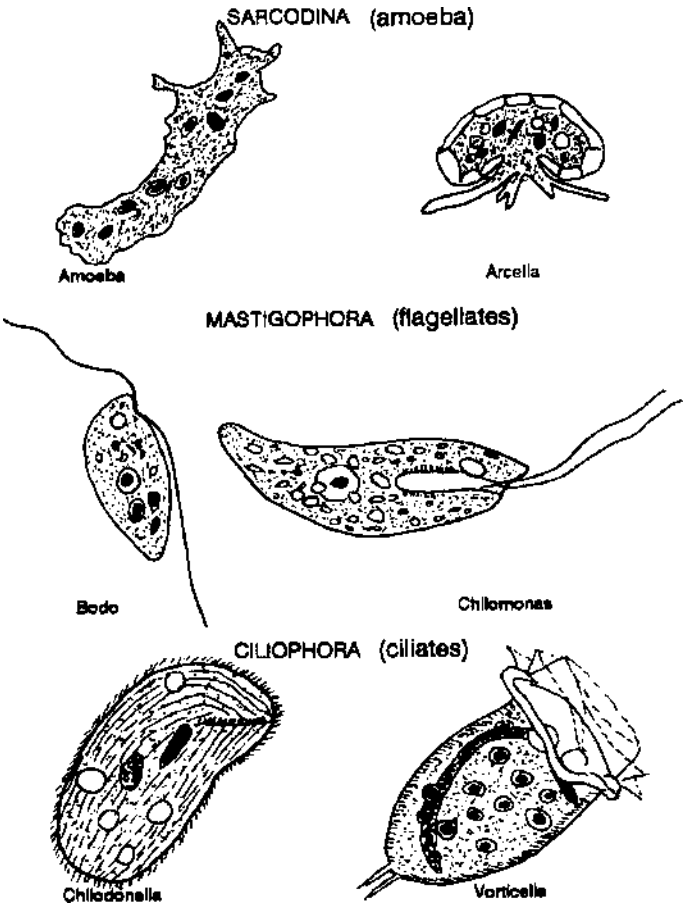

Figure 1.26 Protozoa groups found in water and wastewater.

1.4.4 Viruses

Viruses belong neither to prokaryotes nor to eukaryotes; they carry out no catabolic or anabolic function. Their replication occurs inside a host cell. The infected cells may be animal or plant cells, bacteria, fungi, or algae. Viruses are very small colloidal particles (20–350 nm) and most of them can be observed only with an electron microscope. Figure 1.27 shows the various sizes and shapes of some viruses.

1.4.4.1 Virus Structure. A virus is essentially made of a core of nucleic acid (double-stranded or single-stranded DNA; double-stranded or ssRNA) surrounded by a protein coat called a capsid. Capsids are composed of arrangements of various numbers of protein subunits known as capsomeres. The combination of capsid and nucleic acid core is called nucleocapsid. There are two main classes of capsid symmetry. In helical symmetry, the capsid is a cylinder with a helical structure (e.g., tobacco mosaic virus). In polyhedral symmetry, the capsid is an icosahedron, consisting of 20 triangular faces, 12 corners, and 30 edges (e.g., poliovirus). Some viruses have more complex structures (e.g., bacterial phages), and some (e.g., influenza or herpes viruses) have an envelope composed of lipoproteins or lipids.

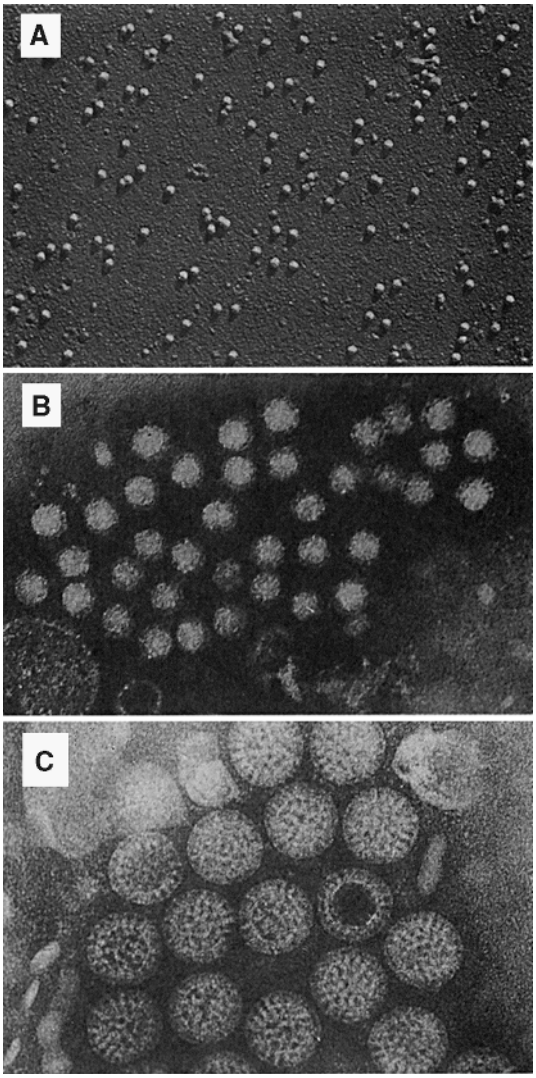

Figure 1.27 Some enteric viruses of public health importance: (a) poliovirus type 1; (b) hepatitis A virus; (c) rotavirus. Courtesy of R. Floyd and J.E. Banatvala.

1.4.4.2 Virus Replication. Bacterial phages have been used as models to elucidate the phases involved in virus replication. The various phases are as follows (Fig. 1.28):

1. *Adsorption.* This is the first step in the replication cycle of viruses. In order to infect the host cells, the virus particle must adsorb to receptors located on the cell surface. Animal viruses adsorb to surface components of the host cell. The receptors may be polysaccharides, proteins, or lipoproteins.
2. *Entry.* This step involves the entry of a virus particle or its nucleic acid inside the host cell. Bacteriophages "inject" their nucleic acid into the host cell. For animal viruses, the whole virion penetrates the host cell by endocytosis.
3. *Eclipse.* During this step, the virus particle is "uncoated" (i.e., stripping of the capsid), and the nucleic acid is liberated.

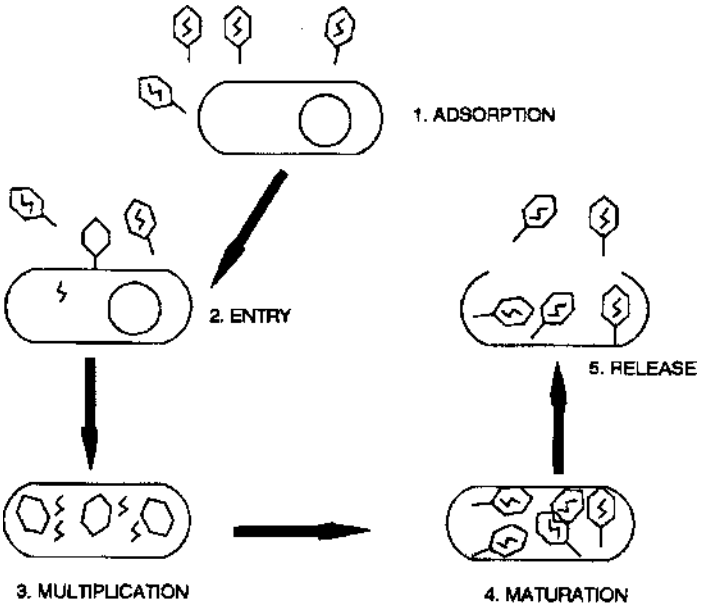

Figure 1.28 Viral lytic cycle.

4. *Replication.* This step involves the replication of the viral nucleic acid.
5. *Maturation.* The protein coat is synthesized and is assembled with the nucleic acid to form a nucleocapsid.
6. *Release of mature virions.* Virus release is generally attributable to the rupture of the host cell membrane.

1.4.4.3 Virus Detection and Enumeration. There are several approaches to virus detection and enumeration:

• *Animal Inoculation*

This was the traditional method for detecting viruses before the advent of tissue cultures. Newborn mice are infected with the virus and are observed for symptoms of disease. Animal inoculation is essential for the detection of enteroviruses such as Coxsackieviruses A.

• *Tissue Cultures*

Viruses are quantified by measuring their effect on established host cell lines, which, under appropriate nutritional conditions, grow and form a monolayer on the inner surface of glass or plastic bottles. There are two main types of host cell lines: (1) *primary cell lines*: These cells are removed directly from the host tissues and can be subcultured for only a limited number of times; and (2) *continuous cell lines*: Animal cells, after serial subculturing, acquire characteristics that are different from the original cell line, allowing them to be subcultured indefinitely; they are derived from normal or cancerous tissues. Cell lines traditionally used in water virology laboratories include HEp-2, HeLa, VERO, or Buffalo Green Monkey (BGM) cells. The BGM cell line is the most popular and perhaps the most sensitive of all cell lines used for the detection of enteroviruses.

Many viruses (enteroviruses, reoviruses, adenoviruses) infect host cells and display a *cytopathic effect*. Others (e.g., rotaviruses, HAV) multiply in the cells but do not produce a cytopathic effect. The presence of the latter needs to be confirmed by other tests, including immunological procedures, monoclonal antibodies (MAbs), or nucleic acid probes. Other viruses (e.g., Norwalk type virus) cannot yet be detected by tissue cultures.

- *Plaque Assay*

A viral suspension is placed on the surface of a cell monolayer and, after adsorption of viruses to the host cells, an overlay of soft agar or carboxymethylcellulose is poured on the surface of the monolayer. Virus replication leads to localized areas of cell destruction called *plaques*. The results are expressed in numbers of *plaque-forming units* (PFU). Bacteriophages are also assayed by a plaque assay method based on similar principles. They form plaques that are zones of lysis of the host bacterial lawn (Fig. 1.29).

- *Serial Dilution Endpoint*

Aliquots of serial dilutions of a viral suspension are inoculated into cultured host cells and, after incubation, viral cytopathic effect (CPE) is recorded. The titer or endpoint is the highest viral dilution (i.e., smallest amount of viruses) capable of producing CPE in 50% of cultures and is referred to as $TCID_{50}$ (tissue culture infectious dose).

- *Most Probable Number (MPN)*

Virus titration is carried out in tubes or 96-well microplates, using three dilutions of the viral suspension. Virus-positive tubes or wells are recorded and the MPN is computed from MPN tables.

1.4.4.4 Rapid Detection Methods

- *Immunoelectron Microscopy*

Viruses are incubated with specific antibodies and examined by electron microscopy for the presence of virus particles aggregated by the antibody. This is a useful technique for examining viruses such as the Norwalk-type agent.

- *Immunofluorescence*

A fluorescent dye-labeled antibody is combined with the viral antigen, and the complex formed is observed with a fluorescence microscope. This approach enables the detection of rotaviruses as fluorescent foci in MA-104 or CaCo-2 cultured cells. This method can be accelerated by using flow cytometry. Immunomagnetic separation has been used to detect rotavirus and HAV in environmental samples.

- *Enzyme Linked Immunosorbent Assay (ELISA)*

A specific antibody is fixed on a solid support and the antigen (virus) is added to form an antigen–antibody complex. An enzyme-labeled specific antibody is then added to the fixed antigen. The presence of the virus is detected by the formation of a colored product upon addition of the enzyme substrate. This enzymatic reaction can be conveniently quantified with a spectrophotometer.

- *Radioimmunoassay (RIA)*

This assay is also based on the binding of an antigen by a specific antibody. The antigen is quantified by labeling the antibody with a radioisotope (e.g., ^{125}I) and measuring the radioactivity bound to the antigen–antibody complex. When viruses growing in host cells

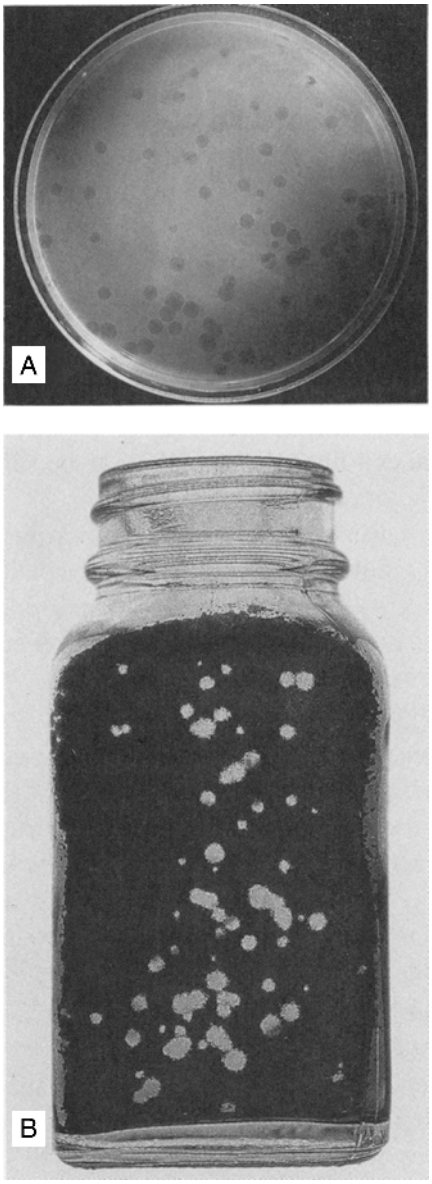

Figure 1.29 Viral enumeration by plaque assay: (a) bacterial phage; (b) animal virus (poliovirus type 1).

are treated with a ^{125}I-labeled antibody, the radioactive foci can be enumerated following contact with a special film. This test, the *radioimmunofocus assay* (RIFA), is used for the detection of HAV.

- *Molecular-Based Methods*

Molecular-based methodology has helped in the detection of viruses that show no growth or grow marginally in tissue cultures (e.g., Norwalk-like viruses, rotaviruses).

Gene probes are pieces of nucleic acid that help identify unknown microorganisms by hybridizing (i.e., binding) to the homologous organism's nucleic acid. For easy detection,

the probes can be labeled with radioactive isotopes such as ^{32}P, fluorescent compounds, or enzymes such as alkaline phosphatase, peroxidase, or β-galactosidase. Nucleic acid probes have been used for the detection of viruses (e.g., polioviruses, HAV) in environmental samples (water, sediments, shellfish). ssRNA probes were used for the detection of HAV in shellfish concentrates. Unfortunately, these probes are not sensitive and detect a minimum of 10^6 HAV particles.

Amplification of the target viral sequences by PCR has also been considered. Presently, a popular method for detecting viruses (enteroviruses, adenoviruses, rotaviruses, astroviruses, Norwalk-like viruses) in environmental samples is the RT-PCR method.

A new method for rapidly detecting HAV in environmental samples is the use of a combined cell culture-molecular beacon assay (Yeh et al., 2008). A molecular beacon is a single-stranded oligonucleotide probe that is labeled with a fluorophore and a quencher at the 5′ and 3′ ends, respectively. The beacon is introduced into permeabilized and fixed host cells that have been infected with HAV, and the fluorescent host cells are visualized 6 h after infection instead of 1 week.

1.4.4.5 Virus Classification. Viruses may be classified on the basis of the host cell they infect. We will briefly discuss the classification of animal, algal, and bacterial phages.

- *Animal Viruses*

Animal viruses are classified mainly on the basis of their genetic material (DNA or RNA), presence of an envelope, capsid symmetry, and site of capsid assembly. All DNA viruses have double-stranded DNA, except members of the parvovirus group, and all RNA viruses have ssRNA, except members of the reovirus group. Tables 1.2 and 1.3 show the major groups of animal viruses. Of great interest to us in this book is the *enteric virus* group, the members of which may be encountered in water and wastewater. This group will be discussed in more detail in Chapter 4.

Retroviruses are a special group of RNA viruses. These viruses make an enzyme, called *reverse transcriptase*, which converts RNA into double-stranded DNA, which integrates into the host genome, and controls viral replication. A notorious retrovirus is HIV, which causes acquired immunodeficiency syndrome (AIDS).

TABLE 1.2. Major Groups of Animal DNA Viruses[a]

Group	Parvoviruses	Papovaviruses	Adenoviruses	Herpesviruses	Poxviruses
Capsid symmetry	Cubic	Cubic	Cubic	Cubic	Complex
Virion: naked or enveloped	Naked	Naked	Naked	Enveloped	Complex coat
Site of capsid assembly	Nucleus	Nucleus	Nucleus	Nucleus	Cytoplasm
Reaction to ether (or other liquid solvent)	Resistant	Resistant	Resistant	Sensitive	Resistant
Diameter of virion (nm)	18–26	45–55	70–90	100	230–300

Adapted from Melnick (1976).

[a]All DNA viruses of vertebrates have double-stranded DNA, except members of the parvoviruses, which have single-stranded DNA.

TABLE 1.3. Major Groups of Animal RNA Viruses[a]

Group	Picornavirus	Reovirus	Rotavirus	Rubella	Arbovirus	Myxovirus	Paramyxovirus	Rhabdovirus
Capsid symmetry	Cubic	Cubic	Cubic	Cubic	Cubic	Helical (or unknown)	Helical (or unknown)	Helical (or unknown)
Virion: naked or enveloped	Naked	Naked	Naked	Enveloped	Enveloped	Enveloped	Enveloped	Enveloped
Site of capsid assembly	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
Reaction to ether (or to other liquid solvent)	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
Diameter of virion (nm)	20–30	75	64–66	60	40	80–120	150–300	60–180

Adapted from Melnick (1976).

[a]All RNA viruses of vertebrates have single-stranded DNA, except members of the reovirus group, which are double-stranded.

• *Algal Viruses*

A wide range of viruses or "virus-like particles" of eukaryotic algae have been isolated from environmental samples. The nucleic acid is generally double-stranded DNA but is unknown in several of the isolates. Viruses infecting *Chlorella* cells are large particles (125-200 nm in diameter), with an icosahedral shape and a linear double-stranded DNA.

Cyanophages were discovered during the 1960s. They infect a number of cyanobacteria (blue-green algae). They are generally named after their hosts. For example, LPP1 cyanophage has a series of three cyanobacterial hosts: *Lyngbia, Phormidium*, and *Plectonema.* They range in size from 20 to 250 nm, and all contain DNA. Cyanopahges have been isolated around the world from oxidation ponds, lakes, rivers, and fish ponds. Cyanophages have been proposed as biological control agents for the overgrowth (i.e., blooms) of cyanobacteria. Although some of the experiments were successful on a relatively small scale, cyanobacteria control by cyanophages under field conditions remains to be demonstrated.

• *Bacterial Phages*

Bacteriophages infect a wide range of bacterial types. A typical T-even phage is made of a *head* (capsid), which contains the nucleic acid core; a *sheath* or "tail," which is attached to the head through a "neck"; and *tail fibers*, which help in the adsorption of the phage to its host cell. The genetic material is mostly double-stranded DNA, but it may be single-stranded DNA (e.g., ΦX174) or ssRNA (e.g., f2, MS2). Phages adsorb to the host bacterial cell and initiate the lytic cycle, which results in the production of phage progeny and the destruction of the bacterial host cell. Sometimes, the phage becomes incorporated in the host chromosome as a prophage. This process, called *lysogeny*, does not lead to the destruction of the host cell. Phages infecting *E. coli* are called *coliphages*. These phages are considered potential indicators of fecal contamination (see Chapter 5). The various families of phages are illustrated in Figure 1.30.

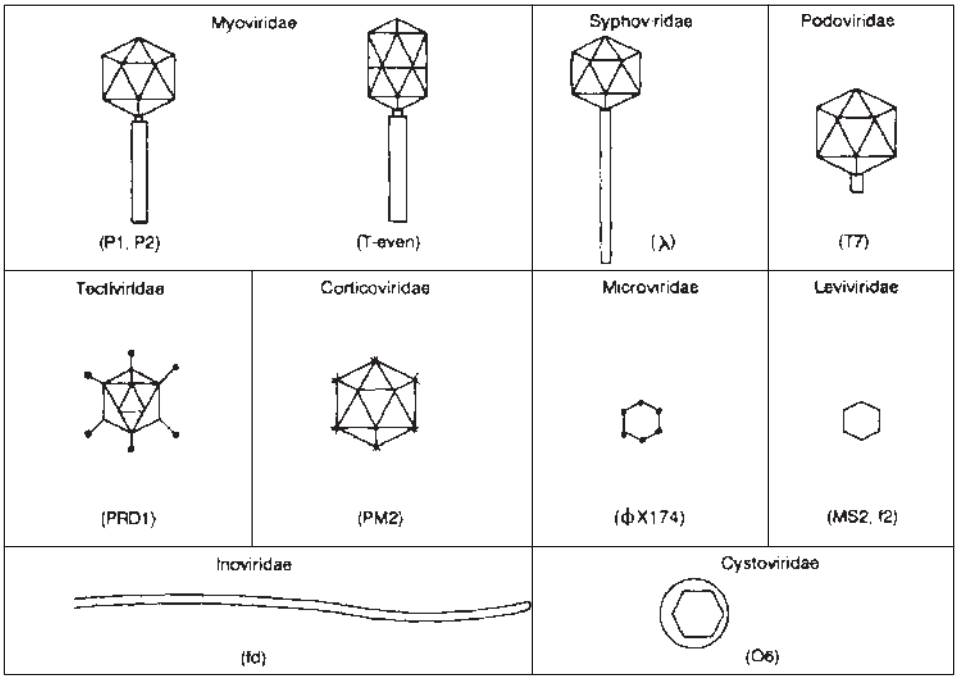

Figure 1.30 Major groups of bacteriophages. Adapted from Jofre (1991).

1.5 WEB RESOURCES

General

http://www.microbes.info/resources/Environmental_Microbiology/ (collection of Web sites on microbes)

Bacteria

http://commtechlab.msu.edu/sites/dlc-me/zoo/ (various good pictures of bacteria, from the Digital Center for Microbial Ecology)
http://commtechlab.msu.edu/sites/dlc-me/zoo/microbes/microbemonth.htm (various good pictures of bacteria, from the Digital Center for Microbial Ecology)
http://www.micrographia.com/specbiol/bacteri/bacter/bact0100.htm (some good pictures of bacteria and cyanobacteria)
http://www.lifesci.ucsb.edu/~biolum/ (bioluminescence)
http://www.buckman.com/eng/micro101/bacteria.htm (bacterial pictures)

Cyanobacteria

http://www-cyanosite.bio.purdue.edu/ (excellent cyanobacteria and general pictures from Purdue University)

Algae

http://www.dipbot.unict.it/sistematica/Index.html (good pictures, in Italian)
http://www.whoi.edu/redtide/ (Woods Hole Institute Harmful algae page)
http://www.nwfsc.noaa.gov// (NOAA harmful algae page)
http://www.nhm.ac.uk/hosted_sites/ina/ (nanoplankton web page)
http://www.geo.ucalgary.ca/~macrae/palynology/dinoflagellates/dinoflagellates.html (U. Calgary page on dinoflagellates)
http://www.psaalgae.org/ (Phycological Society of America)
http://oceancolor.gsfc.nasa.gov/SeaWiFS/ (go to Teacher's Resources)
http://starcentral.mbl.edu/microscope/portal.php (good collection of pictures of microorganisms from the Astrobiology institute, Marine Biological Laboratory, Woods Hole, MA)
http://www.botany.uwc.ac.za/algae/
http://www.microscopy-uk.org.uk/mag/indexmag.html?http://www.microscopy-uk.org.uk/mag/wimsmall/diadr.html (pictures of diatoms, bacteria, protozoa, rotifers)
http://www.micrographia.com/specbiol/alg/diato/diat0200.htm (diatoms pictures)
http://www.xs4all.nl/~hummelrh/index.html (diatoms images)

Viruses

http://www.bing.com/images/search?q=Virus+pictures&FORM=MFEIMG&PUBL=Google&CREA=userid174316c0d0ada7f77e6c09ffca975eda596a (virus pictures)
http://www.virology.net/garryfavweb.html (virology: general)
http://www.rkm.com.au/imagelibrary/index.html (virus color pictures and diagrams)

Protozoa

http://www.uga.edu/~protozoa/ (pictures and information from the Society of Protozoologists)

http://zoology.okstate.edu/zoo_lrc/zool1604/lab/protozoa.htm (excellent collection of web pages on protozoa; good collection of pictures)
http://www.microscopy-uk.org.uk/micropolitan/index.html (excellent pictures, from Microscopy—UK)

Archaea

http://www.ucmp.berkeley.edu/alllife/threedomains.html (Tree of life: The 3 domains)
http://www.ucmp.berkeley.edu/archaea/archaea.html (introduction to archaea; Berkeley)
http://www.earthlife.net/prokaryotes/archaea.html
http://www.daviddarling.info/encyclopedia/A/archaea.html (archaea: general)
http://faculty.washington.edu/leighj/mmarchaea.html
http://www.microbe.org/microbes/archaea.asp

Microbial Genetics

http://evolution.genetics.washington.edu/phylip/software.html (phylogeny software packages)
http://www.ncbi.nlm.nih.gov/About/primer/microarrays.html (microarray primer)
http://www.ncbi.nlm.nih.gov (National Center for Biotechnology Information)
http://www.tigr.org (Institute for Genomic Research)
http://rdp.cme.msu.edu/index.jsp (Ribosomal Database Project)
http://www.ncbi.nlm.nih.gov/Genbank/ (GenBank)
http://www.johnkyrk.com/DNAreplication.html (DNA replication animation)

NCBI Virus Genomes

http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=10239&type=5&name=Viruses
http://www.swbic.org/ (site on molecular biology)
http://www.microbial-ecology.net/probebase/default.asp?mode=lists (online resource for rRNA probes, University of Vienna)

1.6 REVIEW QUESTIONS

1. State whether the following characteristics are seen in prokaryotes or eukaryotes:
   (a) circular DNA
   (b) nuclear membrane
   (c) presence of histones
   (d) mitosis
   (e) binary fission
   (f) produce gametes through meiosis
2. An electron micrograph of a cell shows a cell wall, cytoplasmic membrane, nuclear body without a nuclear membrane, and no endoplasmic reticulum or mitochondria. The cell is:
   (a) a plant cell
   (b) an animal cell
   (c) a bacterium

(d) a fungus

(e) a virus

3. Match the following descriptions with the **best** answer.

___ The movement of water across a membrane from an area of higher water concentration (lower solute concentration) to lower water concentration (higher solute concentration).

__ The net movement of small molecules or ions from an area of higher concentration to an area of lower concentration. No energy is required.

__ Also known as facilitated diffusion, the transport of substances across the membrane. The transport is from an area of higher concentration to lower concentration and no energy is required.

If the net flow of water is out of a cell, the cell is in _____ environment.

If the net flow of water is into a cell, the cell is in _____ environment.

A passive transport

B active transport

C simple diffusion

D osmosis

E a hypotonic

F a hypertonic

G an isotonic

4. In the complement base pairing of nucleotides, adenine can form hydrogen bonds with _____ and guanine can form hydrogen bonds with _____

5. Copies the genetic information in the DNA by complementary base pairing and carries this "message" to the ribosomes where the proteins are assembled. This best describes:

(a) tRNA

(b) mRNA

(c) rRNA

6. Transfer RNA picks up specific amino acids, transfers the amino acids to the ribosomes, and inserts the correct amino acids in the proper place according to the mRNA message. This best describes:

(a) tRNA

(b) mRNA

(c) rRNA

7. Give the ecological role of cell wall, outer membrane, glycocalyx, and gas vacuoles.

8. What type of plasmid is of interest to bioremediation experts?

9. As compared to DNA, why is RNA a good target to probe?

10. Give and explain the stages involved in protein synthesis

11. Explain the main features of fluorescent *in situ* hybridization (FISH).

12. What problems are encountered in PCR when handling environmental samples?

13. What is, essentially, RT-PCR?

14. In PCR, what approaches are used to determine the viability/activity of target bacterial cells or viral infectivity?
15. Give some methods used in genetic fingerprinting.
16. What are some differences between archaea and bacteria?
17. Give the different groups of algae and point out the groups that are exclusively of marine origin?
18. Give the steps involved in the viral lytic cycle.
19. Compare lytic with lysogenic cycles in virus replication.
20. What is the importance of actinomycetes? Discuss some problems caused by actinomycetes in water and wastewater treatment.

1.7 FURTHER READING

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Talbot, G., E. Topp, M.F. Palin, and D.I. Massé. 2008. Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. Water Res. 42: 513–537.

Wolfe, S.L. 1993. *Molecular and Cellular Biology*, Wadsworth Pub. Co., Belmont, CA.