GROUND-WATER MICROBIOLOGY AND GEOCHEMISTRY

Second Edition

Francis H. Chapelle
United States Geological Survey

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CHAPTER 2

MICROORGANISMS PRESENT IN THE GROUND-WATER ENVIRONMENT

The kinds of microorganisms encountered in the biosphere are as varied and diverse as the kinds of environments present on earth. If one considers the diversity of potential habitats in the biosphere (surface sediments in freshwater or saltwater bodies, subsurface sediments in either aerobic or anaerobic environments, the water column of deep or shallow water bodies, hot hydrothermal waters, frozen sediments in the Arctic and Antarctic plains, extreme pressure in deep ocean waters, and the bodies of higher plants and animals), it's little wonder that microorganisms display such astonishing diversity.

In spite of this diversity, there are only three primary lineages, referred to as domains, to which all life on earth (not just microorganisms) belong. These domains are the Archaea, the Bacteria, and the Eucarya (Woese et al., 1990). If we include viruses, which many microbiologists consider to be noncellular life-forms, there are just four basic kinds of microorganisms to consider when studying ground-water systems.

The bacteria are easily distinguished from eucarya on the basis of cellular architecture. The bacteria, also called procaryotes, are characterized by the lack of a true nucleus (*pro* meaning "early" or "primitive," and *karyo* meaning "nucleus") and includes the bacteria and the cyanobacteria. Cyanobacteria were formerly called "blue-green algae," but are now recognized as members of the Domain Bacteria. The eucaryotic cell has a true nucleus (*eu* meaning "true") and includes algae, fungi, and protozoa.

The archaea, which include the methane-producing "bacteria," are morphologically indistinguishable from bacteria and were once considered to be procaryotic microorganisms. Studies in the last 30 years, however, have shown that archaea are not bacteria and that they represent a previously unrecognized domain. Archaea are restricted to anaerobic environments, such as organic-rich sediments and the intestines of higher animals, or hypersaline environments. Geologically, the archaea are important because they inhabit virtually all subsurface environments, are an important source of commercial methane, and greatly impact the chemistry of ground-water systems.

Viruses are distinct from other types of microorganisms in that they are obligate parasites. That is, they do not have the capability to live and reproduce without having a host cell to provide energy. Viruses are important in subsurface microbiology primarily because ground water may transport viruses to wells or other drinking water supplies and thus spread infectious diseases. It is almost certainly true that viruses use subsurface bacteria as hosts and therefore are probably present wherever bacteria are present. That topic, however, has yet to be explored systematically.

2.1 THE BACTERIA

Bacterial microorganisms are characterized by their distinctive and relatively simple cellular structure. Figure 2.1 shows the kinds of structures that are typically observed in bacteria.

The bacterial chromosome consists of a single molecule of DNA that, in spite of many loops and twists, is arranged into a closed circle. This closed circle arrangement is found only in procaryotes and the archaebacteria. The DNA is otherwise identical to that of other organisms and is characterized by its double helix structure. The DNA carries genetic information needed for the cell to carry out metabolism and growth, as well as carrying the information needed for replication. Each bacterial chromosome consists of anywhere between 2,000 and 10,000 units of heredity called genes. Genes are segments of the DNA strand that code for a particular protein or polypeptide. Most of the time, procaryotic cells have just one copy of their chromosomal DNA and are therefore referred to as being haploid. Just before cell division, however, two or more copies of the bacterial chromosome may be present. These masses of DNA are sometimes visible under the microscope and are referred to as nucleoids.

Bacteria may also contain smaller circles of DNA, distinct from chromosomal DNA, that are termed plasmids. Plasmids are not involved in cell replication but

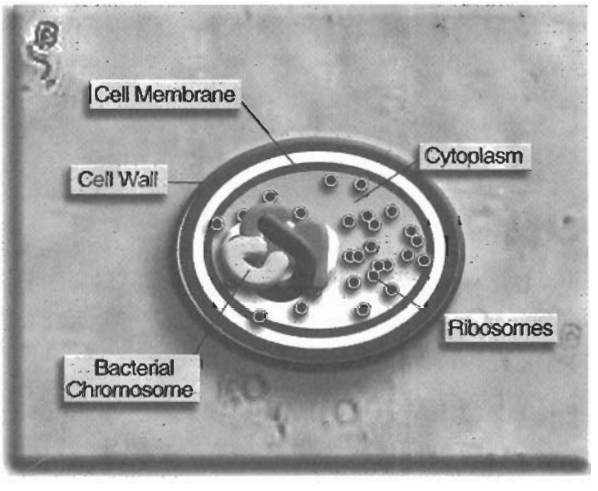

Figure 2.1. Cellular structure of bacteria.

are nevertheless very important. Plasmids code for enzymes or other proteins that have specific functions in helping the microorganism deal with its environment. For example, plasmids often code for proteins that detoxify or otherwise neutralize antibiotics. Plasmids may also code for proteins that aid in the decomposition of particular organic compounds, enabling the bacteria to use those compounds as an energy source. Much research has gone into identifying plasmids that code for the decomposition of toxic chemicals, because such capability could increase the effectiveness of bioremediation strategies. This topic is discussed in greater detail in Chapter 9.

Ribosomes are small, dark structures that are embedded in the cytoplasm of the cell. Ribosomes are protein assembly structures and provide a surface upon which amino acids can be brought together and assembled in the proper sequence. Ribosomes in procaryotes are made from two subunits. The smaller subunit is called the 30S subunit and the larger one is called the 50S subunit. The *S* stands for "Svedberg" units, a measure of the rate of sedimentation in an ultracentrifuge and hence a measure of molecular size. Because the 50S subunit settles faster than the smaller 30S subunit, it is proportionally more massive. Each subunit consists of an RNA molecule, which on its own may be either 23S, 16S, or 5S, and associated proteins that aid in assembling amino acids.

The cytoplasm of procaryotes always contains individual ribosomes. Often, however, ribosomes are arranged into complexes that are called polysomes. These polysomes work simultaneously to assemble different polypeptides which may constitute parts of one protein. Sometimes polysomes are embedded into the cell membrane.

The cell membrane, also termed the cytoplasmic membrane, acts as the boundary between the interior of the cell and the outside environment. As such, the cell membrane has numerous functions that regulate the chemical environment inside and outside the cell. Figure 2.2 shows a schematic diagram of the cell membrane. It consists of approximately 60% protein and 40% phospholipids. The phospholipids are arranged into a bilayer, in which the hydrophobic (non-water-soluble) portions point outward. This arrangement helps the cell to regulate its water balance. Embedded in the phospholipids are proteins that exhibit a variety of arrangements. The purpose of cell membrane proteins is to regulate the transport of chemicals into and out of the cell. Some proteins, for example, act as "ports" for bringing simple sugars into the cell, where they can be utilized for energy. Other proteins act as "switches," transferring electrons in the cell's electron transport system.

The cell membrane is semipermeable and allows some substances, such as water, to cross in response to concentration gradients. This type of transport occurs spontaneously and does not require the cell to expend energy. Other substances are actively carried across the cell membrane by specialized proteins. This type of transport is termed facilitated transport and requires the cell to expend energy.

The cell membrane is enclosed within the cell wall (Fig. 2.1), which gives the cell rigidity and helps to protect it against osmotic stress. A substance called peptidoglycan, which is unique to procaryotic microorganisms, provides much of the cell wall's structural strength. Peptidoglycan is a three-dimensional polymer of sugars and amino acids that are cross-linked with short peptide bridges.

There are two major types of cell walls (Fig. 2.3). The gram-positive cell wall consists of an inner membrane with a relatively thick layer of peptidoglycan cov-

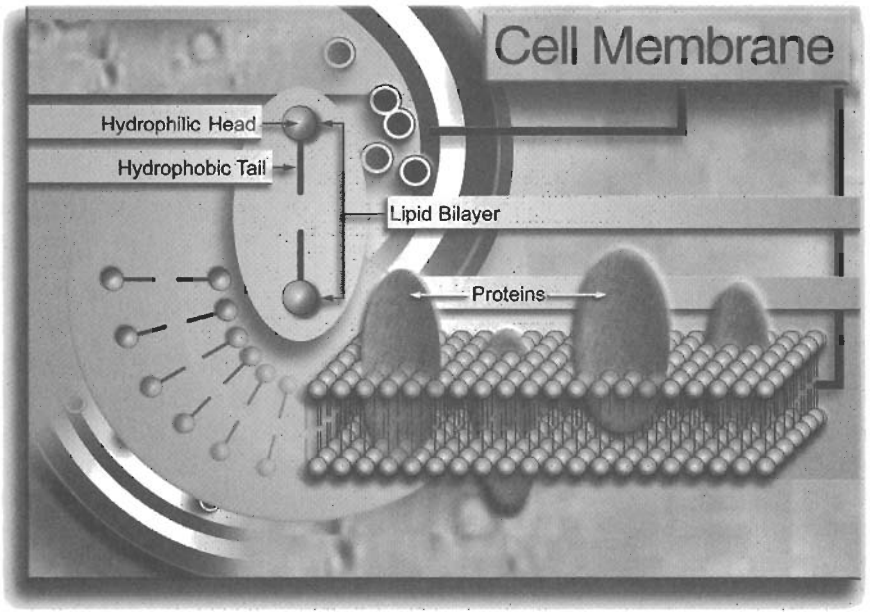

Figure 2.2. Schematic diagram of the cell membrane, sowing the arrangement of proteins and phospolipids.

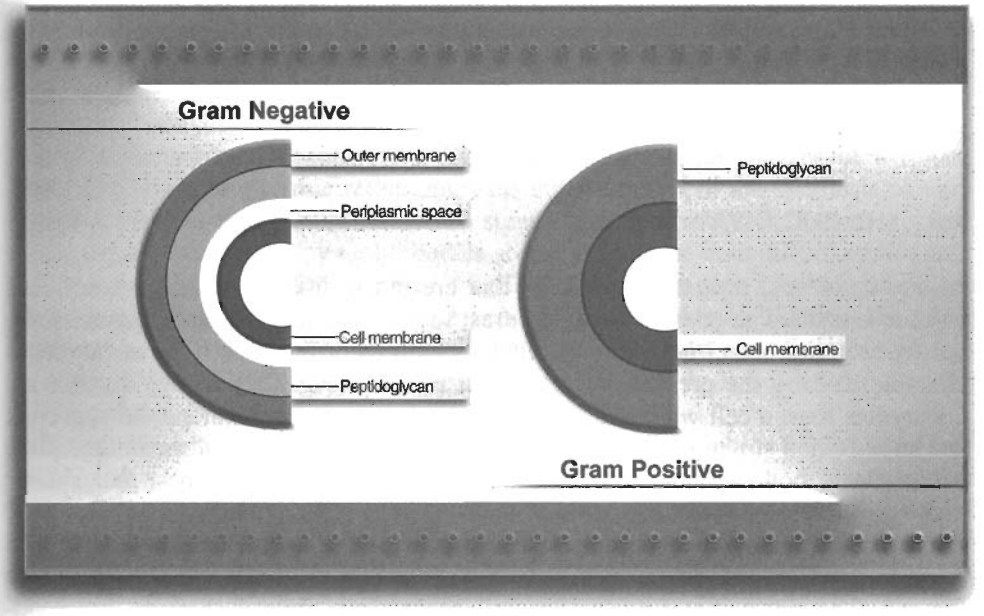

Figure 2.3. Diagram showing the configuration of (a) the gram-negative and (b) the gram-positive cell wall.

ering it. There are also varying amounts of teichoic acids, polymers of sugar alcohols and phosphates, present in gram-positive cell walls. This thick peptidoglycan layer has the characteristic that it retains the crystal violet pigment in Gram's stain, even when washed with ethyl alcohol—hence the term "gram positive."

The gram-negative cell wall (Fig. 2.3) has a layer of phospholipids and lipoproteins outside a thinner peptidoglycan layer. The gram-negative cell wall does not retain Gram's stain when washed with ethyl alcohol. In the gram-negative cell wall, there is a space between the cell membrane and the peptidoglycan layer, termed the periplasmic space. The periplasmic space is absent from gram-positive organisms and reflects a basic difference in how substrate-degrading enzymes are utilized by the two types of microorganisms.

Bacteria often have external coatings on their cell walls. These coatings are called the glycocalyx and consist of either polysaccharides or proteins. If the coating is hard and dense, it is often referred to as a capsule. If the coating is soft and pliable, it is often termed a slime layer. Cells growing on culture media often produce a thick glycocalyx, and this is what gives individual bacterial colonies a smooth appearance. Cells that do not produce abundant glycocalyx often produce colonies that appear rough.

One important function of the glycocalyx is that it facilitates the attachment of bacteria to surfaces in the environment. For example, *Streptococcus mutans,* a bacterium that lives in the mouths of human beings, produces a thick glycocalyx slime designed to allow it to stick to its host's teeth (thus avoiding being swallowed and killed). *S. mutans* produces this polysaccaride glycocalyx from sucrose (refined sugar), so that eating a candy bar is a boon for slime production. The "cottony" feeling in your mouth that becomes noticeable 10 or 15 minutes after eating candy comes from the slime produced from sucrose and used to coat your teeth. This slime, also called plaque, contributes greatly to tooth decay.

In the same way that *S. mutans* uses a glycocalyx to cling to teeth, bacteria in sediments use these coatings to cling to mineral surfaces. This ability helps bacteria gain access to nutrients associated with sediment particles and provides a stable environment for subsequent reproduction.

2.2 THE EUCARYA

Algae, fungi, and protozoa have cells that are much different from bacterial cells and are classified as eucaryotes. In contrast to the relatively simple structures present in bacterial cells, the eucaryotic cell is extremely complex. The eucaryote is characterized by the presence of a distinct nucleus (Fig. 2.4). Many, but not all, eucaryotes have a cell wall, and all eucaryotes have a cell membrane. Also present are large folded structures, called the endoplasmic reticulum, oval membrane disks called Golgi bodies, and organelles called mitochondria, lysosomes, and chloroplasts (Fig. 2.4).

The cell wall in eucaryotes performs many of the same functions as that in bacteria, such as protecting against osmotic shock and giving tensile strength to the cell. The cell walls of eucaryotes are formed from polysaccharides, but peptidoglycan is absent. The cell membrane in eucaryotes is similar to that of procaryotes, the main differences being in the composition of the lipids. The eucaryotic

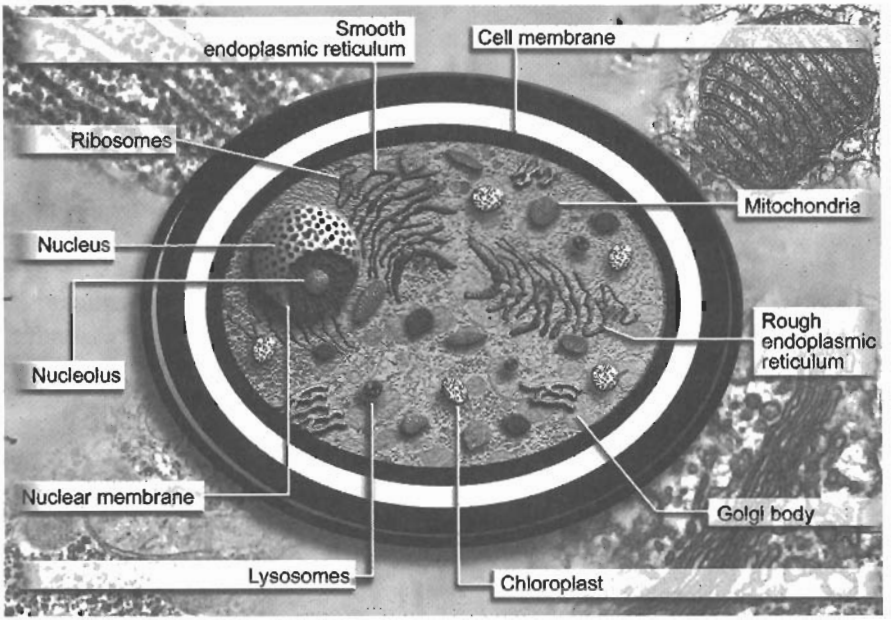

Figure 2.4. Structures present in eucaryotic cells.

cell membrane carries out facilitated and active transport, as in procaryotic cells. Many of the metabolic functions carried out in procaryotic cell membranes, however, have been taken over by specialized organelles in the eucaryote.

In the eucaryotic cell, energy production occurs in the mitochondria, photosynthetic activity is carried out by chloroplasts, and digestive enzymes are stored in lysosomes. All of these functions are carried out in the cell membranes of bacteria. Protein assembly in eucaryotes is carried out by ribosomes, as in procaryotes. However, eucaryotic subunits are larger (40S and 60S) than in procaryotes. Furthermore, eucaryotic ribosomes are often arranged along the surface of a folded membrane, the endoplasmic reticulum. This structure aids in the synthesis of very complex proteins that require numerous steps during assembly. Further processing of enzymes is performed by Golgi bodies separate from the endoplasmic reticulum.

Algae are photosynthetic eucaryotes and have the distinction of producing most of the world's oxygen. As such, algae contain photosynthetic chloroplasts. Algae have cell walls composed of cellulose, pectin, or silica, and may live singly or in colonies. There are six groups of algae recognized: green algae, euglenids, diatoms, dinoflagellates, brown algae, and red algae.

Unlike the photosynthetic algae, the fungi are heterotrophic; that is, they are decomposers and obtain energy and nutrients from preexisting organic carbon sources. The fungi have developed mechanisms for degrading almost any kind of organic carbon compound found in nature. Most fungi are obligate aerobes, meaning that they must have oxygen in order to respire. However, some fungi, notably the yeasts, are capable of fermentation.

The special function of fungi in the environment is to recycle the remains of plant and animal debris. In this way, fungi often compete directly with heterotrophic

bacteria for available resources. The greater complexity of eucaryotic structure, which allows the synthesis of many degrading enzymes lacking in the procaryotes, often gives fungi a competitive advantage over bacteria. Fungi, for example, are particularly adept at degrading lignins in decaying plants, a class of compounds that bacteria attack, but less efficiently. Also, the metabolic flexibility of fungi allow them to live in particularly stressful environments, such as hypersaline lakes. This resistance to osmotic stress is the main reason that molds, which are a type of fungi, are able to grow on preserved foods, such as jams, that are resistant to attack by bacteria.

The protozoa are single-celled heterotrophic eucaryotes that are characterized by extremely complex cell structure. The most familiar protozoa to geoscientists are foraminifera, radiolaria, and dinoflagellates, all of which are prominently represented in the fossil record. Some protozoans are predatory and are able to attack and ingest other microorganisms. Many predatory protozoans are specifically adapted to feeding on bacteria, and this grazing is often a limiting factor in bacterial populations in both surface and ground-water systems. Most protozoa, however, belong to floating communities called zooplankton.

2.2.1 Eucaryotes in Ground-Water Systems

The presence and distribution of eucaryotic microorganisms in ground-water systems has been studied seriously only recently. The first microbiologic investigations of shallow water table aquifers indicated that procaryotes were the dominant microorganisms present and that eucaryotes might be absent altogether (White et al., 1983; Balkwill and Ghiorse, 1985). Subsequent studies, using more sensitive techniques, were able to show the presence of limited populations of eucaryotes in ground-water systems.

Sinclair and Ghiorse (1987) used a most-probable-number counting procedure to document the presence of protozoa in the shallow pristine water-table aquifer at Lula, Oklahoma. Many of the horizons sampled, to a depth of 8 meters, had fewer than 0.2 protozoa/gram of dry sediment. A gravelly bed at a depth of 7.5 meters, however, exhibited between 2 and 5 protozoa/gram of dry sediment. Comparable numbers were found in other sandy horizons. Surface soils from this site, in contrast, were characterized by counts of protozoa in the 102–106 cells per gram dry sediment. So, while protozoa were demonstrably present, they were present in very low numbers. A latter study of buried-valley aquifer sediments in Kansas (Sinclair et al., 1990) showed a similar pattern of protozoa abundance.

While the presence of eucaryotic cells is low in pristine aquifer sediments, there is evidence that, in aquifers contaminated by organic chemicals, the abundance of eucaryotes may be much higher. For example, Madsen et al. (1991), in a study of an aquifer that was contaminated by polyaromatic hydrocarbons, showed that numbers of protozoa were much higher in the plume of contamination than outside the plume. This was interpreted as reflecting much greater growth rates of bacteria in the contaminated zone, which in turn supported a significantly higher population of protozoan grazing on the bacteria. In contrast, little relationship was found between the presence of fungi and contamination. On the basis of this data, Madsen et al. (1991) suggested that the population size of protozoans could be used as evidence for chemical contamination of aquifers.

A similar pattern of higher numbers of protozoa being present in contaminated versus uncontaminated aquifer sediments was demonstrated at a site contaminated with jet fuel (Sinclair et al., 1993). In this case, an engineered bioremediation system designed to add hydrogen peroxide to ground water, and thus to increase rates of aerobic degradation of petroleum hydrocarbons, was in place. A major limitation to this kind of engineered bioremediation system is clogging of sediments due to growth of bacterial biomass. However, at this site, no such clogging was observed. Rather, it appeared that protozoa were grazing on bacteria in the zone of treatment, and that this grazing maintained bacterial numbers below a level that would cause clogging. This grazing did not, however, appear to decrease the efficiency of petroleum hydrocarbon degradation (Sinclair et al., 1993).

Giardia *and* Cryptosporidium In 1993, over 400,000 people in Milwaukee, Wisconsin, became ill with a disease that caused diarrhea and vomiting. Some of the victims, especially those with compromised immune systems, died. Later study showed that the disease was caused by the protozoa *Cryptosporidium parvum,* which had contaminated the city's drinking water supply. Another water-borne protozoa, *Giardia lamblia,* is also known to cause intestinal disease in humans. These cases have led to widespread concern that these protozoa may contaminate ground water as well as surface water supplies.

Both *G. lamblia* and *C. parvum* are protozoan parasites that infect the digestive tract of humans and other mammals. It has been demonstrated that aquatic mammals, such as beavers and muskrats, can serve as zoonotic hosts, maintaining populations of *Giardia* in otherwise pristine surface waters. Domestic mammals, particularly ruminants such as cows, can serve as an infective host for *Cryptosporidium.*

Because both *Giardia* and *Cryptosporidium* inhabit surface water, it is possible that induced recharge of surface water to ground-water systems, either due to pumpage or to bank storage during flood events, can cause contamination of ground water. The vulnerability of ground-water supplies to contamination, therefore, depends on the transport of the protozoa in ground-water systems.

The transport of *Giardia* and *Cryptosporidium* in ground-water systems has not been studied directly. However, the transport of protozoa in a sandy aquifer underlying Cape Cod, Massachusetts, was studied by Harvey et al. (1995). In this study, an indigenous strain of a flagellated protozoa was recovered from aquifer sediments, grown in culture and stained, and then injected into the aquifer with a bromide tracer. A tenfold reduction in protozoa numbers was observed in the first meter of flowpath, and numbers of protozoa became undetectable 3.6 meters downgradient. These data suggest that protozoa can be transported in ground-water systems, but that they are probably filtered out relatively rapidly. While contamination of ground water adjacent to surface-water bodies by *Giardia* and *Cryptosporidium* is possible, such contamination is unlikely to extend significant distances (>10 meters) from the point of recharge.

2.3 THE ARCHAEA

The distinction between bacterial and eucaryotic microorganisms was first defined in terms of the internal structures that were visible with a microscope (Figs. 2.1

and 2.4). On this basis, as well as on the basis of differences between the biochemistry of their enzyme systems, biologists constructed a tree in which all cells could be classified under either the procaryotic or eucaryotic "stems."

This dichotomous classification was questioned in the 1970s by Carl R. Woese and his associates, who were working at the University of Illinois. Woese's group was interested in being able to quantify genetic similarities between microorganisms. In order to do this, they decided to look at the nuceotide sequences of ribosomal RNA (rRNA). The choice of rRNA was based on the fact that it was relatively easy to isolate from bacterial cells and that sequencing discrete portions of it was technically feasible. There are three kinds of rRNA in bacteria. The "large" ribosomal unit has a Svedberg unit value of 23S and is approximately 2,900 nucleotide units long. There is also a very "small" one that is 5S and is only 120 nucleotides long. Intermediate between these two is the 16S rRNA that is about 1,540 nucleodides long. The 23S was too long to be conveniently sequenced in the early 1970s whereas the 5S did not contain enough nucleotides to give much information. Thus, Woese's group settled on the 16S as the rRNA unit of choice and began sequencing the 16S rRNA of many different types of procaryotes and eucaryotes.

As the sequencing proceeded, a surprise emerged. As expected, eucaryotes were most closely related to eucaryotes. Among the bacteria, however, it appeared as if the methane-producing bacteria were distinct from other types of bacteria. In fact, the methanogens were no more closely related to the other bacteria than they were to eucaryotes. The implications of this finding were straightforward. There were not just two but three stems in the tree (Fig. 2.5). Woese named these three stems the eubacteria (true bacteria), the eucaryotes (true nucleus), and the archaebacteria (ancient bacteria). This nomenclature reflected Woese's belief that archaebacteria evolved very early in earth's history. More recently, however, Woese has suggested that using the word *bacteria* in archaebacteria implies is misleading, and proposed the term "Archaea" instead (Woese et al., 1990).

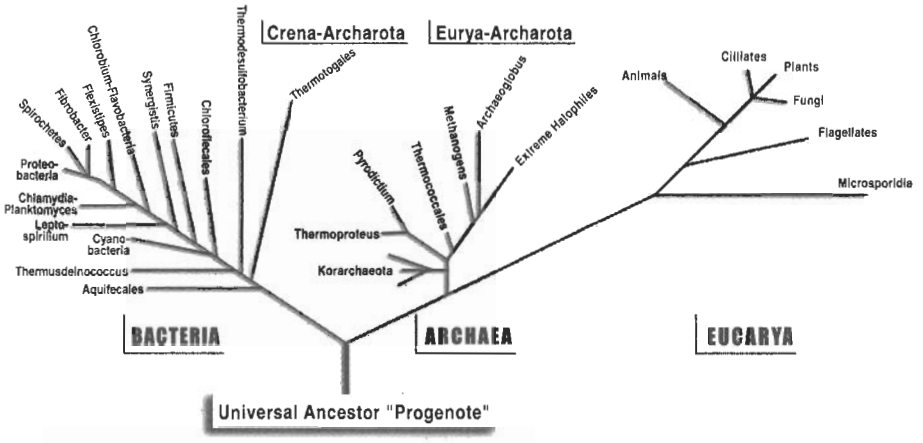

Figure 2.5. Diagram showing the relatedness of the three domains.

In addition to their distinctive 16S rRNA sequences, archaea exhibit other differences from the procaryotes. The lipids of both bacteria and eucaryotes consist mainly of two straight-chain fatty acids bound at one end to a glycerol molecule by an ester linkage (-CO-O-). In archaea, on the other hand, the glycerol and the acid chains have an ether (-O-) link. In addition, the cell walls of bacteria contain peptidoglycan, whereas the cell walls of archaea contain pseudopeptidoglycan or just protein.

The archaea include three distinct kingdoms. Kingdom Crenarchaeota consists mainly of thermophilic (heat-loving) organisms. Kindom Euryarchaeota includes the methanogens (microbes that produce methane from carbon dioxide and hydrogen), extreme halophiles (microbes that live in concentrated salt brines), and thermoacidophiles (microbes that live under extreme conditions of heat and low pH). Interestingly, a third kingdom, Kingdom Korarchaeota, has been proposed based solely on the 16S rRNA sequences of uncultured microorganisms found in terrestrial hot springs. Of these types, the methanogens most commonly affect the geochemistry of ground water. Early in the earth's history, methanogens could have existed almost anywhere. Today they are restricted to environments where oxygen has been excluded and where hydrogen and carbon dioxide are available. Because subsurface environments are often anoxic, they are extensively colonized by methanogens.

2.4 THE VIRUSES

Viruses are very small infectious agents. They are too small to be seen with a light microscope, and their presence was discovered indirectly. In 1886, an American bacteriologist, A. E. Mayer, described a mottling disease of the tobacco plant, called "tobacco mosaic disease." He noted that the disease could be transmitted by injecting the sap of infected plants into healthy plants. A few years later, O. Iwanowski discovered that the causative agent of tobacco mosaic disease was filterable; that is, the ground-up extract of diseased plants could infect healthy plants even when the extract was passed through a very fine filter. Iwanowski's experiments were reproduced by Martinus Beijerinck in 1898. Clearly, the infectious agent was not a bacterium. These experiments had the effect of extending microbiology beyond the study of bacteria and eucaryotes to the study of infectious agents, viruses, which were too small to be seen.

Exactly what kind of infectious agent viruses were was largely a mystery in the early part of the twentieth century. A major contribution to virology was made in 1935 when the American Wendell Stanley showed that the tobacco mosaic virus could be crystallized. This discovery led to studies of viral size and shape using X-ray defraction techniques. It was not until the advent of the electron microscope, however, that viral particles could be directly observed.

What is a virus? The best way to answer this question is to say what it is not. Viruses are not cells, for example, because they cannot reproduce independently and because they have no independent metabolism. One of the classic (and unresolved) debates in microbiology is whether or not viruses are living organisms. When it was first shown that "inanimate" crystals of the tobacco mosaic virus could cause the disease, many people argued that viruses were merely toxic chem-

icals. On the other hand, these particular toxic chemicals appeared to have the ability to reproduce themselves; was this not a clear characteristic of life? Not necessarily, came the rejoinder. Many crystalline substances "grow" from supersaturated solutions. Was this not "reproduction"? This debate has never been settled to everyone's satisfaction, and most people have come to the conclusion that there is not much to be gained by arguing about it. It does point out, however, some of the unique features of viruses.

At the simplest level, a virus is simply a genome, genetic information stored on DNA or RNA, wrapped in an exterior coat of protein. In the tobacco mosaic virus (Fig. 2.6a), a single strand of RNA is surrounded by protein subunits (called capsomeres) that link together to form the protein coat (called a capsid). Viral particles are called virions and are characterized by a regular geometrical arrangement of the capsomeres. Common shapes of virions are spheres (Fig. 2.7), cylinders, wedges, or prisms. Some virions are covered by an envelope and are termed enveloped; others have no envelope and are termed naked.

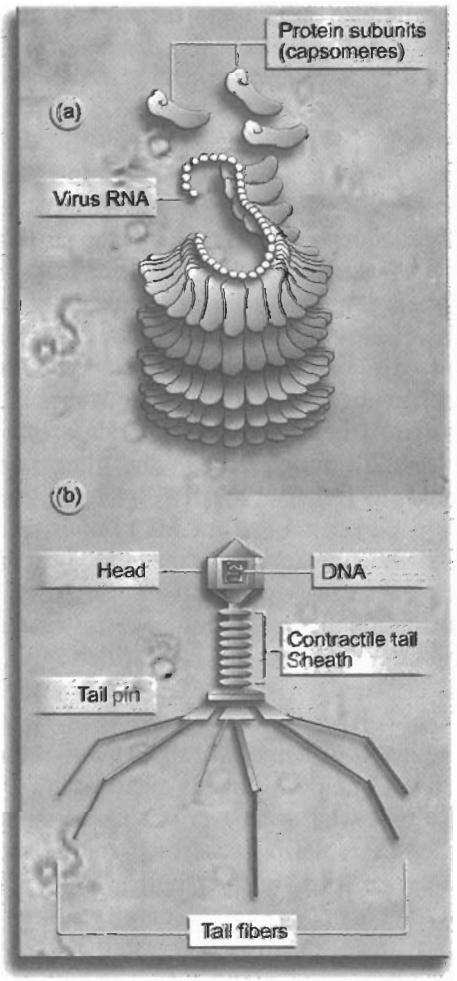

Figure 2.6. Structures present in (a) tobacco mosaic virus, and (b) a typical bacteriophage.

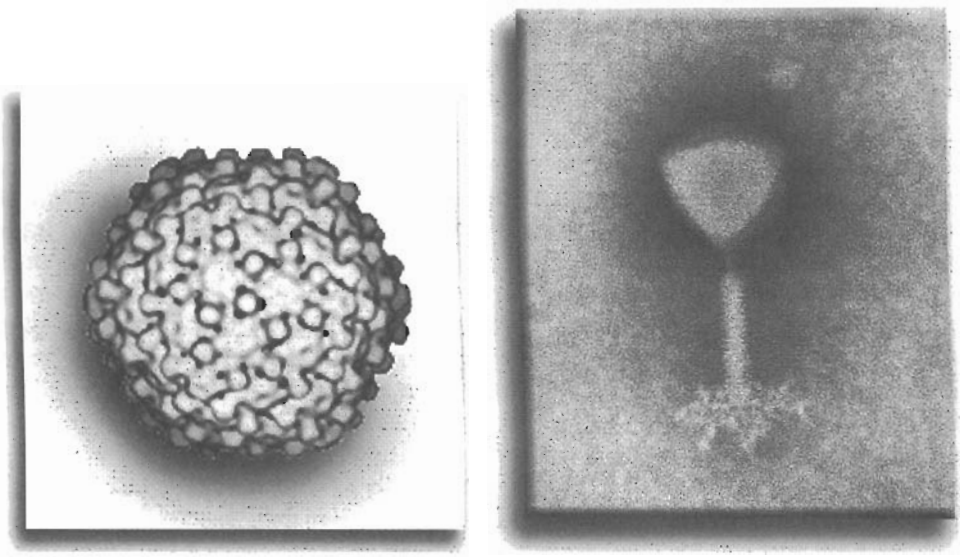

Figure 2.7 Electron micrographs of (a) a circular virus and (b) bacteriophage T-14.

Viruses that are parasitic to bacteria are called bacteriophages and have a somewhat more complicated stucture (Fig. 2.6b). The head of the virus contains the nucleic acid genome (in this case, DNA) that is set upon the contractile tail sheath. At the base of the tail sheath is the tail pin and the tail fibers. These stuctures enable bacteriophages, such as the T4 bacteriophage, to attack a bacterium. First, the tail fibers find and attach to a target protein on the cell wall. Second, the cell wall is pierced by the tail pin. Finally, the tail sheath contracts and injects DNA into the bacterium. The DNA promptly takes over the cell's metabolism and redirects it to manufacture more virus particles.

It is convenient to classify viruses according to the nucleic acid that makes up its genome. The DNA viruses include the herpesvirus, which causes herpes simplex types I and II, and adenoviruses, which cause symptoms of the common cold. The RNA viruses include enteroviruses, which cause polio and gastrointestinal illness; rhinoviruses, which also cause symptoms of the common cold; and the HIV virus, which causes the deadly disease AIDS in humans.

2.4.1 Viral Ecology

Given the history of how viruses came to be studied by microbiologists, it is entirely understandable that most of what is known about viruses deals with how they infect humans, animals, and plants. Very little attention has been given to the role that viruses play in the ecology of natural systems.

In the late 1980s, however, microbiologists made the surprising discovery that a milliliter of ordinary seawater often contained as many as 10^8 viral particles (Proctor and Furhman, 1990; Suttle, 1993). Further studies showed that viruses were intimately involved in the ecology of photosynthetic cyanobacteria that form the base of marine ecosystems. Specifically, viruses infect cyanobacteria, hijack

their cellular metabolism into reproducing more viral particles, and kill them (Proctor and Furhman, 1990). For cyanobacteria, life is a race to reproduce before they are infected and killed by the viruses. For viruses, on the other hand, "life" consists of trying to locate a cyanobacteria, attach to specific proteins on the bacterial cell wall, and injecting their DNA. If, after a few hours, a viron cannot locate and infect a host, it loses its ability to attach to bacterial cells and, in effect, "dies."

The ecological advantage for a viron infecting a cyanobacteria is obvious—it allows the viron to reproduce. However, the ecological advantage for the cyanobacteria is less obvious. Studies have shown that cyanobacteria introduced into virus-free seawater do not thrive, as one might expect. Rather, their rate of growth slows down and stops. The reason for this puzzling behavior, it turns out, is due to the cycling of nutrients. In natural seawater, the death of individual cyanobacteria due to viral infection is a principal source of essential nutrients such as nitrogen and phosphorus for growing cells. Without that source of nutrients, the cyanobacteria can't grow (Suttle, 1999). Thus, while viral infection is a disaster for individual cyanobacteria, it actually benefits the health of the overall cyanobacterial population. This is a classic example of a host–parasite relationship that has benefits for both species.

Other than the studies of seawater cited above, very little is known about the ecology of virus–host interactions in terrestrial environments or in ground-water systems. Nevertheless, by analogy to the ecology of seawater, it may be anticipated that such relationships exist, and that they may be integral parts of the ecology of these systems.

2.4.2 Viruses in Ground-Water Systems

The study of viruses in water science has focused largely on the mobility of pathogenic viruses from sewage effluents. Infectious hepatitis is the most closely studied disease that has been documented as being transmitted by a water-borne virus. Many epidemics of hepatitis have been traced to fecal contamination of drinking water or fecal contamination of shellfish that are subsequently consumed by humans. Although the disease hepatitis has been closely studied, not much is known about the virus itself. This is largely because of the extreme difficulty in studying the hepatitis virus in the laboratory.

There is considerable debate as to the possibility of pathogenic viruses being transported in ground water. Fecal contamination of shallow water table aquifers could result in the transmission of infectious hepatitis. However, solid evidence that such transmission has occurred is generally lacking. Studies have shown, however, that viruses can persist in ground-water environments. For example, Keswick et al. (1982) showed that polioviruses, coxsackieviruses, and rotaviruses survive much longer in subsurface environments—on the order of weeks or months—than had generally been assumed. A field study by Wellings et al. (1975) gave direct evidence that viruses could survive up to 28 days in ground-water systems. These survival times are sufficient for viruses to move through shallow aquifers to wells from, for example, septic system effluents.

In one such study, a septic tank was seeded with a model enterovirus, and the survival and transport of virons in the tank itself and in the septic drainfield was monitored for three months (Scandura and Sobsey, 1997). The results showed that

the numbers of the model viron decreased rapidly in the septic tank itself as effluent washed out into the drainfield. Furthermore, the model viron was detected just one day later in a monitoring well placed near the drainfield outfall. Monitoring data showed that virons were transported at least 15 meters downgradient of the outfall.

The development of polymerase chain reaction (PCR) technology has greatly aided the study of human viruses in wastewater and ground water. These methods, which are described in greater detail in Chapters 5 and 6, increase the detection sensitivity for specific viruses and decrease the time required for detection. Standard cell culture methods for the detection of human pathogenic viruses in water samples is expensive and time-consuming, requiring up to a month for confirmed positive results (APHA, 1989). With PCR technology, target nucleic acids can be amplified using specific oligonucleotide primers. Using repeated cycles of PCR, a 10^6-fold amplification of a single copy of target DNA can be completed in a few hours. The decreased time and increased sensitivity of PCR allow the detection of small amouts of DNA and RNA in water samples (Reynolds et al., 1997). Studies using PCR technology have shown that enteroviruses can be detected in well water that has been exposed to sewage effluent (Abbaszadegan et al., 1993).

Because land application is widely used to dispose of sewage effluents, the delivery and transport of viruses to ground water is a matter of environmental concern (Berg, 1983). These studies indicate that viron survival in water is affected by physical (light, temperature, adsorption, aggregation) processes, chemical (pH, ionic strength, redox) processes, and biologic (types of virons, bacterial activity, protozoan activity) processes. Solar radiation, for example, was shown to greatly increase the rate of inactivation of poliovirus and coliphage T4 viruses—a fact of importance for land application of sewage effluents. Similarly, it was shown that increased ionic strength increased the inactivation rate of poliovirus, and that the adsorption of viruses onto soil particles was pH dependent.

Because the kinds of cells needed for the replication of human pathogenic viruses are either absent or present in low concentrations in ground-water systems, it is unlikely that human pathogens such as poliovirus are active in ground-water systems for long periods of time (>3 months). While this seems perfectly logical, firm data demonstrating this to be the case is not available. Additional studies on the persistence of viruses in subsurface environments are needed to verify this. In addition, studies on the role of viruses in the ecology of subsurface environments are warranted as well.

2.5 BACTERIA IN GROUND-WATER SYSTEMS

This book is concerned largely with bacteria—that is, procaryotic microorganisms—and how they influence the chemistry of ground water. In this context, the archaea, even though they are not procaryotic microorganisms, will be considered together with bacteria. While eucaryotic microbes are present in ground-water systems, their abundance is typically three or four orders of magnitude less than that of bacteria. Also, because most eucaryotes are restricted to oxygenated environments, they are excluded from many deep subsurface environments. Similarly, viruses are present in some subsurface environments. Because they have no independent metabolism, however, their impact on ground-water chemistry is probably

limited to the effects that they have on indigenous bacteria. Thus, the emphasis on bacteria and bacterial processes in this book reflects the relative importance of the different types of microorganisms on the chemistry of ground water.

2.5.1 Classifying Bacteria

The classification of living organisms is called taxonomy. The purpose of bacterial taxonomy is really twofold. First, its purpose is to be able to distinguish between different bacteria. Second, and equally important, its purpose is to identify similarities between different bacteria so that phylogenic relationships can be determined. Because of these two aims, classifications of bacteria are hierarchical; that is, groups of closely related species are arranged into genera, similar genera are arranged into families, families into orders, and so on. The names given to the microorganisms reflect this hierarchical classification scheme.

Bacteria are named according to a binomial nomenclature, with each distinct species given a name consisting of two words. Take, for example, the bacterium *Escherichia coli*. The first part of the name, *Escherichia* is the genus to which the microorganism belongs and is always capitalized. The second part of the name, *coli,* is the name of the species and is not capitalized. It is proper usage to italicize or underline the proper names of microorganisms. The root of the genus name is either a Latin or a Greek word that may in some way be descriptive of the organism or be in honor of a particular person. It is common practice to abbreviate the names of microbes to the first letter of the genus, followed by the species name. *Escherichia coli,* therefore, is commonly referred to as *E. coli. E. coli* belongs to a family of related genera that inhabit the intestines of higher animals and are therefore called the Enterobacteriaceae, or "intestine bacteria." The Enterobacteriaceae belong to the order Eubacteria, or "true bacteria." This order includes the greatest number of bacterial species that inhabit ground-water systems.

Criteria Used to Classify Bacteria Bacteria are classified on the basis of cell morphology, type of cell wall, cell growth, biochemical transformations carried out by the cell, nutrition, and sequences of rRNA and DNA. All of these characteristics, of course, are determined by the genetic information stored in the cell's chromosomal DNA. Because of this, similarities and differences of base sequences in chromosomal DNA between different strains of bacteria is the basis of modern bacterial taxonomy. However, it is still useful to characterize bacteria based on morphological characteristics that can be determined under the light microscope and physiological characteristics that can be determined by growth on different substrates.

The gram stain is used to determine cell shape and type of cell wall. In this process, the bacteria are obtained in pure culture, smeared onto a glass slide, and fixed (heated) so that the smear will adhere to the slide. The fixed smear is subjected to the following solutions in this order: crystal violet, iodine solution, ethyl alcohol (a decolorizing agent), and safranin. Bacteria that have peptidoglycan as their outer cell wall retain the crystal violet, are stained a deep purple color by this procedure, and are termed gram positive. Bacteria that have an outer cell wall consisting of lipopolysaccharides and proteins do not retain the crystal violet stain, are colored red by the counter stain safranin, and are termed gram negative.

The shape and arrangement of the cells (Fig. 2.8) are also observed using the Gram stain technique. A coccus (plural, cocci) is a spherical cell (Fig. 2.8a), a bacillus (plural, bacilli) is a rod-shaped cell (Fig. 2.8b), a spirillum (plural, spirilla) is a helical rod (Fig. 2.8c), and a vibrio is a V-shaped cell (Fig. 2.8d). It is common to refine these basic shapes by terming different cells long rods, short rods, or whatever term the individual observer deems important. The arrangement of cells is also important. Some cells, notably those of the genus *Streptococcus,* are characterized by cells arranged in chains. Others, such as *Micrococcus,* are typically found in grape-like clusters. Some bacteria tend to occur in pairs or in clusters of four, called tetrads. All of these characteristic cell arrangements are useful in taxonomy.

Motility in bacteria is conferred by the presence of extremely thin, hair-like appendages, called flagella, that protrude from the cell wall (Fig. 2.9). Because flagella confer motility and because not all species of bacteria are flagellated, it follows that there are motile and nonmotile species. As such, the presence or absence of motility is a convenient characteristic for the classification of bacteria.

Flagella are hooked structures that are embedded in granular bodies just beneath the cell wall in the cytoplasm. As the flagellum is rotated, much like a motorboat's propeller, the bacterium is propelled forward, allowing it to "swim." There are many different arrangements of flagella found in nature. If a single flagellum is found on one or both ends of a rod-shaped bacterium, it is termed a polar flagellum. All bacteria with a polar flagellum are classified into the order Pseudomonadales.

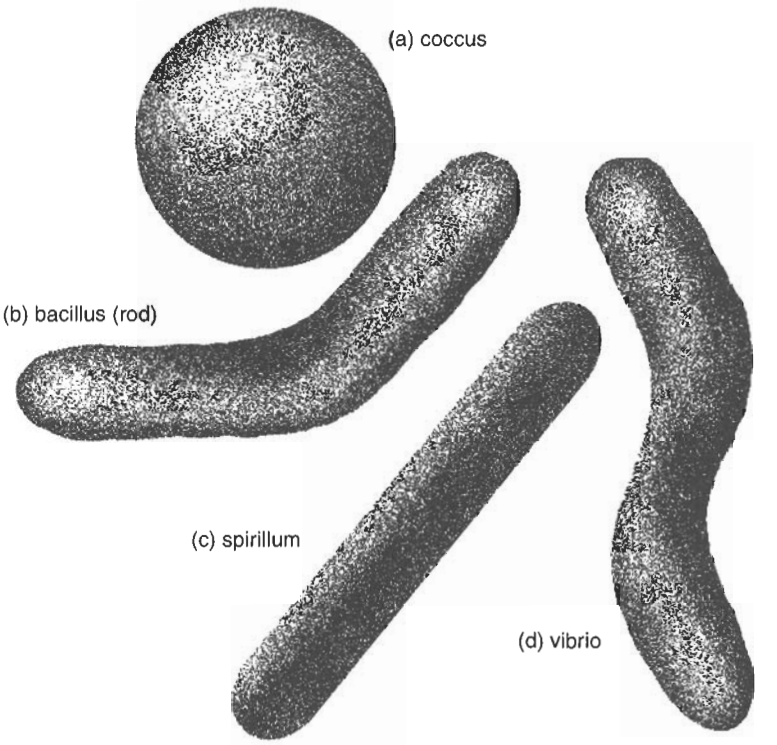

Figure 2.8. Common shapes exhibited by bacterial cells.

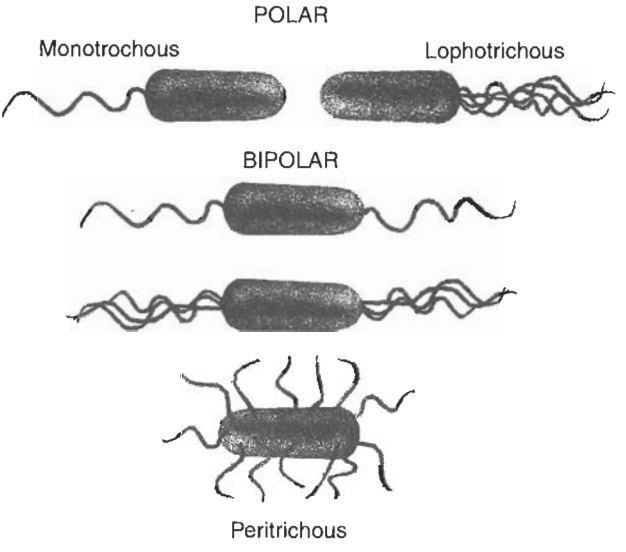

Figure 2.9. Different arrangements of flagella in bacterial cells.

Other types of rod-shaped bacteria have many flagella arranged over much of the cell. These are termed peritrichous flagella, and all bacteria showing this arrangement are classified in the order Eubacteriales.

While morphology can vary significantly in bacteria (Fig. 2.10 and Fig. 2.11) other classification criteria are useful. Nutrition, or the types of substances used for carbon and energy sources, is another logical basis upon which to classify bacteria. Heterotrophs are organisms that use organic carbon as energy and carbon sources.

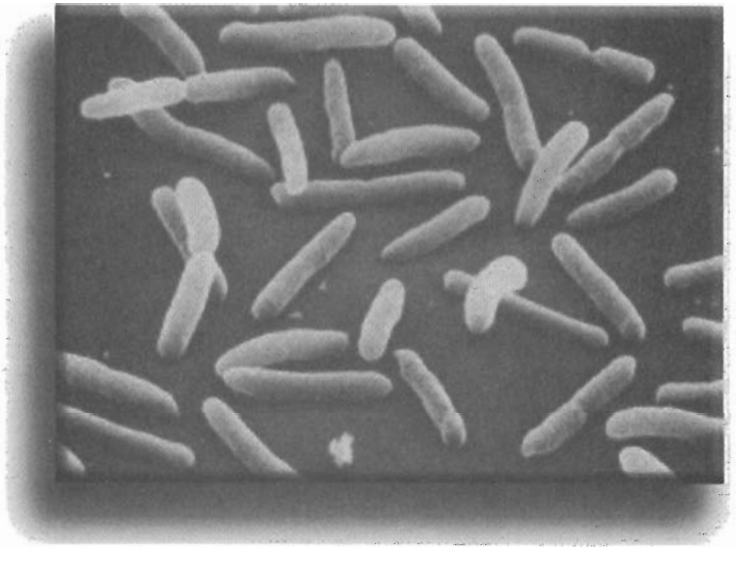

Figure 2.10 Micrographs of rod-shaped bacteria.

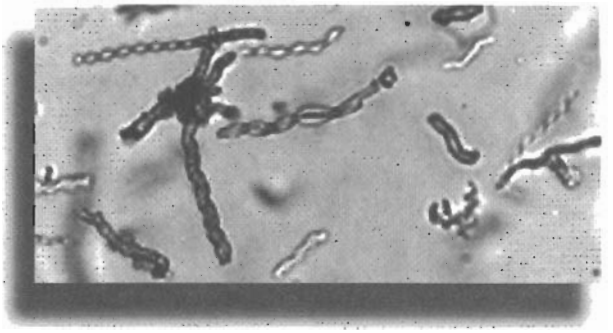

Figure 2.11. Micrograph of the iron-oxidizing bacteria *Gallionella.*

Lithotrophs use inorganic carbon, such as carbon dioxide or bicarbonate, as carbon sources and an external source of energy. Chemolithotrophs are a type of lithotroph that obtain energy by oxidizing reduced inorganic chemicals, such as ferrous iron or sulfides. Photolithotrophs are lithotrophs that are able to obtain energy from light.

Metabolism, the ways in which bacteria utilize their nutrients, is also used in classification. In order to obtain energy from a substrate, microorganisms remove electrons and transfer them to other chemicals that serve as electron acceptors. The use of inorganic chemicals, such as oxygen, ferric iron, or sulfate as electron acceptors is called respiration. The use of organic chemicals as an electron acceptor is called fermentation. Bacteria that use oxygen as an electron acceptor are called aerobes. Bacteria that can use only oxygen as an electron acceptor are called obligate aerobes. Bacteria that use oxygen when it is available but are also capable of fermentation are termed facultative anaerobes. Microorganisms that grow only in the absence of oxygen are called obligate anaerobes.

The most direct means for taxonomy is the composition and structure of chromosomal DNA that codes for all of the characteristics—size, shape, nutrition, and metabolism—exhibited by microorganisms. One common method of comparing the DNA of different microorganisms is to compare the relative abundance of the nucleotides guanine plus cytosine (G + C) to total nucleotide abundance. For example, based just on G + C, the enteric bacteria *Escherichia, Shigella,* and *Salmonella* (48–52% G + C) can be easily distinguished from most species of *Pseudomonas* (66–68% G + C).

Measuring G + C has the advantage that it is relatively easy to do. However, because most of the information in DNA is coded by the sequence of nucleotides, not just by their relative abundance, this method is not ideal. The order that nucleotides occur in a genome contains much more information and is a more direct way to classify bacteria. The attempts to use DNA to classify bacteria date to the 1960s when investigators began experimenting with DNA homology (Schildkraut et al., 1961). DNA homology is the degree to which DNA of one bacterial strain matches another, and in theory, at least, is an ideal way to classify bacteria. This method has been used with some success for microbial taxonomy (Grimont, 1988). In practice, however, DNA homology has some serious drawbacks. Whereas this method is very good at distinguishing differences between closely related species,

it gives very little information about the relationship of more distantly related species, and does not help place strains within higher taxa, such as genus, family, order, division, and kingdom.

For these reasons, RNA present in bacteria also have been extensively used for classification purposes. Originally, the approach was to separate and sequence the 16S ribosomal RNA (rRNA) from bacterial cells (Woese, 1981). Because there are many fewer nucleotides in the 16S rRNA (about 1,500 base pairs) than in chromosomal DNA itself (about 4.5 million base pairs), this was a much more manageable task. This tool has been especially useful for determining the "relatedness" of different eucaryotic, bacterial, and archaeal species. With the advent of polymerase chain reaction (PCR), cloning, and sequencing technology (Chapter 5), it has become possible to analyze bacterial DNA for specific 16S rRNA genes and use these as a basis for taxonomic classification. This has been greatly facilitated by the development of large databases that include phylogenetically ordered alignments of rRNA for large numbers of microorganisms (Maidak et al., 1994). It is now possible to take an unknown bacterial isolate, amplify a fragment of its 16S rRNA gene, sequence a portion of the fragment, and compare the resulting sequence to a database. It is usually possible to identify a microorganism to the genus level by sequencing between 300 and 500 base pairs. These PCR-based methods are now the most reliable ways for identifying and classifying bacteria found in natural and human-created environments (Amann et al., 1995).

2.5.2 Gram-Negative Bacteria Found in Ground-Water Systems

Gram-negative bacteria are found extensively in ground-water systems. For example, in a shallow water table aquifer in Oklahoma (Balkwill and Ghiorse, 1985), about two-thirds of the bacteria isolated were gram negative. In a carbonate aquifer in the Atlantic Coastal Plain (Chapelle et al., 1988), about 70% of the isolates recovered were gram negative. Finally, in clastic aquifers of the Atlantic Coastal Plain (Balkwill, 1989), anywhere from 60% to 90% of the isolates recovered were gram negative. This group of microorganisms, therefore, are important components of the terrestrial subsurface flora.

Aerobic Gram-Negative Rods Aerobic gram-negative rods are widely distributed in soils and shallow ground-water systems. Most of these bacteria have an enzyme called cytochrome oxidase that enables the organism to transfer electrons onto molecular oxygen, thus using oxygen as a terminal electron acceptor. Such microorganisms are termed oxidase positive. In the shallow aquifer near Lula, Oklahoma (Balkwill and Ghiorse, 1985), all of the bacteria isolated and characterized were oxidase positive, meaning that they carried this particular enzyme. In deeper anaerobic aquifers, as one might expect, the presence of gram-negative, aerobic rods becomes less common.

Members of the genera *Pseudomonas, Azotobacter, Rhizobium, Alcaligenes, Flavobacterium,* and *Bordetella* are representative of aerobic gram-negative rods. Pseudomonas appears to be particularly common in ground-water systems. Of 29 strains of bacteria identified to the genus level in the carbonate Floridan aquifer system of South Carolina, 11 could be assigned to the genus *Pseudomonas* (Chapelle et al., 1988). At the Lula, Oklahoma, site (Balkwill and Ghiorse, 1985), 3 of the 6