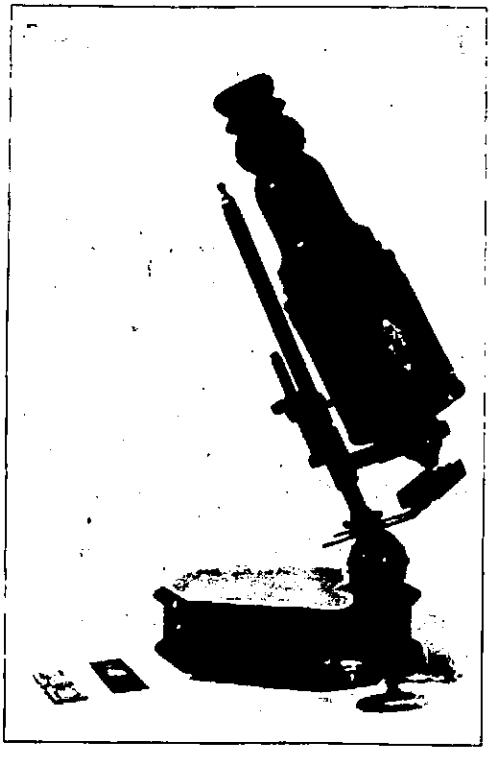
PART ONE
INTRODUCTION TO MICROBIOLOGY



The introduction of agar for pure culture study of microorganisms

The discovery of the world of microorganisms came about as investigators developed microscopes and used them to examine droplets of natural fluids. Menageries of microbes were revealed from a variety of specimens. Initially these observations were a source of great curiosity. During the period from 1600 to 1800, considerable information accumulated about the occurrence of these microscopic forms of life. Great debates emerged as to the origin of these microbes. The controversy centered on the question of whether they arose from nonliving materials, i.e., spontaneous generation.

Simultaneous with the development of evidence over hundreds of years to disprove spontaneous generation was the growing acceptance of the concept that these microorganisms were the cause of many conditions that occur in everyday life, ranging from food spoilage to diseases of humans, other animals, and plants. In the latter part of the nineteenth century a major problem confronted investigators searching for evidence to prove that a specific kind of microorganism was responsible for a disease or spoilage. How could they isolate in pure culture the microorganism suspected of causing the change and prove that it was the causal agent? What the researchers needed was a solid nutrient substance upon which a specimen could be spread so that individual microbial cells would be distant from each other. Upon incubation, each cell would reproduce, resulting in a mass of identical cells (a colony). A small portion of the colony could then be transferred to a fresh medium and be maintained as a pure culture for subsequent experiments, i.e., the pure culture technique.

Robert Koch (1843–1885) was particularly concerned with the need to develop a technique for the isolation of microorganisms in pure culture in order to establish the causative agent of a disease. He experimented with slices of sterile potatoes as the solid surface upon which to grow colonies of bacteria. This proved unsatisfactory for a variety of reasons. He tried gelatin as a solidifying agent. This had the desirable feature of being a transparent gel, but it had the serious disadvantage of becoming liquid above 25°C, which is below the optimum temperature for the growth of human disease-producing bacteria.

The solution to this problem was provided in 1883 by a German housewife, Fannie E. Hesse, who spent part of her time working in the laboratory of her husband, Walther Hesse. Hesse, a physician, was a former student of Robert Koch. Frau Hesse suggested to her husband that he use agar, a polysaccharide of algal origin, as a substitute for gelatin in microbiological media. She had gained experience with the characteristics of agar in the process of making jelly; the agar increased the consistency. His experiments with agar as a substitute for gelatin were dramatically successful. This observation was of such significance that Hesse promptly reported the experiments with agar to Robert Koch. Koch immediately recognized the great value of agar as a solidifying agent for microbiological media and adopted its use. Agar goes into solution (1.5 percent) at 100°C and solidifies at 45°C. Upon jelling at 45°C, it remains solid at elevated temperatures—temperatures just below 100°C. This feature makes it possible to incubate the inoculated media at almost any desired temperature and still have the medium remain solid.

It is a remarkable fact that agar, introduced for use as a solidifying agent in microbiological media just about 100 years ago, has not been replaced. Agar is as important now as it was then. The manner in which it was discovered adds credence to one of Louis Pasteur's observations: "Chance favors the prepared mind."

Preceding page. A compound microscope made by John Marshall of London about 1700 after a design by Robert Hooke. A condensing lens on a jointed arm allowed the instrument to be tilted on a ball-and-socket joint. *(Courtesy of the Armed Forces Institute of Pathology, Washington, D.C.)*

Chapter 1 The Scope of Microbiology

OUTLINE

Microbiology is the study of living organisms of microscopic size, which include bacteria, fungi, algae, protozoa, and the infectious agents at the borderline of life that are called viruses. It is concerned with their form, structure, reproduction, physiology, metabolism, and classification. It includes the study of their distribution in nature, their relationship to each other and to other living organisms, their effects on human beings and on other animals and plants, their abilities to make physical and chemical changes in our environment, and their reactions to physical and chemical agents.

Microorganisms are closely associated with the health and welfare of human beings; some microorganisms are beneficial and others are detrimental. For example, microorganisms are involved in the making of yogurt, cheese, and wine; in the production of penicillin, interferon, and alcohol; and in the processing of domestic and industrial wastes. Microorganisms can cause disease, spoil food, and deteriorate materials like iron pipes, glass lenses, and wood pilings.

Most microorganisms are unicellular. In unicellular organisms all the life processes are performed by a single cell. In the so-called higher forms of life, organisms are composed of many cells that are arranged in tissues and organs to perform specific functions. Regardless of the complexity of an organism, the cell is the basic structural unit of life. All living cells are fundamentally similar.

The word *cell* was first used more than two centuries ago by an Englishman, Robert Hooke (1635–1703), in his descriptions (1665) of the fine structure of cork and other plant materials. The honeycomblike structure he observed in a thin slice of cork (see Fig. 1-1) was due to the cell walls of cells that were once

Figure 1-1. Robert Hooke's drawing of a thin slice of cork as he observed it under the microscope. This drawing was included in a report made to the Royal Society (London) in 1665. He is credited as being the first person to use the word *cell*. *(Courtesy of National Library of Medicine.)*

living. But the concept of the cell as the structural unit of life—the **cell theory**—is credited to two Germans, Matthias Schleiden and Theodor Schwann, who in 1838–1839 described cells as the basic structural and functional units of all organisms. Schleiden and Schwann recognized that all cells, no matter what the organism, are very similar in structure. As the concept of the cell as the basic unit of life gained acceptance, investigators speculated on the nature of the substance contained within the cell. **Protoplasm** (Greek *proto*, "first"; *plasm*, "formed substance", introduced to characterize the living material of a cell), is a colloidal organic complex consisting largely of protein, lipids, and nucleic acids. These substances are enclosed by membranes or cell walls; and the protoplasm always contains nuclei or an equivalent nuclear substance (see Fig. 1-2). Developments in electron-microscope techniques have made it possible to reveal the complex intricacies of intracellular organization (see Fig. 1-3).

All biological systems have the following characteristics in common: (1) the ability to reproduce; (2) the ability to ingest or assimilate food substances and metabolize them for energy and growth; (3) the ability to excrete waste products; (4) the ability to react to changes in their environment—sometimes called **irritability**; and (5) susceptibility to mutation. In the study of microbiology, we encounter "organisms" which may represent the borderline of life. These are the viruses, which are simpler in structure and composition than single cells. Viruses provide an exciting challenge and an opportunity to gain a better un-

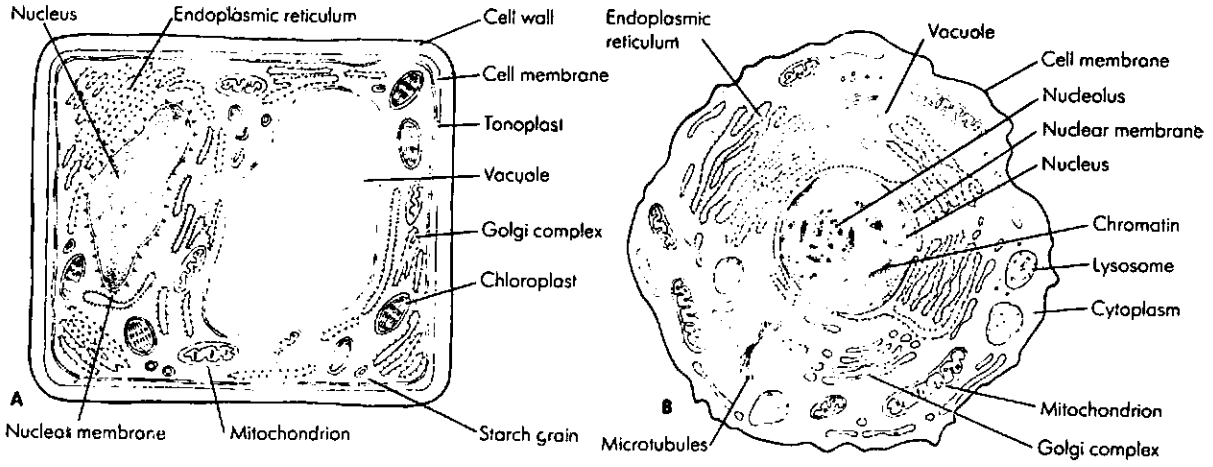
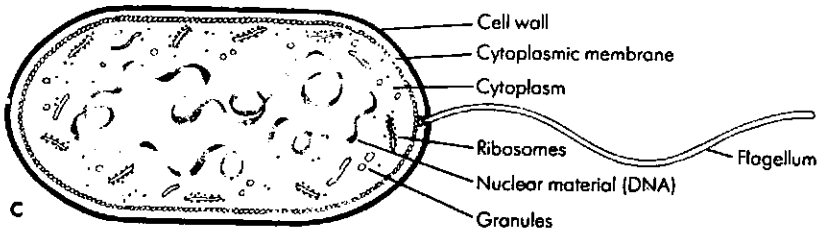


Figure 1-2. Generalized diagram of typical cell structures. (A) Plant cell. (B) Animal cell. (C) Bacterial cell. *(Erwin F. Lessel, illustrator.)*



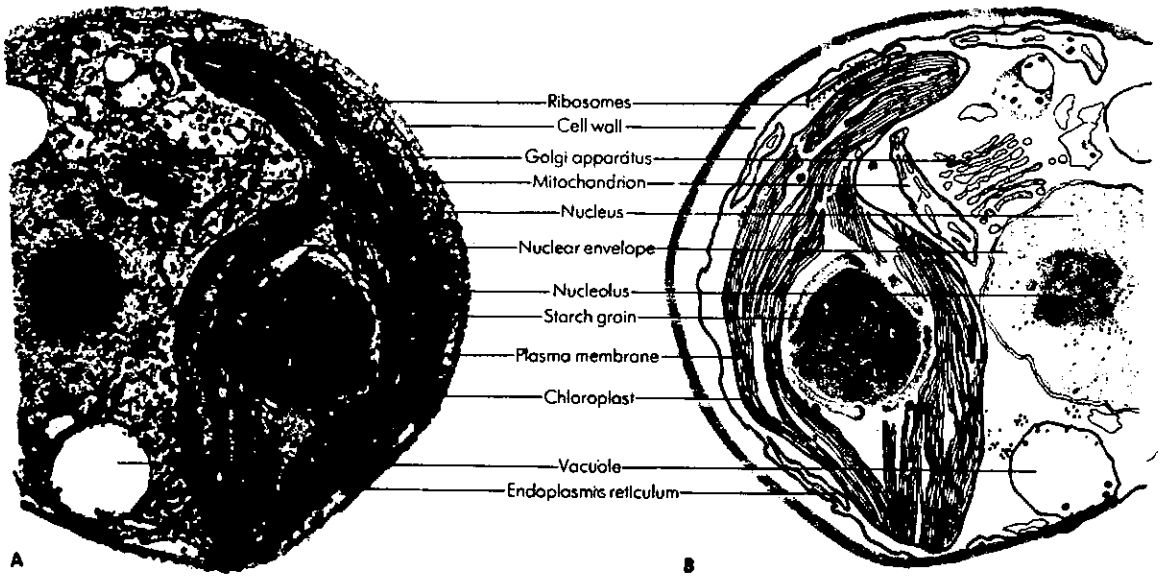


Figure 1-3. (A) Electron micrograph of the alga *Chlamydomonas reinhardii* (X15,000), a eucaryotic cell. *(Courtesy of George E. Palade, The Rockefeller University, by permission of Holt, New York, publishers of Ariel G. Leowy and Philip Seikovitz, Cell Structure and Function, 1969.)* (B) Schematic representation of (A). *(Erwin F. Lessel, illustrator.)*

derstanding of the nature of complex organic substances that may bridge the gap between the living and the nonliving worlds.

Viruses are obligate parasites; that is, they are obligated to grow within an appropriate host cell—plant, animal, or microbe. They cannot multiply outside a host cell. However, when a virus enters an appropriate living cell, it is able to direct the synthesis of hundreds of identical viruses, using the cell's energy and biochemical machinery. A virus is made up of substances unique to life: **nucleic acids** (chemicals that make up genetic material) and **proteins** (complex nitrogenous substances found in various forms in animals and plants).

MICROBIOLOGY AS A FIELD OF BIOLOGY

Biologists are known for their differing opinions as to how the huge field of biology can best be subdivided. Historically, the divisions followed the major groups of life, as in zoology (animals), botany (plants), entomology (insects), and microbiology (microorganisms). Another manner of subdividing the subject matter of biology is based on the level at which the study is conducted: for example, studies at the level of molecular constituents of the cell (molecular biology); studies at the level of the cell (cell biology); studies at the level of the intact organism (organismal biology); and studies of groups of organisms (population biology). Still another approach is to establish divisions on the basis of form and function, as in morphology or anatomy, physiology, metabolism, and genetics. In some colleges and universities, the study of microbiology is carried out in a department of biology; in others, it is in a department of microbiology or a department of molecular biology.

Irrespective of where microbiology is placed in the broad field of biology, microorganisms have some characteristics which make them ideal specimens for the study of numerous fundamental life processes. This is possible because, at the cellular level, many life processes are performed in the same manner whether they be in microbe, mouse, or human.

Microorganisms are exceptionally attractive models for studying fundamental life processes. They can be grown conveniently in test tubes or flasks, thus requiring less space and maintenance than larger plants and animals. They grow rapidly and reproduce at an unusually high rate; some species of bacteria undergo almost 100 generations in a 24-h period. The metabolic processes of microorganisms follow patterns that occur among higher plants and animals. For example, yeasts utilize glucose in essentially the same manner as cells of mammalian tissue; the same system of enzymes is present in these diverse organisms. The energy liberated during the breakdown of glucose is "trapped" and made available for the work to be performed by the cells, whether they be bacteria, yeasts, protozoa, or muscle cells. In fact, the mechanism by which organisms (or their cells) utilize energy is fundamentally the same throughout the biological world. The source of energy does, of course, vary among organisms. Plants are characterized by their ability to use radiant energy, whereas animals require chemical substances as their fuel. In this respect some microorganisms are like plants, others like animals; and some have the unique ability of using either radiant energy or chemical energy and thus are like both plants and animals. Furthermore, some microorganisms, the bacteria in particular, are able to utilize a great variety of chemical substances as their energy source—ranging from simple inorganic substances to complex organic substances.

In microbiology we can study organisms in great detail and observe their life processes while they are actively metabolizing, growing, reproducing, aging, and dying. By modifying their environment we can alter metabolic activities, regulate growth, and even change some details of their genetic pattern—all without destroying the organisms.

For example, bacteriophages, which are viruses that infect and reproduce in bacteria, demonstrate the complete sequence of host-parasite reactions and provide a model by which virus-host cell reactions can be postulated for infections in higher plants and animals. Bacteriophages have been of inestimable value in elucidating many biological phenomena, including those concerned with genetics.

Microorganisms have a wider range of physiological and biochemical potentialities than all other organisms combined. For example, some bacteria are able to utilize atmospheric nitrogen for the synthesis of proteins and other complex organic nitrogenous compounds. Other species require inorganic or organic nitrogen compounds as the initial building blocks for their nitrogenous constituents. Some microorganisms synthesize all their vitamins, while others need to be furnished vitamins. By reviewing the nutritional requirements of a large collection of microorganisms, it is possible to arrange them from those with the simplest to those with the most complex requirements. The increasing complexity of nutritional requirements in such an arrangement is also a reflection of the decreasing synthetic capacity of the organisms so arranged. In addition, this kind of arrangement provides information about the steps in the synthesis of various metabolites, e.g., from atmospheric oxygen to inorganic nitrogen salts to amino acids. The biochemist has used microorganisms having varying degrees of synthetic ability to investigate pathways of synthesis.

In his presidential address to the Society of American Bacteriologists (now The American Society for Microbiology) in 1942, the late Selman A. Waksman (Fig. 1-4) observed:

Figure 1-4. Selman A. Waksman (1888–1973), world's foremost authority on soil microbiology and codiscoverer of the antibiotic streptomycin.

There is no field of human endeavor, whether it be in industry or agriculture, or in the preparation of food or in connection with problems of shelter or clothing, or in the conservation of human or animal health and the combating of disease, where the microbe does not play an important and often dominant role.

Waksman, longtime professor of microbiology at Rutgers University, in 1952 was awarded the Nobel prize in physiology or medicine for the part he played in the discovery of the antibiotic streptomycin, which is produced by a soil bacterium.

THE PLACE OF MICROORGANISMS IN THE LIVING WORLD

In biology as in any other field, classification means the orderly arrangement of units under study into groups of larger units. Present-day classification in biology was established by the work of Carolus Linnaeus (1707–1778), a Swedish botanist. His books on the classification of plants and animals are considered to be the beginning of modern botanical and zoological nomenclature, a system of naming plants and animals. Nomenclature in microbiology, which came much later, was based on the principles established for the plant and animal kingdoms.

Until the eighteenth century, the classification of living organisms placed all organisms into one of two kingdoms, plant and animal. As previously stated, in microbiology we study some organisms that are predominantly plantlike, others that are animallike, and some that share characteristics common to both plants and animals. Since there are organisms that do not fall naturally into either the plant or the animal kingdom, it was proposed that new kingdoms be established to include those organisms which typically are neither plants nor animals.

Haeckel's Kingdom *Protista*

One of the earliest of these proposals was made in 1866 by a German zoologist, E. H. Haeckel. He suggested that a third kingdom, *Protista*, be formed to include those unicellular microorganisms that are typically neither plants nor animals. These organisms, the protists, include bacteria, algae, fungi, and protozoa. (Viruses are not cellular organisms and therefore are not classified as protists.) Bacteria are referred to as lower protists; the others—algae, fungi, and protozoa—are called higher protists.

Procaryotic and Eucaryotic Protists

Haeckel's kingdom *Protista* left some questions unanswered. For example, what criteria could be used to distinguish a bacterium from a yeast or certain microscopic algae? Satisfactory criteria were unavailable until late in the 1940s when more definitive observation of internal cell structure was made possible with the aid of the powerful magnification provided by electron microscopy. It was discovered that in some cells, for example typical bacteria, the nuclear substance was not enclosed by a nuclear membrane. In other cells, such as typical algae and fungi, the nucleus was enclosed in a membrane. This discovery—the absence of membrane-bound internal structures in one group of protists (bacteria) and the presence of membrane-bound structures in all the others (fungi, algae, and protozoa)—was a discovery of fundamental significance. Further research has revealed additional differences in the internal structure of these cells.

These two cell types, as characterized in Table 1-1, have been designated

Table 1-1. Features Distinguishing Procaryotic from Eucaryotic Cells

Feature	Procaryotic Cells	Eucaryotic Cells
Groups where found as unit of structure	Bacteria	Algae, fungi, protozoa, plants, and animals
Size range of organism	1–2 by 1–4 μm or less	Greater than 5 μm in width or diameter
Genetic system		
Location	Nucleoid, chromatin body, or nuclear material	Nucleus, mitochondria, chloroplasts
Structure of nucleus	Not bounded by nuclear membrane; one circular chromosome	Bounded by nuclear membrane; more than one chromosome
	Chromosome does not contain histones; no mitotic division	Chromosomes have histones; mitotic nuclear division
	Nucleolus absent; functionally related genes may be clustered	Nucleolus present; functionally related genes not clustered
Sexuality	Zygote nature is merozygotic (partial diploid)	Zygote is diploid
Cytoplasmic nature and structures		
Cytoplasmic streaming	Absent	Present
Pinocytosis	Absent	Present
Gas vacuoles	Can be present	Absent
Mesosome	Present	Absent
Ribosomes	70S,* distributed in the cytoplasm	80S arrayed on membranes as in endoplasmic reticulum; 70S in mitochondria and chloroplasts
Mitochondria	Absent	Present
Chloroplasts	Absent	May be present
Golgi structures	Absent	Present
Endoplasmic reticulum	Absent	Present
Membrane-bound (true) vacuoles	Absent	Present
Outer cell structures		
Cytoplasmic membranes	Generally do not contain sterols; contain part of respiratory and, in some, photosynthetic machinery	Sterols present; do not carry out respiration and photosynthesis
Cell wall	Peptidoglycan (murein or mucopeptide) as component	Absence of peptidoglycan
Locomotor organelles	Simple fibril	Multifibrilled with "9 + 2" microtubules

Table 1-1. (*continued*)

Feature	Procaryotic Cells	Eucaryotic Cells
Pseudopodia	Absent	Present in some
Metabolic mechanisms	Wide variety, particularly that of anaerobic energy-yielding reactions; some fix nitrogen gas; some accumulate poly-β-hydroxybutyrate as reserve material	Glycolysis is pathway for anaerobic energy-yielding mechanism
DNA base ratios as moles % of guanine + cytosine (G + C %)	28 to 73	About 40

* S refers to the Svedberg unit, the sedimentation coefficient of a particle in the ultracentrifuge.
NOTE: Definitions of technical words are provided in the glossary at the back of the book.

procaryotic and eucaryotic organisms of each cell type are called procaryotes and eucaryotes, respectively.

Bacteria are procaryotic microorganisms. The eucaryotic microorganisms in-

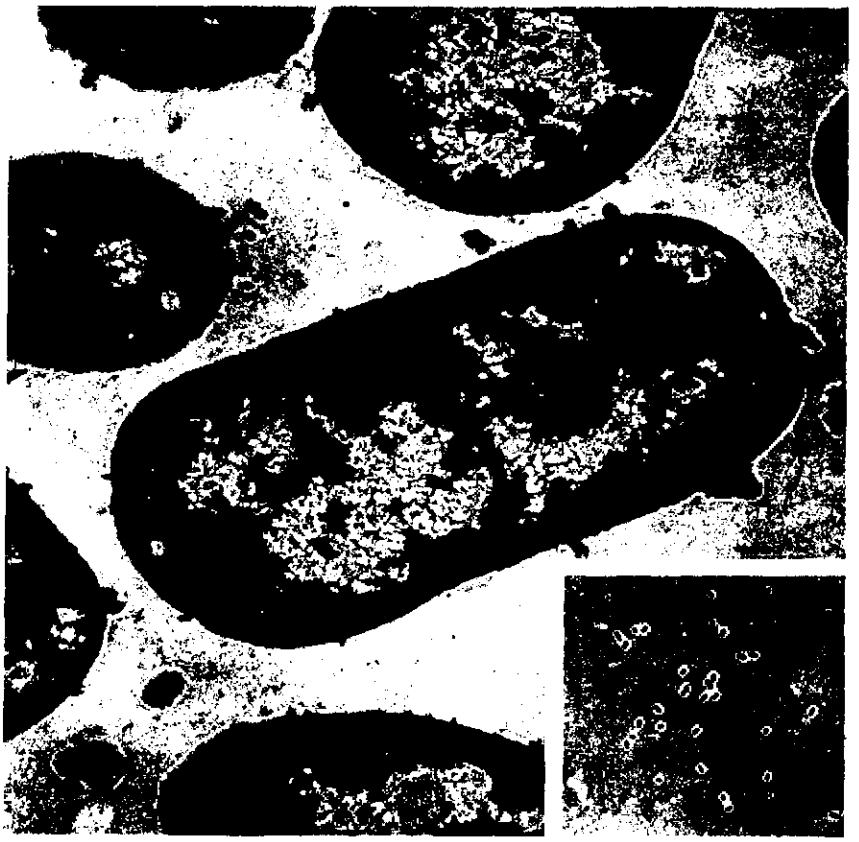


Figure 1-5. The bacterium *Escherichia coli*, a typical procaryotic cell. Note the absence of any discrete intracellular organelle structures. The light area represents nuclear material; the dark area is ribosomal material. (*Courtesy I. D. J. Burdett and R. G. E. Murray, J. Bacteriol* **119**:*1039, 1974.*) Inset: *E. coli* cells as seen by light microscopy.

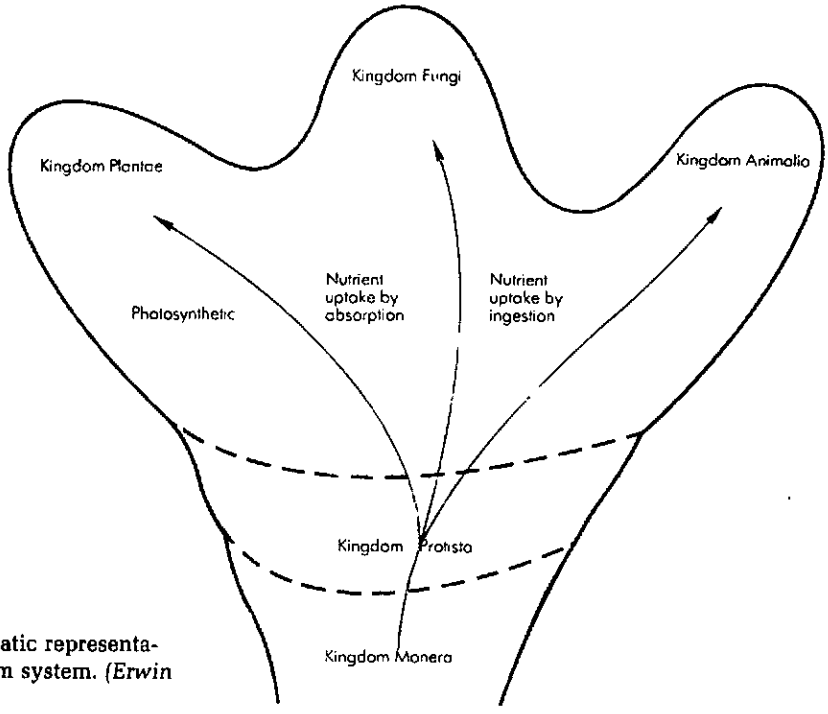


Figure 1-6. A simplified schematic representation of Whittaker's five-kingdom system. *(Erwin F. Lessel, illustrator.)*

clude the protozoa, fungi, and algae. (Plant and animal cells are also eucaryotic.) Viruses are left out of this scheme of classification. Examples of typical procaryotic and eucaryotic cells are shown in Figs. 1-2, 1-3, and 1-5.

Whittaker's Five-Kingdom Concept

A more recent and comprehensive system of classification, the five-kingdom system, was proposed by R. H. Whittaker (1969). This system of classification, shown in Fig. 1-6, is based on three levels of cellular organization which evolved to accommodate three principal modes of nutrition: photosynthesis, absorption, and ingestion. The procaryotes are included in the kingdom *Monera*; they lack the ingestive mode of nutrition. Unicellular eucaryotic microorganisms are placed in the kingdom *Protista*; all three nutritional types are represented here. In fact, as shown in Fig. 1-6, the nutritional modes are continuous: the mode of nutrition of the microalgae is photosynthetic; the mode of nutrition of the protozoa is ingestive; and the mode of nutrition in some other protists is absorptive, with some overlap to the photosynthetic and ingestive modes. The multicellular and multinucleate eucaryotic organisms are found in the kingdoms *Plantae* (multicellular green plants and higher algae), *Animalia* (multicellular animals), and *Fungi* (multinucleate higher fungi). Their diversified nutritional modes lead to a more diversified cellular organization. Microorganisms are found in three of the five kingdoms: *Monera* (bacteria and cyanobacteria), *Protista* (microalgae and protozoa), and *Fungi* (yeasts and molds).

Kingdom *Procaryotae* after *Bergey's Manual of Systematic Bacteriology*

Bergey's Manual of Systematic Bacteriology places all bacteria in the kingdom *Procaryotae* which in turn is divided into 4 divisions as follows:

Division 1 Gracilicutes
Procaryotes with a complex cell-wall structure characteristic of Gram-negative bacteria

Division 2 Firmicutes
Procaryotes with a cell-wall structure characteristic of Gram-positive bacteria

Division 3 Tenericutes
Procaryotes that lack a cell wall

Division 4 Mendosicutes
Procaryotes that show evidence of an earlier phylogenetic origin than those bacteria included in Divisions 1 and 2 (above)

Bergey's Manual is the international standard for bacterial taxonomy. More detailed descriptions of groups of bacteria are given in Part Four of this book.

A comparable manual of classification does not exist for fungi, algae, or protozoa. There are, however, schemes of classification for each group that have wide acceptance and usage. An international system for classification and nomenclature of viruses is in the process of development.

GROUPS OF MICROORGANISMS

The major groups of protists are briefly described below. Although viruses are not protists or cellular organisms, they are included for two reasons: (1) the techniques used to study viruses are microbiological in nature; and (2) viruses are causative agents of diseases, hence, diagnostic procedures for their identification are employed in the clinical microbiological laboratory as well as the plant pathology laboratory.

Algae are relatively simple organisms. The most primitive types are unicellular. Others are aggregations of similar cells with little or no differentiation in structure or function. Still other algae, such as the large brown kelp, have a complex structure with cell types specialized for particular functions. Regardless of size or complexity, all algal cells contain chlorophyll and are capable of photosynthesis. Algae are found most commonly in aquatic environments or in damp soil.

Viruses are very small noncellular parasites or pathogens of plants, animals, and bacteria as well as other protists. They are so small that they can be visualized only by the electron microscope. Viruses can be cultivated only in living cells.

Bacteria are unicellular procaryotic organisms or simple associations of similar cells. Cell multiplication is usually by binary fission.

Protozoa are unicellular eucaryotic organisms. They are differentiated on the basis of morphological, nutritional, and physiological characteristics. Their role in nature is varied, but the best-known protozoa are the few that cause disease in human beings and animals.

Fungi are eucaryotic lower plants devoid of chlorophyll. They are usually multicellular but are not differentiated into roots, stems, and leaves. They range in size and shape from single-celled microscopic yeasts to giant multicellular

mushrooms and puffballs. We are particularly interested in those organisms commonly called molds, the mildews, the yeasts, and the plant pathogens known as rusts. True fungi are composed of filaments and masses of cells which make up the body of the organism, known as a mycelium. Fungi reproduce by fission, by budding, or by means of spores borne on fruiting structures that are quite distinctive for certain species.

Some morphological and characteristic features of these various microbial groups are shown in Fig. 1-7 and Table 1-2.

Microbiologists may specialize in the study of certain groups of microorganisms. Strictly speaking, bacteriology is the study of bacteria, but the term is

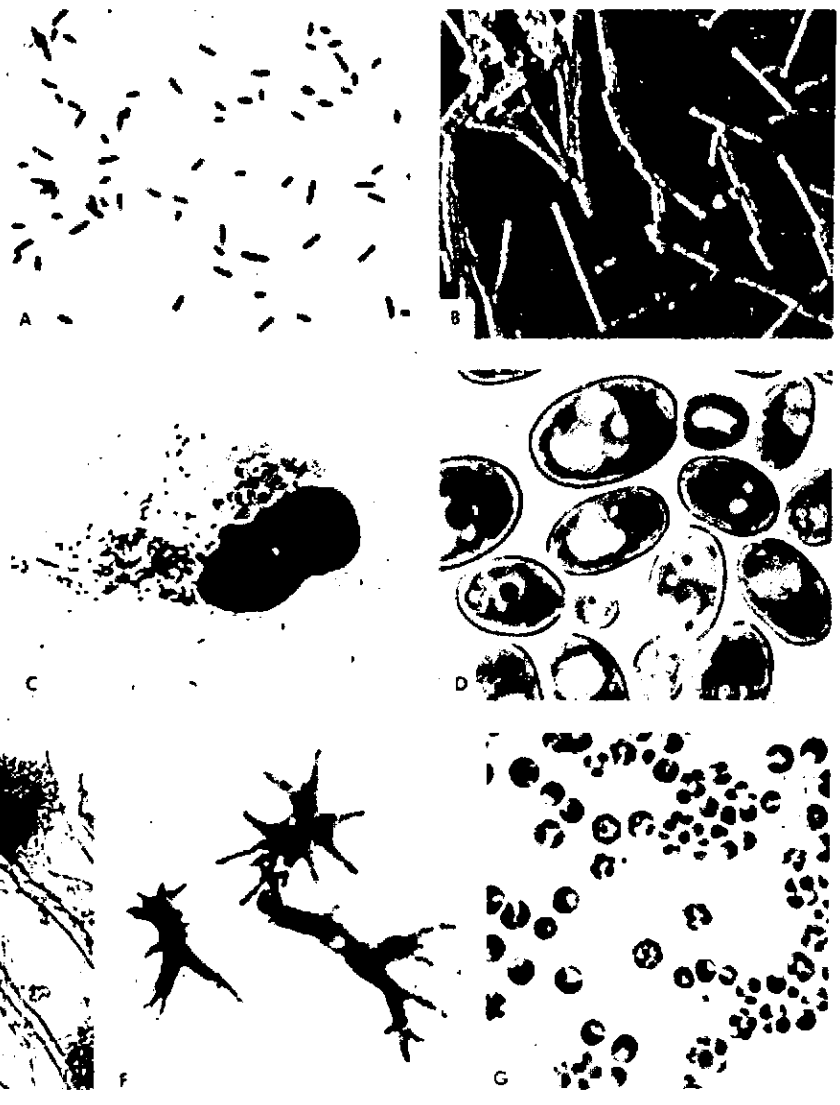
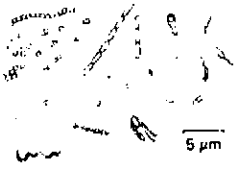
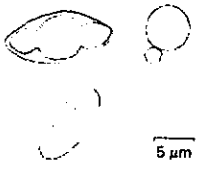
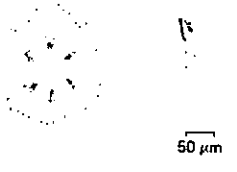
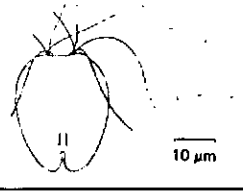


Figure 1-7. Morphological features of various groups of microorganisms. (Note that this illustration is only intended to convey the impression of morphological diversity. No size relationship between groups can be obtained from it. The wide range in microbial sizes does not permit both constancy in magnification and showing of meaningful morphological details at the same time.) (A) *Escherichia coli* (X1,000). (B) Tobacco mosaic virus (X100,000). *(Hitachi, Ltd., Tokyo.)* (C) *Rickettsia tsutsugamushi* in cytoplasm of infected cell (X940). *(N.J. Kramis and The Rocky Mountain Laboratory, U.S. Public Health Service.)* (D) *Candida utilis* (X2,000 approx.). *(Courtesy of G. Svihla, J. L. Dainko, and F. Schlenk, J Bacteriol,* **85**:399, 1963.) (E) *Aspergillus* sp. *(Courtesy of Douglas F. Lawson.)* (F) Amoeba. *(Carolina Biological Supply Co.)* (G) *Chlorella infusionum* (X1,000). *(Courtesy of Robert W. Krauss.)*

Table 1-2. Some Characteristics of Major Groups of Microorganisms *(Erwin F. Lessel, illustrator)*

Group	Morphology	Size	Important Characteristics	Practical Significance
Bacteria		Typical: 0.5–1.5 μm by 1.0–3.0 μm Range: 0.2 by 100 μm	Procaryotic; unicellular, simple internal structure; grow on artificial laboratory media; reproduction asexual, characteristically by simple cell division	Some cause disease; some perform important role in natural cycling of elements which contributes to soil fertility; useful in industry for manufacture of valuable compounds; some spoil foods and some make foods
Viruses		Range: 0.015–0.2 μm	Do not grow on artificial laboratory media—require living cells within which they are reproduced; all are obligate parasites; electron microscopy required to see viruses	Cause diseases in humans, other animals, and plants; also infect microorganisms
Fungi: Yeasts		Range: 5.0–10.0 μm	Eucaryotic; unicellular; laboratory cultivation much like that of bacteria; reproduction by asexual cell division, budding, or sexual processes	Production of alcoholic beverages; also used as food supplement; some cause disease
Fungi: Molds		Range: 2.0–10.0 μm by several mm	Eucaryotic; multicellular, with many distinctive structural features; cultivated in laboratory much like bacteria; reproduction by asexual and sexual processes	Responsible for decomposition (deterioration) of many materials; useful for industrial production of many chemicals, including penicillin; cause diseases of humans, other animals, and plants
Protozoa		Range: 2.0–200 μm	Eucaryotic; unicellular; some cultivated in laboratory much like bacteria; some are intracellular parasites; reproduction by asexual and sexual processes	Food for aquatic animals; some cause disease
Algae		Range: 1.0 μm to many feet	Eucaryotic; unicellular and multicellular; most occur in aquatic environments; contain chlorophyll and are photosynthetic; reproduction by asexual and sexual processes	Important to the production of food in aquatic environments; used as food supplement and in pharmaceutical preparations; source of agar for microbiological media; some produce toxic substances

often used as a synonym for microbiology. Protozoology is the study of protozoa; a special branch of protozoology called parasitology deals exclusively with the parasitic or disease-producing protozoa and other parasitic micro- and macroorganisms. Mycology is the study of fungi such as yeasts and molds. Virology is the science that deals with viruses. Phycology is the study of algae. Further specialization in some aspect of the biology of a particular group of organisms is not uncommon; e.g., bacterial genetics, algal physiology, and bacterial cytology.

DISTRIBUTION OF MICROORGANISMS IN NATURE

Microorganisms occur nearly everywhere in nature. They are carried by air currents from the earth's surface to the upper atmosphere. Even those indigenous to the ocean may be found many miles away on mountaintops. Microbes are found in the bottom of the ocean at its greatest depths. Fertile soil teems with them. They are carried by streams and rivers into lakes and other large bodies of water; and if human wastes containing harmful bacteria are discharged into streams, diseases may be spread from one place to another. Microorganisms occur most abundantly where they find food, moisture, and a temperature suitable for their growth and multiplication. Since the conditions that favor the survival and growth of many microorganisms are those under which people normally live, it is inevitable that we live among a multitude of microbes. They are in the air we breathe and the food we eat. They are on the surfaces of our bodies, in our alimentary tracts, and in our mouths, noses, and other body orifices. Fortunately most microorganisms are harmless to us; and we have means of resisting invasion by those that are potentially harmful.

APPLIED AREAS OF MICROBIOLOGY

Microorganisms affect the well-being of people in a great many ways. As we have already stated, they occur in large numbers in most natural environments and bring about many changes, some desirable and others undesirable. The diversity of their activities ranges from causing diseases in humans, other animals, and plants to the production and deposition of minerals, the formation of coal, and the enhancement of soil fertility.

There are many more species of microorganisms that perform important roles in nature than there are disease-producing species.

A summary of the major fields of applied microbiology appears in Table 1-3.

MICROBIOLOGY AND THE ORIGIN OF LIFE

Many explanations have been offered for the origin of life on earth. One of the more acceptable of these proposals suggests that life originated in the sea following millions of years of a chemical evolutionary process. According to this hypothesis the inorganic compounds of the atmosphere, under the influence of ultraviolet light, electrical discharges, and/or high temperatures, interacted to form organic compounds which precipitated into the sea, where they accumulated. These organic compounds, subjected to additional physical effects of the environment, combined to form amino acids. The amino acids interacted to form peptides, polypeptides, and other more complex organic substances which served as the precursors of the first form of life.

Table 1-3. Major Fields of Applied Microbiology

Field	Some Applied Areas
Medical microbiology	Causative agents of disease; diagnostic procedures; diagnostic procedures for identification of causative agents; preventive measures
Aquatic microbiology	Water purification; microbiological examination; biological degradation of waste; ecology
Aeromicrobiology	Contamination and spoilage; dissemination of diseases
Food microbiology	Food preservation and preparation; foodborne diseases and their prevention
Agricultural microbiology	Soil fertility; plant and animal diseases
Industrial microbiology	Production of medicinal products such as antibiotics and vaccines; fermented beverages, industrial chemicals; production of proteins and hormones by genetically engineered microorganisms
Exomicrobiology	Exploration for life in outer space
Geochemical microbiology	Coal, mineral and gas formation; prospecting for deposits of coal, oil, and gas; recovery of minerals from low-grade ores

The time scale of chemical evolution, biological evolution, and the emergence of microbial life is shown in Fig. 1-8.

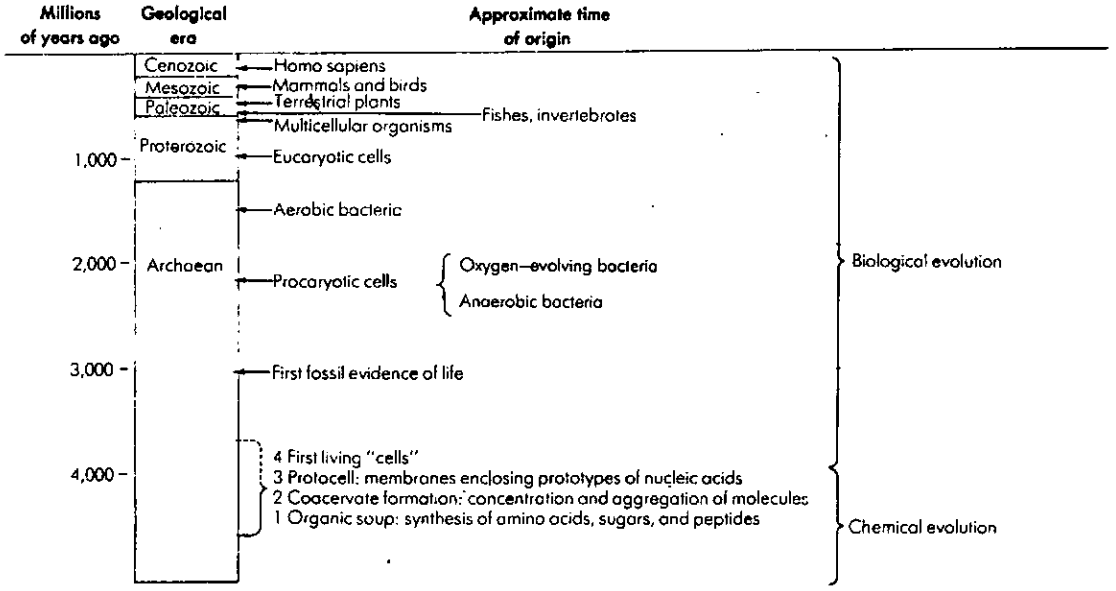


Figure 1-8. Time scale of the chemical evolution, the biological evolution, and the occurrence of microbial life.

QUESTIONS

1 List the characteristics common to all biological systems.
2 Why are microorganisms useful as subjects for research in the field of biology?
3 Explain why a knowledge of microbiology is useful in understanding life processes in higher plants and animals.
4 How did the term *protists* arise? What organisms do we refer to by use of this term? What is the difference between lower protists and higher protists?
5 Discuss the differences between procaryotic and eucaryotic cells.
6 How do viruses differ from other microorganisms?
7 What is the basis of the five-kingdom classification scheme according to Whittaker? Give a reason why it is so widely accepted in the biological community.
8 Discuss the place of microorganisms in Whittaker's five-kingdom classification scheme.
9 Why is *Bergey's Manual of Systematic Bacteriology* so important to bacteriologists?
10 Where are microorganisms found in nature? How may they be transferred from place to place?
11 Name several applied areas of microbiology. Describe the importance of microorganisms in each of these applied fields.

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Chapter 2 The History of Microbiology

OUTLINE

History is the story of the achievements of men and women, but it records relatively few outstanding names and events. Many important contributions were made by people whose names have been forgotten and whose accomplishments have been lost in the longer and deeper shadows cast by those who caught the fancy of the chroniclers. It has been said that in science the credit goes to the one who convinces the world, not to the one who first had the idea. So, in the development of microbiology, the outstanding names are often of those who convinced the world—who developed a technique, a tool, or a concept that was generally adopted, or who explained their findings so clearly or dramatically that the science grew and prospered.

Antony van Leeuwenhoek's lucid reports on the ubiquity of microbes enabled Louis Pasteur 200 years later to discover the involvement of these creatures in fermentation reactions and allowed Robert Koch, Theobald Smith, Pasteur, and many others to discover the association of microbes with disease. Koch is remembered for his isolation of the bacteria that cause anthrax and tuberculosis and for the rigid criteria he demanded before a specific bacterium be held as the cause of a disease. His important contributions to the creation of the science of microbiology won him the 1905 Nobel prize.

The building of the Panama Canal dramatized Walter Reed's studies of the epidemiology of yellow fever, but historians remember that Theobald Smith's work on transmission of Texas fever pointed the way for Walter Reed's subsequent work.

in diagnosis by laboratory methods. G. F. I. Widal and August von Wassermann presented those who followed them with tools and ideas with which to work. Paul Ehrlich's discovery of a chemical compound that would destroy the syphilis spirochete in the human body without injury to tissue cells paved the way for future developments in the use of chemicals in treating disease. For this he shared the Nobel prize in 1908 with Elie Metchnikoff, who discovered a system in the human body that combated infection.

Though of relatively short duration, the history of microbiology is filled with thrilling achievements. We have won many battles with microorganisms and have learned not only to make them work for us but also to control some of those that work against us.

THE MICROSCOPE

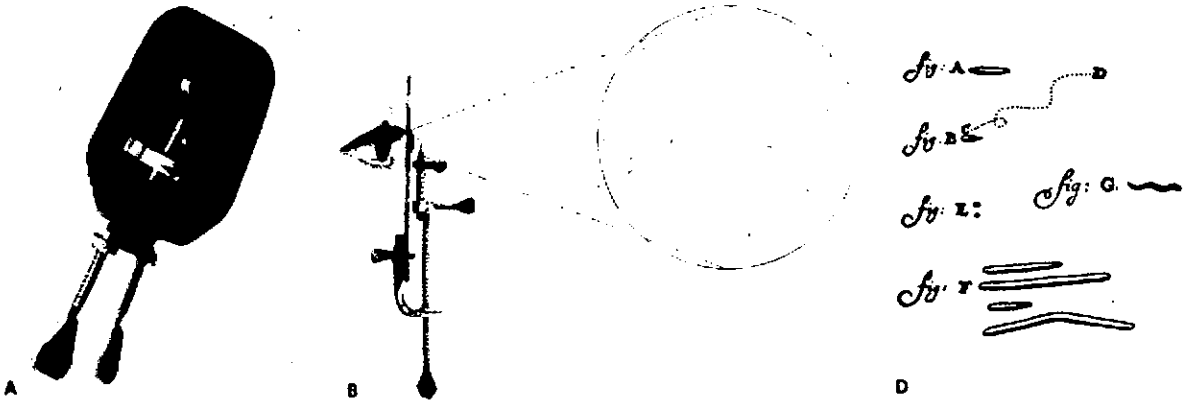
Microbiology began when people learned to grind lenses from pieces of glass and combine them to produce magnifications great enough to enable microbes to be seen. During the thirteenth century Roger Bacon (1220–1292) postulated that disease is produced by invisible living creatures. This suggestion was made again by Girolamo Fracastoro of Verona (1483–1553) and Anton von Plenciz in 1762, but these people had no proof. As early as 1658, a monk named Athanasius Kircher (1601–1680) referred to "worms" invisible to the naked eye in decaying bodies, meat, milk, and diarrheal secretions. Although his description lacked accuracy, Kircher was the first person to recognize the significance of bacteria and other microbes in disease. In 1665 Robert Hooke's description of cells in a piece of cork established the fact that the bodies of "animals and plants, complex as they may appear, are yet composed of a few elementary parts frequently repeated"—a quotation not from Hooke but from Aristotle's description of the cellular structure of living things back in the fourth century B.C.

Although he was probably not the first to see bacteria and protozoa, Antony van Leeuwenhoek, who lived in Delft, Holland, from 1632 to 1723, was the first to report his observations with accurate descriptions and drawings (Fig. 2-1). Leeuwenhoek had the means and opportunity to pursue his hobby of lens grinding and microscope making. During his lifetime he made more than 250 microscopes consisting of home-ground lenses mounted in brass and silver, the most powerful of which would magnify about 200 to 300 times (Fig. 2-2). These microscopes bear little resemblance to the compound light microscope of today, which is capable of magnifications of 1,000 to 3,000 times. However, the lenses of Leeuwenhoek's microscopes were well made and Leeuwenhoek had the openness of mind that is so very important in an investigator. His descriptions of protozoa were so accurate that many of the forms he described are easily recognized today.

Leeuwenhoek carefully recorded his observations in a series of letters to the British Royal Society. In one of the first letters, dated September 7, 1674, addressed to Henry Oldenburg, Secretary of the Royal Society, he described the "very little animalcules" which we recognize as free-living protozoa. On October 9, 1676, he wrote:

In the year 1675, I discovered living creatures in rain water which had stood but a few days in a new earthen pot, glazed blue within. This invited me to view this

Figure 2-1. Antony van Leeuwenhoek (1632–1723), a Dutch student of natural history whose hobby was making microscopes, is shown here with one of the more than 250 microscopes that he made. His best lenses were capable of magnifications up to ×270, and he was the first person to report descriptions of microorganisms in detail. *(Courtesy of Lambert-Hudnut, Division Warner-Lambert Pharmaceutical Company.)*



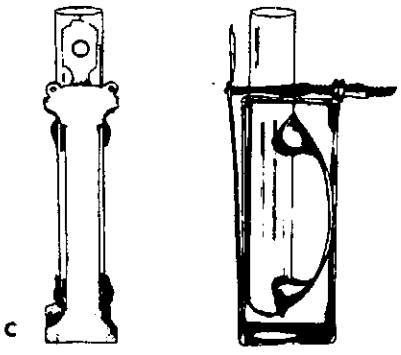


Figure 2-2. (A) The Leeuwenhoek microscope. Replica of a simple microscope made in 1673 by Leeuwenhoek. *(From the collection of the Armed Forces Institute of Pathology, Washington, D.C.)* (B) Side view of a Leeuwenhoek microscope, illustrating the manner in which observations of specimens were made. (C) Front and side views of Leeuwenhoek's brass aquatic microscope. Lenses could be slotted in at the front and focused by the butterfly nut at the rear. *(Erwin F. Lessel, illustrator.)* (D) Leeuwenhoek's sketches of bacteria from the human mouth, from letter of September 17, 1683. Note particularly shapes of cells and relative sizes. The dotted line between **C** and **D** indicates motility (movement) of a bacterium. *(Courtesy of C. Dobell, Antony van Leeuwenhoek and His "Little Animals," Dover, New York, 1960.)*

water with great attention, especially those little animals appearing to me ten thousand times less than those . . . which may be perceived in the water with the naked eye.

He described his little animals in great detail, leaving little doubt that he saw bacteria, fungi, and many forms of protozoa. For example, he reported that on June 16, 1675, while examining well water into which he had put a whole pepper the day before:

I discovered, in a tiny drop of water, incredibly many very little animalcules, and these of divers sorts and sizes. They moved with bendings, as an eel always swims with its head in front, and never tail first, yet these animalcules swam as well backwards as forwards, though their motion was very slow.

His enthusiastic letters were read with interest by the British scientists, but the importance of his discoveries evidently went unappreciated. The talents and astuteness of this remarkable man can best be appreciated by reading Dobell's biography of Leeuwenhoek.

Before the time of Pasteur, microorganisms were studied mainly to satisfy curiosity concerning their characteristics and their relationships to higher living forms, without awareness of their importance in fermentation and disease.

SPONTANEOUS GENERATION VERSUS BIOGENESIS

The discovery of microbes spurred interest in the origin of living things, and argument and speculation grew. As far as human beings were concerned, the Greek explanation that the goddess Gaea was able to create people from stones and other inanimate objects had been largely discarded. But even the astute Aristotle (384–322 B.C.) taught that animals might originate spontaneously from the soil, plants, or other unlike animals, and his influence was still strongly felt in the seventeenth century. About 40 B.C., Virgil (70–19 B.C.) gave directions for the artificial propagation of bees. This was but one of many fanciful tales of a similar nature that persisted into the seventeenth century. For example, it was accepted as a fact that maggots could be produced by exposing meat to warmth and air, but Francesco Redi (1626–1697) doubted this. Proof that his skepticism was well founded came from an experiment in which he placed meat in a jar covered with gauze. Attracted by the odor of the meat, flies laid eggs on the covering, and from the eggs maggots developed. Hence the experiment established the fact that the origin of the maggots was the flies and not the meat. This experiment and others involving mice and scorpions appear to have settled the matter so far as these forms of life were concerned. But microbes were another matter; surely such minute creatures needed no parents!

There appeared champions for and challengers of the theory that living things can originate spontaneously, each with a new and sometimes fantastic explanation or bit of experimental evidence. In 1749, while experimenting with meat exposed to hot ashes, John Needham (1713–1781) observed the appearance of organisms not present at the start of the experiment and concluded that the bacteria originated from the meat. About the same time, Lazaro Spallanzani (1729–1799) boiled beef broth for an hour and then sealed the flasks. No microbes appeared following incubation. But his results, confirmed in repeated

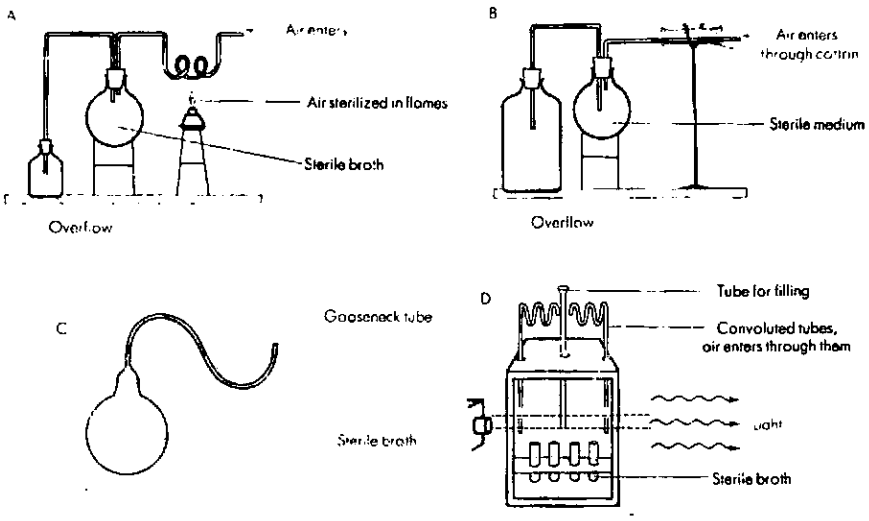


Figure 2-3. The theory of spontaneous generation was disproved with the devices illustrated here, all of which eliminated airborne bacteria. Schwann heat-sterilized the air which flowed through the glass tube to his culture flask (A). Schröder and von Dusch filtered the air entering the culture flask through cotton (B). Simple goose-necked flasks (C) were devised by Pasteur. Tyndall constructed a dust-free incubation chamber (D).

experiments, failed to convince Needham, who insisted that air was essential to the spontaneous production of microscopic beings and that it had been excluded from the flasks by sealing them. This argument was answered some 60 or 70 years later independently by two other investigators, Franz Schulze (1815–1873) and Theodor Schwann (1810–1882). Schulze passed air through strong acid solutions into boiled infusions, whereas Schwann passed air into his flasks through red-hot tubes (Fig. 2-3A). In neither case did microbes appear. But the die-hard advocates of spontaneous generation were still not convinced. Acid and heat altered the air so that it would not support growth, they said. About 1850, H. Schröder and T. von Dusch performed a more convincing experiment by passing air through cotton into flasks containing heated broth (Fig. 2-3B). Thus the microbes were filtered out of the air by the cotton fibers so that growth did not occur, and a basic technique of plugging bacterial culture tubes with cotton stoppers was initiated.

The concept of spontaneous generation was revived for the last time by Felix-Archimede Pouchet (1800–1872), who published in 1859 an extensive report "proving" its occurrence. But Pouchet reckoned without the ingenious, tireless, and stubborn Pasteur (1822–1895). Irritated by Pouchet's logic and data, Pasteur performed experiments that ended the argument for all time. He prepared a flask with a long, narrow gooseneck opening (Fig. 2-3C). The nutrient solutions were heated in the flask, and air—untreated and unfiltered—could pass in or out; but the germs settled in the gooseneck, and no microbes appeared in the solution.

Pasteur reported his results with a great flourish at the Sorbonne in Paris on April 7, 1864. His flasks would yield no sign of life, he said:

> For I have kept from them, and am still keeping from them, that one thing which is above the power of man to make; I have kept from them the germs that float in the air. I have kept from them life.

In his exuberance, Pasteur sent a few darts at those he disagreed with:

There is no condition known today in which you can affirm that microscopic beings come into the world without germs, without parents like themselves. They who allege it have been the sport of illusions, of ill-made experiments, vitiated by errors which they have not been able to perceive and have not known how to avoid.

Finally, John Tyndall (1820–1893) conducted experiments in a specially designed box to prove that dust carried the germs (Fig. 2-3D). He demonstrated that if no dust was present, sterile broth remained free of microbial growth for indefinite periods.

FERMENTATION

Louis Pasteur (Fig. 2-4) began his brilliant career as professor of chemistry at the University of Lille, France. A principal industry of France being the manufacture of wines and beer, Pasteur studied the methods and processes involved in order to help his neighbors produce a consistently good product. He found that fermentation of fruits and grains, resulting in alcohol, was brought about by microbes. By examining many batches of "ferment," he found microbes of different sorts. In good lots one type predominated, and in the poor products another kind was present. By proper selection of the microbe, the manufacturer might be assured of a consistently good and uniform product. Pasteur suggested that the undesirable types of microbes might be removed by heating—not enough to hurt the flavor of the fruit juice, but enough to destroy a very high percentage of the microbial population. He found that holding the juices at a temperature of 62.8°C (145°F) for half an hour did the job. Today pasteurization is widely used in fermentation industries, but we are most familiar with it in the dairy industry.

Figure 2-4. Louis Pasteur in his laboratory. *(Courtesy of Institut Pasteur, Paris.)*

THE GERM THEORY OF DISEASE

Even before Pasteur had proved by experiment that bacteria are the cause of some diseases, many observant students had expressed strong arguments for the germ theory of disease. Fracastoro of Verona suggested that diseases might be due to invisible organisms transmitted from one person to another. In 1762 von Plenciz not only stated that living agents are the cause of disease but suspected that different germs were responsible for different diseases. That the concept of parasitism was becoming quite general is reflected in the following bit of doggerel written by Jonathan Swift (1667–1745) early in the eighteenth century:

> So naturalists observe, a flea
> Hath smaller fleas that on him prey;
> And these have smaller fleas to bit 'em;
> And so proceed ad infinitum.

This is better known in the colloquial version:

> Big bugs have little bugs,
> Upon their backs to bit 'em;
> And little bugs have smaller ones,
> And so ad infinitum.

Oliver Wendell Holmes (1809–1894), a successful physician as well as a scholar, insisted that puerperal fever, a disease of childbirth, was contagious and that it was probably caused by a germ carried from one mother to another by midwives and physicians. He wrote *The Contagiousness of Puerperal Fever* in 1842. At approximately the same time, the Hungarian physician Ignaz Philipp Semmelweis (1818–1865) was pioneering in the use of antiseptics in obstetrical practice. Deaths due to infections associated with childbirth were reduced in the cases handled according to his instructions, which minimized chances for infection. As part of his crusade he published *The Cause, Concept and Prophylaxis of Childbed Fever* in 1861. Still, most physicians ignored his advice, and it was not until about 1890, when the work of Joseph Lister in England had become known, that the importance of antisepsis was fully appreciated by the medical profession.

Pasteur's success in solving the problem of fermentation led the French goverment to request that he investigate pebrine, a silkworm disease that was ruining an important French industry. For several years Pasteur struggled with this problem, heartaches and disappointments following one after another. Eventually he isolated the parasite causing the disease. He also showed that silkworm farmers could eliminate the disease by using only healthy, disease-free caterpillars for breeding stock.

Turning from silk to wool, Pasteur next tackled the problem of anthrax, a disease of cattle, sheep, and sometimes human beings. He grew the microbes in laboratory flasks after isolating them from the blood of animals that had died of the disease. Meanwhile Robert Koch (1843–1910) was busy with the anthrax problem in Germany. Koch, a quiet, meticulous physician, sometimes neglected his medical practice to play with the fascinating new science of bacteriology. It was he who discovered the typical bacilli with squarish ends in the blood of cattle that had died of anthrax. He grew these bacteria in cultures in his laboratory, examined them microscopically to be sure he had only one kind present,

and then injected them into other animals to see if these became infected and developed clinical symptoms of anthrax. From these experimentally infected animals he isolated microbes like those he had originally found in sheep that died of anthrax. This was the first time a bacterium had been proved to be the cause of an animal disease. (Pebrine is caused by a protozoan rather than by a bacterium.) This series of observations led to the establishment of **Koch's postulates**, which provided guidelines to identify the causative agent of an infectious disease. Koch's postulates are: (1) A specific organism can always be found in association with a given disease. (2) The organism can be isolated and grown in pure culture in the laboratory. (3) The pure culture will produce the disease when inoculated into a susceptible animal. (4) It is possible to recover the organism in pure culture from the experimentally infected animal.

LABORATORY TECHNIQUES AND PURE CULTURES

As we have previously stated, microorganisms occur in nature in extremely large populations made up of many different species. In order to study the characteristics of a particular species it is first necessary to separate it from all other species. Laboratory procedures have been developed that make it possible to isolate microorganisms representing each species and to grow (cultivate) each of the species separately. The growth of a mass of cells of the same species in a laboratory vessel (such as a test tube) is called a **pure culture**.

Pure cultures of bacteria were first obtained by Joseph Lister in 1878 using serial dilutions in liquid media. With a specially constructed syringe he diluted a fluid (probably milk) containing a mixture of bacteria until a single organism was delivered into a container of sterile milk. After incubation, bacteria in this container were of a single kind, identical to the parent cell. Lister named the organism *Bacterium lactis*.

Meanwhile Koch was carefully refining methods for the study of bacteria. He found that by smearing bacteria on a glass slide and adding certain dyes to them, individual cells could be seen more clearly with the microscope. He added gelatin and other solidifying materials such as agar to media in order to obtain isolated growths of organisms known as **colonies**, each of which contained millions of individual bacterial cells packed tightly together. From these colonies, pure cultures could be transferred to other media. The development of a liquefiable solid-culture medium was of fundamental importance.

Using techniques he had devised, Koch studied with painstaking care material taken from patients with pulmonary tuberculosis. After performing a series of rigid tests, as he had done with the anthrax bacillus, he announced the discovery of the microorganism that causes tuberculosis.

The importance of pure cultures to the development of the science of microbiology cannot be overestimated, since by using pure-culture techniques the microorganisms responsible for many infections, certain fermentations, nitrogen fixation in soil, and other activities were isolated and identified. However, strict adherence to pure-culture techniques and Koch's postulates sometimes led investigators up dead-end streets. Early investigators did not know about viruses, nor did they know about the cooperation of two or more microorganisms in causing disease or in bringing about a desirable fermentation such as we find in the ripening of cheese. Today we are as much interested in mixed microbial

populations and the effects they produce as we are in pure cultures. Further advances in marine microbiology, rumen microbiology, microbiology of the intestinal tract, and many other systems will depend upon understanding first the physiology of individual microorganisms in pure culture and then upon the ecological relationships of the total microbial populations in a given environment.

PROTECTION AGAINST INFECTION: IMMUNITY

Pasteur continued to make discoveries concerning the cause and prevention of infectious diseases. About 1880 he isolated the bacterium responsible for chicken cholera and grew it in pure culture. Here again, the practical Pasteur made use of the fundamental techniques devised by the more theoretical Koch. To prove that he really had isolated the organisms responsible for chicken cholera, Pasteur arranged for a public demonstration where he repeated an experiment (Fig. 2-5) that had been successful in many previous trials. He inoculated healthy chickens with his pure cultures, but to his dismay, the chickens failed to get sick and die! Reviewing each step of the experiment, Pasteur found that he had accidentally used cultures several weeks old instead of the fresh ones grown especially for the demonstration. Some weeks later he repeated the experiment, using two groups of chickens. One of these groups had been inoculated at the first demonstration with the old cultures that had proved ineffective, and the second had not been previously exposed. Both groups received bacteria from fresh young cultures. This time the chickens in the second group got sick and died, but those in the first group remained hale and hearty. This puzzled Pasteur, but he soon found the explanation. In some way bacteria could lose their ability to produce disease, i.e., their virulence, after standing and growing old. But these attenuated (having decreased virulence) bacteria still retained their capacity for stimulating the host to produce substances, i.e. antibodies, that protect against subsequent exposure to virulent organisms.

This demonstration explained the principle involved in Edward Jenner's successful use of cowpox virus, in 1798, to immunize people against smallpox (Fig. 2-6). Pasteur next applied this principle to the prevention of anthrax, and again

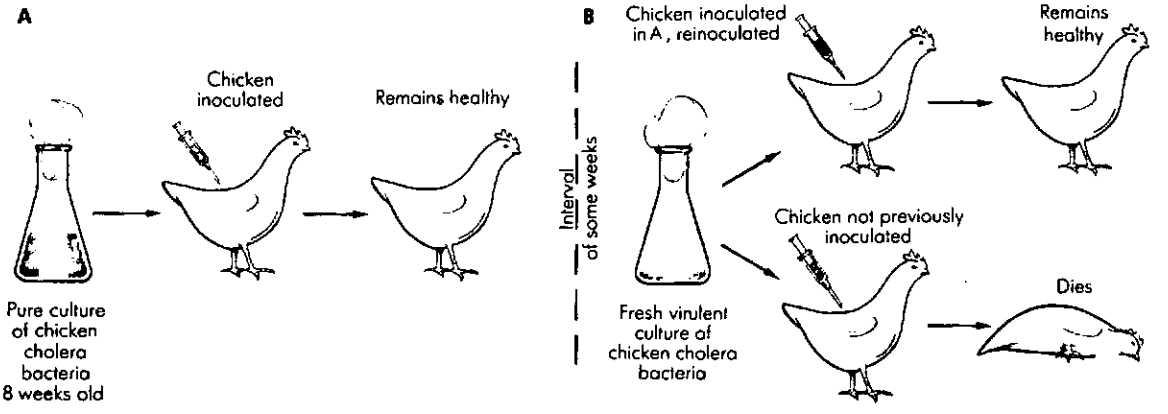


Figure 2-5. The principle of immunization was demonstrated by Pasteur when he inoculated chickens with cultures of chicken cholera bacteria several weeks old and the chickens remained healthy. They did not become sick when inoculated with a fresh culture several weeks later although this fresh culture killed chickens that had not received the attenuated (old) culture.

Figure 2-6. Edward Jenner vaccinating (inoculating) James Phipps with cowpox material, which resulted in development of resistance to smallpox infection. *(Courtesy of Culver Pictures, New York.)*

it worked. He called the attenuated cultures vaccines, a term derived from the Latin *vacca*, meaning "cow." Pasteur was honoring Jenner when he applied the term *vaccination* to immunization with attenuated cultures of bacteria that had no connection with cows.

Pasteur's fame was by now well established throughout France, and the belief became prevalent that he could work miracles with bacteria and the control of infections. It was not surprising, then, that he was given an even greater challenge: he was asked to work on a disease affecting human beings. As he was a chemist and not a physician, studying a human disease might prove risky. But Pasteur again accepted the challenge to be of service to humanity and set out to make a vaccine for hydrophobia, or rabies, a disease transmitted to people by bites of dogs, cats, and other animals. Because it was invariably fatal, when a boy named Joseph Meister was bitten by a mad wolf, his family did not hesitate to take the one chance in thousands that Pasteur could make a vaccine that would save him.

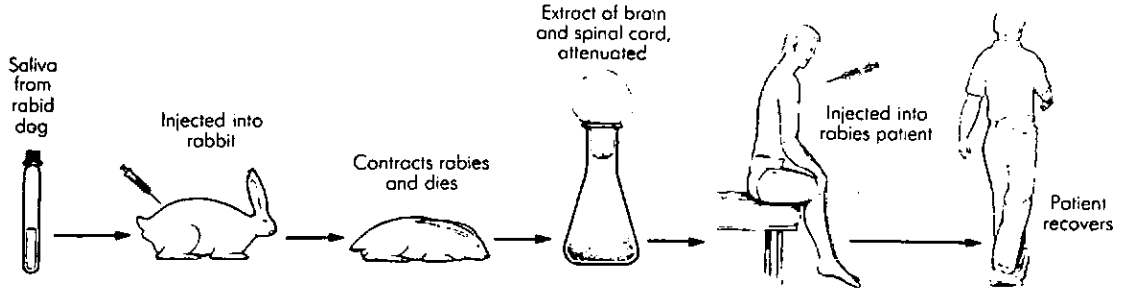


Figure 2-7. Rabies vaccine is made by inoculating a rabbit with saliva from a rabid dog. Virus in the extract of the rabbit's spinal cord is attenuated before injection into a patient.

It had been established that the rabies virus was too minute to be seen even with a microscope; it had never been grown in laboratory culture, and it was not a bacterium. The disease could be produced in rabbits by inoculating them with saliva from mad dogs. Then the brain and spinal cord could be removed from the infected rabbits, dried for several days, pulverized, and mixed with glycerin. Injecting this mixture into dogs protected them against rabies (Fig. 2-7). But vaccinating dogs was quite different from treating a sick boy. Perhaps the worried Pasteur was as surprised as anyone else when after the crucial trial, which took several weeks, Joseph Meister did not die. As with Jenner's vaccination for smallpox, the principles of the preventive treatment of rabies have not changed.

WIDENING HORIZONS

Medical Microbiology

The success of Pasteur and Koch brought honors and accolades from their appreciative countrymen. Koch became Professor of Hygiene and Director of the Institute for Infective Diseases, which was founded for him at the University of Berlin. France showed its gratitude by establishing the Pasteur Institute in Paris in 1888. To each of these men came scholars from all over the world, and these students later carried the spirit and knowledge of Koch and Pasteur to America and throughout Europe.

New bacteria were being discovered with increasing frequency, and their disease-producing capacities were proved by Koch's postulates. After Edwin Klebs in 1883 and Frederick Loeffler in 1884 discovered the diphtheria bacillus and demonstrated that it produced its poisons in a laboratory flask, Emil von Behring and Shibasaburo Kitasato devised a method of producing immunity to infections caused by these organisms by injecting their toxins (poisons) into animals so that an antitoxin (a substance that neutralizes toxin) would develop. Similarly, Kitasato cultivated *Clostridium tetani*, the cause of tetanus (lockjaw), and with von Behring made antitoxin for the prevention and treatment of this disease. For his work on serum therapy, von Behring was awarded the Nobel prize in physiology or medicine in 1901. During this time, D. E. Salmon and Theobald Smith demonstrated that immunity to many infections could be produced by inoculation with killed cultures of microorganisms.

Working in Pasteur's laboratory, Elie Metchnikoff (Fig. 2-8A), a Russian, described how certain leukocytes (white blood cells) could ingest (eat) disease-producing bacteria in the body. He called these special defenders against infection phagocytes ("eating cells") and the process phagocytosis (Fig. 2-8B). Metchnikoff formulated the theory that the phagocytes were the body's first and most important line of defense against infection.

In Germany, one of Koch's students had a different concept of how the body destroys bacteria. Paul Ehrlich explained immunity on the basis of certain soluble substances in the blood. Today we know that there is much merit in both arguments and that both mechanisms play their parts. Ehrlich made another important discovery that opened the door to future developments in chemotherapy and antibiotics: he found that the 606th compound tested, an organic chemical compound containing arsenic, would destroy the syphilis microbe in the body. This was the first chemotherapeutic substance scientifically discovered and evaluated. The period from 1880 to1900 was indeed a golden time for

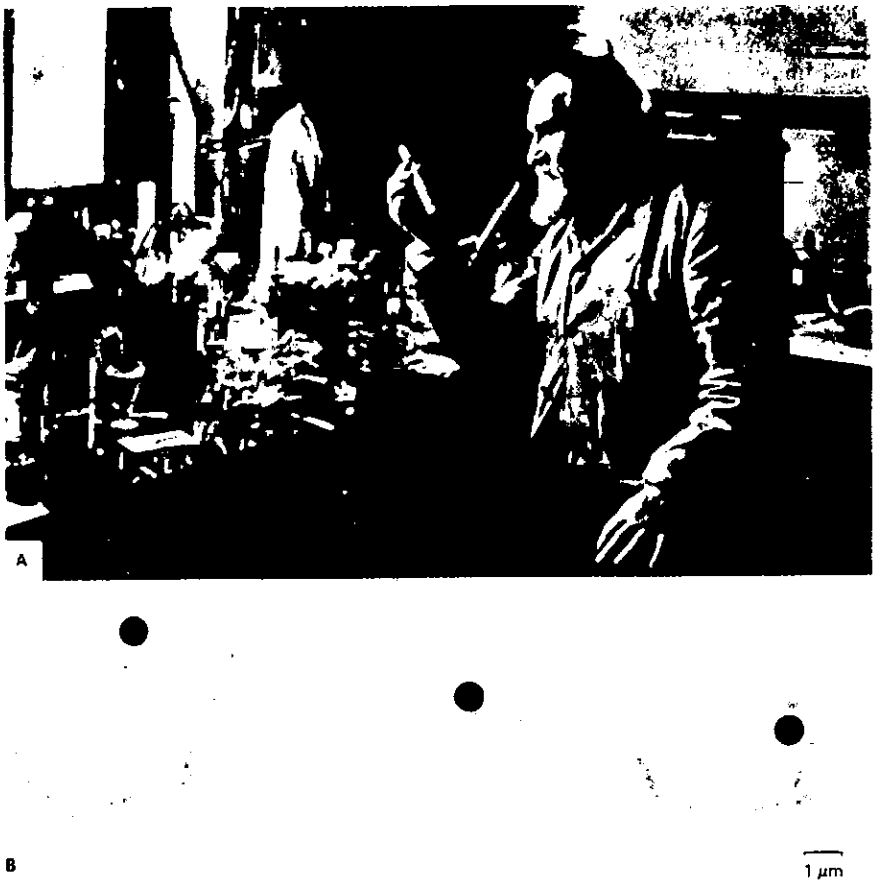


Figure 2-8. (A) Elie Metchnikoff was the first person to recognize the role of phagocytes in combating bacterial infections. *(Courtesy of René Dubos.)* (B) The process of phagocytosis, the ingestion of particulate matter by certain cells, shown in three steps. Phagocytosis is a natural defense mechanism against disease. *(Erwin F. Lessel, illustrator.)*

microbiology because the infant science grew to adolescence during those years. As shown in Table 2-1, most of the causative agents of bacterial diseases that had plagued the world for centuries were isolated and identified.

The students of Koch and Pasteur continued to discover the causative agents of diseases and new methods for diagnosis; e.g., the Widal test for typhoid fever and the Wassermann test for syphilis made diagnosis of these diseases accurate and quick.

In the 1860s, while all these things were happening on the continent of Europe, an English surgeon, Joseph Lister, was trying to combat the microbes that caused postoperative and wound infections. Deaths from these infections were frequent in the nineteenth century. Disinfectants as such were unknown, but since carbolic acid would kill bacteria, Lister used a dilute solution of this acid to soak surgical dressings. Wounds protected in this way did not become infected, and healing took place rapidly. So remarkable was Lister's success that the technique was quickly accepted, and this antiseptic surgical practice established the principles of present-day aseptic ("without infection") techniques.

Table 2-1. Discovery of Causative Agents of Microbial Diseases Following Establishment of Germ Theory of Disease

Date	Disease of Infection	Causative Agent*	Discoverer†
1876	Anthrax	*Bacillus anthracis*	Koch
1879	Gonorrhea	*Neisseria gonorrhoeae*	Neisser
1880	Typhoid fever	*Salmonella typhi*	Eberth
1880	Malaria	*Plasmodium* spp.	Laveran (C. Alphonse)
1881	Wound infections	*Staphylococcus aureus*	Ogston
1882	Tuberculosis	*Mycobacterium tuberculosis*	Koch
1882	Glanders	*Pseudomonas mallei*	Loeffler and Schütz
1883	Cholera	*Vibrio cholerae*	Koch
1883	Diphtheria	*Corynebacterium diphtheriae*	Klebs
1884	Diphtheria	*Corynebacterium diphtheriae*	Loeffler
1885	Swine erysipelas	*Erysipelothrix rhusiopathiae*	Loeffler
1885	Tetanus	*Clostridium tetani*	Nicolaier
1886	Bacterial pneumonia	*Streptococcus pneumoniae*	Fraenkel
1887	Meningitis	*Neisseria meningitidis*	Weichselbaum
1887	Malta fever	*Brucella* spp.	Bruce
1888	Equine strangles	*Streptococcus* spp.	Schütz
1889	Chancroid	*Hemophilus ducreyi*	Ducrey
1892	Gas gangrene	*Clostridium perfringens*	Welch and Nuttall
1894	Plague	*Yersinia pestis*	Kitasato and Yersin
1895	Fowl typhoid	*Salmonella gallinarum*	Moore
1896	Botulism (food poisoning)	*Clostridium botulinum*	Van Ermengem
1897	Bang's disease (bovine abortion)	*Brucella abortus*	Bang
1898	Dysentery	*Shigella dysenteriae*	Shiga
1898	Pleuropneumonia of cattle	*Mycoplasma mycoides*	Nocard and Roux
1905	Syphilis	*Treponema pallidum*	Schaudin and Hoffmann
1906	Whooping cough	*Bordetella pertussis*	Bordet and Gengou
1909	Rocky Mountain spotted fever	*Rickettsia rickettsii*	Ricketts
1912	Tularemia	*Francisella tularensis*	McCoy and Chapin

* Present name of causative agent; original name, in many instances, was different.

† In some instances the individual simply observed the causative agent; in other instances the investigator isolated the agent in pure culture.

Microbiologists began to appear in America at the turn of the century. Some had studied under Koch, and others had visited the Pasteur Institute or watched Lister operate in a mist of carbolic acid vapors or a spray of bichloride of mercury. Whether they actually received their training in the "laboratories of the masters," first- and second-generation bacteriologists in Europe and America were greatly influenced by them. These Americans who actually studied under Pasteur and Koch—William Henry Welch at Johns Hopkins, Harold Clarence Ernst at Harvard, F. G. Novy at Michigan, and H. L. Russell at Wisconsin—were, as P. F. Clark, longtime professor at the University of Wisconsin, said, "responsible directly or indirectly for the development of bacteriology in the United States." Their students and their students' students constitute the who's who of those who built the science of microbiology in America.

Agricultural, Industrial, and Food Microbiology

In sketching the history of bacteriology, it is natural to emphasize developments that have strikingly affected the health of people. But discoveries like those of Pasteur and Koch were almost immediately applied to the fields of agriculture and industry. Here we can describe only briefly a few of these applications and the scientists who originated them.

The field of soil microbiology was opened in the late 1800s by the Russian Sergei Winogradsky, who showed the importance of bacteria in taking nitrogen from the atmosphere, combining it with other elements, and making it available as plant food and hence as animal food. In 1888, the mutually beneficial, or symbiotic, relationship between bacteria and the leguminous plants, such as clover and alfalfa, was shown by H. Hellriegel and H. Wilfarth. In 1901, Martinus Willem Beijerinck (1851–1931), a famous Dutch microbiologist, found the free-living nitrogen-fixing bacterium *Azotobacter* and described its usefulness in promoting soil fertility (see Chap. 25).

Emil Christian Hansen (1842–1909), a Dane, opened the way to the study of industrial fermentations. He developed the pure-culture study of yeasts and bacteria used in vinegar manufacture, and pure cultures known as starters were soon used to encourage the study of fermentation processes. For example, L. Adametz (1889), an Austrian, used pure cultures in cheese manufacturing, and H. W. Conn in Connecticut and H. Weigmann in Germany developed pure-culture starters for butter production (1890–1897).

Late in the nineteenth century, T. J. Burrill, working in Illinois, found that in pears a disease known as fire blight was caused by a bacterium. This discovery opened a new area for microbiology, namely plant pathology—the study of plant diseases. Additional discoveries followed. In 1886 A. E. Mayer described a mottling disease of the tobacco plant and transmitted it to healthy plants by transferring sap from an infected plant. About the same time, Erwin F. Smith of the U.S. Department of Agriculture transmitted the disease peach yellows from diseased to healthy plants by the process of budding. With such leads as these to work on, Dmitrii Iwanowski demonstrated the viral nature of the infective agents of these plant diseases. Transmission of virus diseases of plants by insect was suggested by an observant Japanese farmer named Hashimoto in 1894 and independently in 1907 by the American workers A. B. Ball, A. Adams, and J. C. Shaw. Proof that insects could harbor viruses and transmit them from diseased to healthy plants was not provided until 1915, by E. Smith and P. A. Bonquet. The tobacco mosaic virus was isolated in crystalline form in 1935 by Wendell M. Stanley and John H. Northrup. For their valuable contributions to knowledge of the nature of viruses and the crystallization of virus protein, they were awarded the Nobel prize in chemistry in 1946.

From these few examples we can see that the science of microbiology grew up in less than a quarter of a century. Its early years were exciting, and by the beginning of the twentieth century, people in the street and on the farm knew of bacteria and were learning what these organisms could do and how they could be controlled.

The historical events leading to the establishment of microbiology as a science are summarized in Table 2-2.

Table 2-2. A Chronological Arrangement of Events Important in the History of Microbiology

Era	Investigator	Contribution
1500–1600	Girolamo Fracastoro (1483–1553)	Theory that invisible living seeds caused disease
1600–1700	Francesco Redi (1626–1697)	Performed experiments to disprove spontaneous generation
	Antony van Leeuwenhoek (1632–1723)	First to observe and accurately record and report microorganisms
1700–1800	John Needham (1713–1781)	Performed experiments, results supported concept of spontaneous generation
	Lazaro Spallanzani (1729–1799)	Did experiments, results disproved spontaneous generation
	Edward Jenner (1749–1823)	Discovered vaccination for smallpox using cowpox vaccine
1800–1900	Theodor Schwann (1810–1882)	Performed experiments, results disproved spontaneous generation
	Franz Schultze (1815–1873)	Performed experiments, results disproved spontaneous generation
	Justus von Liebig (1803–1873)	Supported concept of physicochemical theory of fermentation
	Jacob Henle (1809–1885)	Established principles for germ theory of disease
	Oliver Wendell Holmes (1809–1894)	Stressed contagiousness of puerperal fever; that agent was carried from one mother to another by doctors
	Ignaz Philipp Semmelweis (1818–1865)	Introduced use of antiseptics
	Louis Pasteur (1822–1895)	Established germ theory of fermentation and germ theory of disease, developed immunization techniques
	Florence Nightingale (1820–1910)	Organized hospitals which minimized cross-infection
	Joseph Lister (1827–1912)	Developed aseptic techniques; isolated bacteria in pure culture
	Thomas J. Burrill (1839–1916)	Discovered bacterial disease of plants
	John Tyndall (1820–1893)	Developed fractional sterilization to kill spores (Tyndallization)
	Fanny Hesse (1850–1934)	Suggested use of agar as a solidifying material for microbiological media
	Robert Koch (1843–1910)	Developed pure culture technique and Koch's postulates; discovered causative agents of anthrax and tuberculosis
	Paul Ehrlich (1854–1915)	Developed modern concept of chemotherapy and chemotherapeutic agents
	Elie Metchnikoff (1845–1916)	Discovered phagocytosis
	Hans Christian Gram (1853–1933)	Developed important procedure for differential staining of bacteria, the Gram stain
	Sergei N. Winogradsky (1856–1953)	Discovered nitrogen-fixing bacteria in soil
	William Henry Welch (1850–1934)	One of first great American microbiologists; discovered relation of clostridia to gas gangrene
	Theobald Smith (1859–1934)	Early American microbiologist; discovered transmission of Texas fever by cattle tick
1900–1910	Walter Reed (1851–1902)	Reported transmission of yellow fever by mosquito
	Jules Bordet (1870–1961) and Octave Gengou (1875–1957)	Discovered complement-fixation reaction

Table 2-2. (*continued*)

Era	Investigator	Contribution
	August von Wassermann (1866–1925)	Introduced complement-fixation test for syphilis
	Martinus Willem Beijerinck (1851–1931)	Utilized principle of enrichment cultures; confirmed finding of first virus
	Frederick W. Twort (1877–1950) Felix H. d'Herelle (1873–1949)	Independently discovered bacteriophages, viruses that destroy bacteria
	Howard T. Ricketts (1871–1910)	Reported Rocky Mountain spotted fever transmitted by wood tick and Mexican typhus transmitted by body louse

MICROBIOLOGY AND MODERN BIOLOGY: MOLECULAR BIOLOGY

As new laboratory techniques and experimental procedures were developed, our knowledge of the characteristics of microorganisms accumulated rapidly. Extensive information about the biochemical activities of microorganisms became available. An analysis of the data suggested that there was much in common among different microorganisms—the differences were likely to be variations on a major central biochemical pathway. At about the same time there was a growing recognition of the unity of the biochemical life processes in microorganisms and higher forms of life, including human beings. Consequently it became attractive to use microorganisms as a tool to explore fundamental life processes. Microorganisms offer numerous advantages for this kind of research: they reproduce (grow) very rapidly, they can be cultured (grown) in small or vast quantities conveniently and rapidly, their growth can be manipulated easily by chemical or physical means, and their cells can be broken apart and the contents separated into fractions of various particle sizes. These characteristics, as well as others, make microorganisms a very convenient research model for determining exactly how various life processes take place in terms of specific chemical reactions and the specific structures involved. Scientists from many disciplines recognized the usefulness of microorganisms as experimental models. Thus it was not surprising that physicists, geneticists, chemists, and biologists joined with microbiologists in what is now known as molecular biology. Salvador E. Luria (Fig. 2-9), professor of biology at the Massachusetts Institute

Figure 2-9. Salvador E. Luria, Professor of Biology at the Massachusetts Institute of Technology, was awarded the Nobel prize in 1969 for his research in molecular biology.

of Technology and one of the major contributors to this field, defines molecular biology as follows:

> It is the program of interpreting the specific structures and functions of organisms in terms of molecular structure.

The results and rewards from this field of research have been spectacular. The contributions include elucidation of enzyme structure and mode of action, cellular regulatory mechanisms, energy metabolism, protein synthesis, structure of viruses, function of membranes, and the structure and function of nucleic acids (including DNA). Most of the basic knowledge about DNA and genetic processes at the molecular level has been obtained through research with bacteria and bacteriophages (viruses that infect bacteria). The significance of these discoveries in molecular biology to all of biology is underscored by the fact that numerous Nobel prizes have been awarded to researchers for their work in this field.

MICROBIOLOGY AND SOCIETY

From the foregoing account it is clear that microbiology has become increasingly important to our society, and microbiology has emerged as one of the most important branches of the life sciences. Microbiologists have made significant contributions to basic biological sciences as well as in the applied areas of public health and medical sciences, agriculture, industry, and environmental sciences.

The most dramatic current development in applied microbiology is the ability to alter an organism's genetic makeup, commonly referred to as genetic engineering. The detailed knowledge that has been obtained about the structure and function of DNA, together with the discovery of enzymes that "cut, unzip, or rebuild" the molecule, has made it possible to alter the DNA structure of microorganisms. New pieces of DNA can be inserted into a DNA molecule in a process called recombination. Thus a microorganism can be engineered, through modification of its DNA, to produce new substances, such as human proteins. Bacteria have been genetically modified to produce human insulin and interferon, for example. Genetically engineered microorganisms hold great potential for the production of drugs and vaccines, for improvement of agricultural crops, and for other products and processes.

QUESTIONS

1. Why was the belief in spontaneous generation an obstacle to the development of the science of microbiology? What is the relationship between the germ theory of fermentation and the germ theory of disease?
2. What is meant by the pure-culture concept? Does it exist in natural environments? Explain.
3. How did Leeuwenhoek's work influence the contributions of Pasteur and Koch?
4. In what way are the contributions of Ehrlich and Fleming related?
5. In what way did Koch's postulates influence the development of microbiology?

6 Why is the period 1880–1900 significant for the emergence of microbiology as a science?
7 Why was the introduction of the use of agar important to microbiology?
8 For what contribution to microbiology is each of the following remembered: (*a*) H. W. Conn, (*b*) Erwin F. Smith, (*c*) Emil Christian Hansen, and (*d*) T. J. Burrill?
9 Name an important contribution to microbiology made by each of the following: (*a*) Jenner, (*b*) Metchnikoff, (*c*) Lister, and (*d*) Welch.
10 Describe the process of phagocytosis.
11 How did Ehrlich's concept of disease differ from the phenomenon of phagocytosis?
12 Many researchers in the biological sciences use microorganisms as a model system to explore life processes. Explain why this is so.
13 For what contributions are the following microbiologists remembered: (*a*) Winogradsky, (*b*) Hellriegel and Wilfarth, and (*c*) Beijerinck?
14 List some of the applied fields of microbiology and make a general statement of the importance of microorganisms in each field.
15 What, in your judgment, were the five most important major discoveries between 1800 and 1900 which contributed to the establishment of microbiology as a science?
16 Prior to the 1930s universities and colleges had departments of bacteriology. Since that time there has been a shift toward a change in name to departments of microbiology. What is the explanation for this change?

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Chapter 3 The Characterization, Classification, and Identification of Microorganisms

OUTLINE

Characterization, classification, and identification are major objectives in all branches of the biological sciences.

Classification is a means of bringing order to the bewildering variety of organisms in nature. Once we learn the characteristics of an organism we can compare it with other organisms to discover similarities and differences. The human mind tends to arrange similar things together in groups and to distinguish these groups from one another.

In order to identify and classify microorganisms, we must first learn their **characteristics.** It is usually not feasible to study the characteristics of a single microorganism, because of its small size; therefore, we study the characteristics of a **culture**—a population of microorganisms. If we study the characteristics of a culture containing many microorganisms (usually millions or billions of cells of only one kind), it is as if we are studying the characteristics of a single organism.

As stated earlier, a culture that consists of a single kind of microorganism (one living species), regardless of the number of individuals, in an environment free of other living organisms is called a **pure culture.** (Strictly speaking, this is an **axenic culture**; however, microbiologists customarily refer to such a culture as a pure culture. In the strict, technical sense a pure culture is one grown from a single cell.)

Determining the characteristics of microorganisms is not only prerequisite for classification but is done for other reasons as well. As we have already pointed out, microorganisms play many important, indeed essential, roles in nature. It is therefore desirable to determine the characteristics of species that enable these activities to occur.

MAJOR CHARACTERISTICS OF MICROORGANISMS

The major characteristics of microorganisms fall into the following categories:

1 ***Morphological characteristics.*** Cell shape, size, and structure; cell arrangement; occurrence of special structures and developmental forms; staining reactions; and motility and flagellar arrangement
2 ***Chemical composition.*** The various chemical constituents of the cells
3 ***Cultural characteristics.*** Nutritional requirements and physical conditions required for growth, and the manner in which growth occurs
4 ***Metabolic characteristics.*** The way in which cells obtain and use their energy, carry out chemical reactions, and regulate these reactions
5 ***Antigenic characteristics.*** Special large chemical components (antigens) of the cell, distinctive for certain kinds of microorganisms
6 ***Genetic characteristics.*** Characteristics of the hereditary material of the cell (deoxyribonucleic acid, or DNA); and occurrence and function of other kinds of DNA that may be present, such as plasmids
7 ***Pathogenicity.*** The ability to cause disease in various plants or animals or even other microorganisms
8 ***Ecological characteristics.*** Habitat and the distribution of the organism in nature and the interactions between and among species in natural environments

Morphological Characteristics

Unlike other kinds of microbial characteristics, determination of morphological features usually requires studying individual cells of a pure culture. Microorganisms are very small and their size is usually expressed in micrometers (μm). One μm is equivalent to 0.001 millimeter (mm) or about 0.00004 in; consequently, routine examination of microbial cells requires the use of a high-power microscope, usually at a magnification of about 1,000 diameters.

The use of electron microscopy provides magnification of thousands of diameters and makes it possible to see fine details of cell structure. Numerous techniques are available for the microscopic examination of microorganisms. The technique selected depends upon the information which is being sought. Some of the techniques are described in Chap. 4.

Chemical Characteristics

Microbial cells consist of a wide variety of organic compounds. When cells are broken apart and their components subjected to chemical analysis, each kind of microorganism is found to have a characteristic chemical composition. Both qualitative and quantitative differences in composition occur among various species. For example, the occurrence of lipopolysaccharide in cell walls is characteristic of Gram-negative bacteria but not Gram-positive bacteria; on the other hand, many Gram-positive bacteria have cell walls that contain teichoic acids, compounds not found in Gram-negative bacteria. Fungal and algal cell walls are very different in composition from those of bacteria. A major distinction among viruses is made on the basis of the kind of nucleic acid they possess, namely ribonucleic acid (RNA) or deoxyribonucleic acid (DNA).

Cultural Characteristics

Each kind of microorganism has specific growth requirements. Many microorganisms can be grown in or on a culture medium (a mixture of nutrients used in the laboratory to support growth and multiplication of microorganisms). Some microorganisms can grow in a medium containing only inorganic compounds, whereas others require a medium containing organic compounds (amino acids, sugars, purines or pyrimidines, vitamins, or coenzymes). Some

require complex natural substances (peptone, yeast autolysate, blood cells, or blood serum), and some cannot as yet be grown in an artificial laboratory medium and can be propagated only in a living host or living cells. For example, rickettsias require a host in which to grow, such as an animal, a fertilized chicken egg (chick embryo), an arthropod, or a culture of mammalian tissue cells. The host serves as a very complex "medium" for such nutritionally demanding microorganisms.

In addition to specific nutrients, each kind of organism also requires specific physical conditions for growth. For example, some bacteria grow best at high temperatures and cannot grow below 40°C; others grow best in the cold and cannot grow above 20°C; still others, such as bacteria pathogenic to humans, require a temperature close to that of the human body (i.e., 37°C). The gaseous atmosphere required for growth is also important; for instance, some bacteria require oxygen for growth; oxygen is lethal to others and they can grow only in its absence. Light may be another important physical condition: certain bacteria, such as cyanobacteria, require light as a source of energy, whereas others may be indifferent to light or may even find it deleterious to their growth.

Each kind of microorganism grows in a characteristic manner. For example, growth in a liquid medium may be abundant or sparse; it may be evenly dispersed throughout the medium, or it may occur only as a sediment at the bottom or only as a thin film or **pellicle** at the top. On solid media, microbes grow as **colonies**—distinct, compact masses of cells that are macroscopically visible. Colonies are characterized by their size, shape, texture, consistency, color, and other notable features.

Metabolic Characteristics

The life processes of the microbial cell are a complex integrated series of chemical reactions collectively referred to as **metabolism**. The variety of these reactions affords many opportunities to characterize and differentiate various groups of microorganisms. For instance, some organisms may obtain energy by absorbing light, others by oxidizing various organic or inorganic compounds, and others by redistributing the atoms within certain molecules so that the molecules become less stable. Organisms also differ in the ways in which they synthesize their cell components during growth. The various chemical reactions of an organism are catalyzed by proteins called **enzymes**, and the complement of enzymes possessed by one kind of organism, as well as the ways in which those enzymes are regulated, can differ significantly from that of other organisms.

Antigenic Characteristics

Certain chemical compounds of microbial cells are called **antigens**. Antigenic characterization of a microorganism has great practical importance. If microbial cells enter the animal body, the animal responds to their antigens by forming specific blood serum proteins called **antibodies**, which bind to the antigens. Antibodies are highly specific for the antigens that induce their formation. Because different kinds of microorganisms have different types of antigens, antibodies are widely used as tools for the rapid identification of particular kinds of microorganisms.

The numerous applications of antigen-antibody reactions will be discussed in detail in Chap. 34. For the present this might be explained as a "lock and key system." Because of the highly specific nature of the reaction, if we know

the identity of one part of the system (antigen or antibody) we can identify the other. For example, if we take typhoid bacterium antibody and mix it with a suspension of unknown bacterial cells, and a positive reaction occurs, we can conclude that the cells are those of the typhoid organism. If no reaction occurs, then these bacterial cells are of some species other than the typhoid bacterium.

Genetic Characteristics

The double-stranded chromosomal DNA of each kind of microorganism has certain features that are constant and characteristic for that organism and useful for its classification:

1 ***DNA base composition.*** It is important to note that the DNA molecule is made up of base pairs: guanine-cytosine and adenine-thymine. Of the total number of nucleotide bases present in the DNA, that percentage represented by guanine plus cytosine is termed the moles % G + C value (or more briefly, mol% G + C). Values for various organisms range from 23 to 75. Some examples are listed in Table 3-1.
2 ***The sequence of nucleotide bases in the DNA.*** This sequence is unique for each kind of organism and is the most fundamental of all the characteristics of an organism; consequently, it has great significance for microbial classification.

In addition to chromosomal DNA, plasmid DNA may sometimes be present in microbial cells. Plasmids are circular DNA molecules that are capable of autonomous replication within bacterial cells, and their presence can confer special characteristics on the cells that contain them, such as the ability to make toxins(toxigenicity), to become resistant to various antibiotics, or to use unusual chemical compounds as nutrients.

Pathogenicity

The ability to cause disease, or pathogenicity, of some microorganisms is certainly a dramatic characteristic and it stimulated much of the early work with microorganisms. Although we now know that relatively few species of microorganisms cause disease, certain microorganisms are pathogenic for animals or plants, and some microorganisms may cause disease in other microorganisms.

Table 3-1. Some Examples of the DNA Base Composition of Bacteria

Species	Moles % G + C Content of DNA
Azospirillum brasilense	70–71
A. lipoferum	69–70
Campylobacter fetus	32–35
C. jejuni	31
Klebsiella pneumoniae	56–58
K. terrigena	57
Neisseria gonorrhoeae	50–53
N. elongata	53–54
Pseudomonas aeruginosa	67
P. cichorii	59
Wolinella recta	42–46
W. succinogenes	46–49

SOURCE: Krieg, N. R. (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 1, Williams & Wilkins, Baltimore, 1984.

For example, bacteria known as bdellovibrios are predatory on other bacteria, and viruses called bacteriophages can infect and destroy bacterial cells.

Ecological Characteristics

The habitat of a microorganism is important in characterizing that organism. For example, microorganisms normally found in marine environments generally differ from those in freshwater environments. The microbial population of the oral cavity differs from that of the intestinal tract. Some kinds of microorganisms are widely distributed in nature, but others may be restricted to a particular environment. The relation of an organism to its environment is often complex and may involve special characteristics of the organism that are not yet known.

MICROBIAL CLASSIFICATION, NOMENCLATURE, AND IDENTIFICATION

Once the characteristics of microorganisms have been determined and appropriately catalogued, the process of classification can begin.

Classification

In microbiology, taxa are initially constructed from strains. A strain is made up of all the descendents of a pure culture; it is usually a succession of cultures derived from an initial colony. Each strain has a specific history and designation.

Taxonomic Groups (Taxa)

For example, strain ATCC 19554 is a strain of spirilla isolated originally from pond water in Blacksburg, Virginia in 1965 by Wells and Krieg, and cultures of this strain are maintained at the American Type Culture Collection (ATCC), Rockville, Maryland. Cultures of the same species that were isolated from other sources would be considered different strains.

The basic taxonomic group (taxon) is the species, i.e., a collection of strains having similar characteristics. Bacterial species consist of a special strain called the type strain together with all other strains that are considered sufficiently similar to the type strain as to warrant inclusion in the species. The type strain is the strain that is designated to be the permanent reference specimen for the species. Unfortunately, it is not always the strain that is most typical of all the strains included in the species, but it is the strain to which all other strains must be compared to see if they resemble it closely enough to belong to the species. Therefore, type strains are particularly important and special attention is given to their maintenance and preservation, particularly by national reference collections such as the ATCC in the United States or the National Collection of Type Cultures in England. Many other culture collections are maintained throughout the world.

In the definition just given for a bacterial species, the phrase "considered sufficiently similar to the type strain" indicates that the definition contains an element of subjectivity. In other words, the criteria which one taxonomist believes to constitute "sufficient similarity" may be quite different from those used by another taxonomist. At present there are no specific criteria for a bacterial species that are universally accepted. However, certain criteria based on DNA homology experiments (described later in this chapter) are probably more widely accepted today than any others and eventually may lead to a unifying concept for defining a species.

Just as a bacterial species is composed of a collection of similar strains, a

bacterial genus is composed of a collection of similar species. One of the species is designated the type species, and this serves as the permanent example of the genus; that is, other species must be judged to be sufficiently similar to the type species to be included with it in the genus. Unfortunately, there is even less agreement about the criteria for a bacterial genus than there is for a bacterial species.

Taxonomic groups of higher rank than genus are listed below, and the same considerations about subjectivity apply here as well:

Family	A group of similar genera
Order	A group of similar families
Class	A group of similar orders
Division	A group of similar classes
Kingdom	A group of similar divisions

The Goals of Classification

Taxonomists strive to make classifications that have the following two qualities:

1 ***Stability.*** Classifications that are subject to frequent, radical changes lead to confusion. Every attempt should be made to devise classifications that need only minor changes as new information becomes available.
2 ***Predictability.*** By knowing the characteristics of one member of a taxonomic group, it should be possible to assume that the other members of the same group probably have similar characteristics. If this cannot be done, the classification has little value.

General Methods of Classifying Bacteria

Three methods are used for arranging bacteria into taxa:

The Intuitive Method. A microbiologist who is thoroughly familiar with the properties of the organisms he or she has been studying for several years may decide that the organisms represent one or more species or genera. The trouble with this method is that the characteristics of an organism that seem important to one person may not be so important to another, and different taxonomists may arrive at very different groupings. However, some classification schemes based on the intuitive method have proved to be quite useful.

Numerical Taxonomy. In an effort to be more objective about grouping bacteria, a scientist may determine many characteristics (usually 100 to 200) for each strain studied, giving each characteristic equal weight. Then using a computer he or she calculates the % similarity (%S) of each strain to every other strain. For any two strains, this is:

$$\%S = \frac{NS}{NS + ND}$$

where NS is the number of characteristics that are the same (positive or negative) for the two strains, and ND is the number of characteristics that are different. (The method is sometimes made more rigorous by making NS equal to the number of *positive* characteristics that are the same for the two strains, since what organisms can do may be more important than what they cannot do.) Those strains having a high %S to each other are placed into groups; those

groups having a high %S to each other are in turn placed into larger groups, and so on (see Fig. 3-1). The degree of similarity needed to rank a group as a species, genus, or other taxon is a matter of judgment on the part of the taxonomist. This method of classification has great practical usefulness as well as being relatively unbiased in its approach; it also yields classifications that have a high degree of stability and predictability.

Genetic Relatedness. The third and most reliable method of classification is

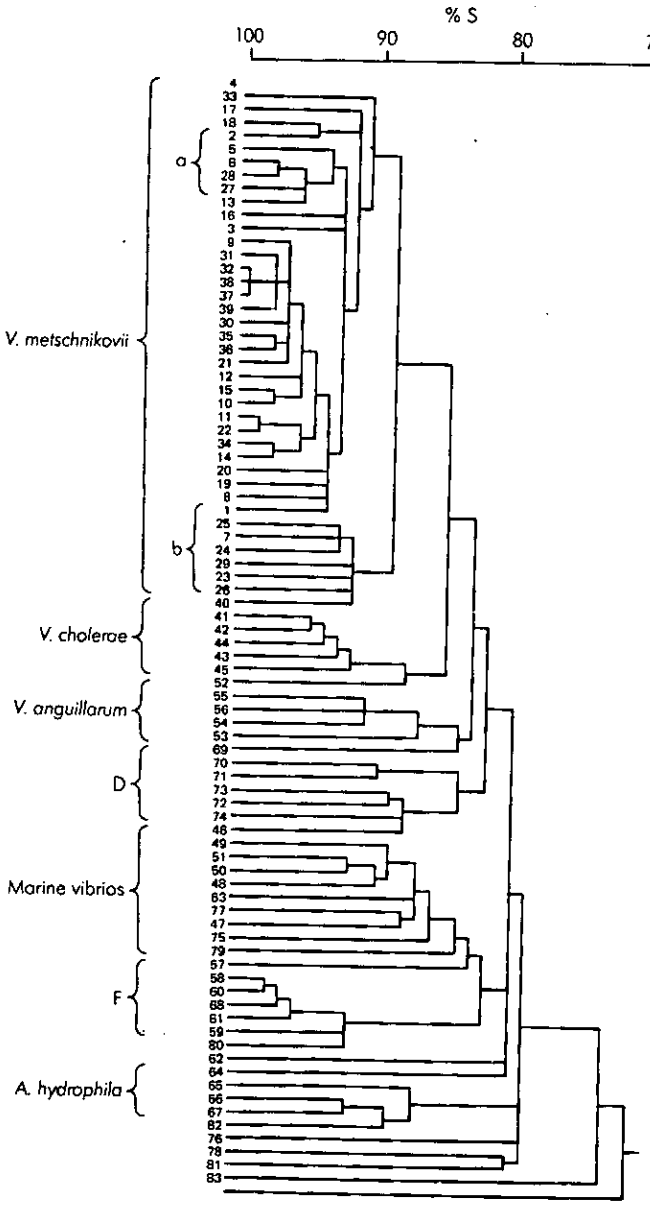

Figure 3-1. Diagram showing the arrangement of 83 strains of oxidase-positive, vibriolike bacteria according to a numerical taxonomic study. Some of the resulting groups represent species (e.g., *Vibrio metschnikovii, V. cholerae*, etc.); others are designated only by vernacular names (Marine vibrios, Group D, etc.). *Courtesy of J. V. Lee, T. J. Donovan, and A. L. Furniss, Int J Syst Bacteriol* **28**:99–111, 1978.)

based on the degree of genetic relatedness between organisms. This method is the most objective of all and is based on the most fundamental aspect of organisms, their hereditary material (DNA). In the 1960s the development of that branch of science known as molecular biology provided techniques by which the DNA of one organism could be compared with that of other organisms. At first only crude comparisons could be made, based on mol% G + C values. It is true that two organisms of the same or similar species that are very closely related will have very similar mol% G + C values, and it is also true that two organisms having quite different mol% G + C values are not very closely related. *However, it is important to realize that organisms that are completely unrelated may have similar mol% G + C values.* Therefore, much more precise methods of comparison were needed—namely, methods by which the DNA molecules from various organisms could be compared with respect to the sequence of their component nucleotides. This sequence is the most fundamental characteristic of an organism. Modern techniques have now made it possible to make such a comparison. The basic principles can be described briefly as follows:

1 ***DNA homology experiments.*** The double-stranded DNA molecules from two organisms are heated to convert them to single strands. The single strands from one organism are then mixed with those from the other organism and allowed to cool. If the two organisms are closely related, heteroduplexes will form. In other words, a strand from one organism will pair with a strand from the other organism (see Fig. 3-2). If the two organisms are not closely related, no heteroduplexes will form. This method is most useful at the species level of classification.

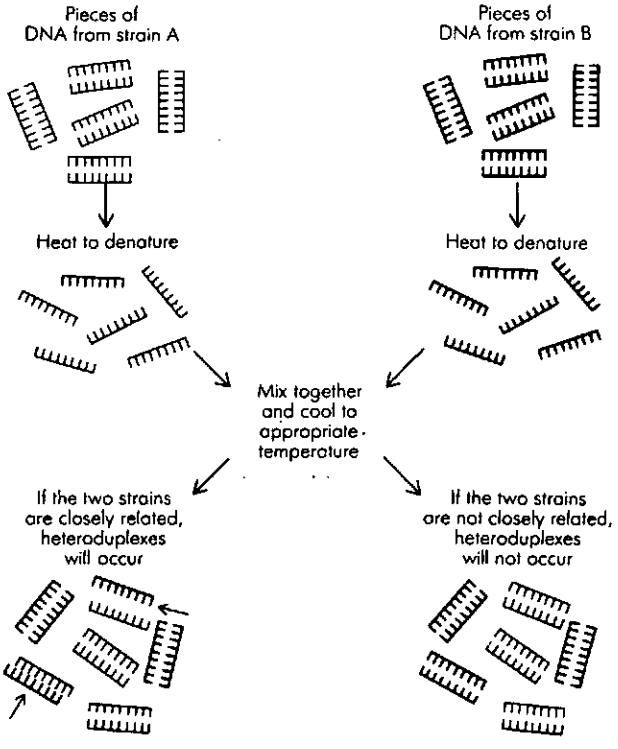

Figure 3-2. Schematic diagram illustrating the basic principle behind DNA homology experiments.

2 ***Ribosomal RNA homology experiments and ribosomal RNA oligonucleotide cataloging.*** Two organisms may not be so closely related as to give a high level of DNA homology, yet they may still have some degree of relatedness. Ribosomes, the small granular-appearing structures within the cell which manufacture proteins, are composed of proteins and RNA. The ribosomal RNA (rRNA) is coded for by only a small fraction of the DNA molecule, the rRNA cistrons. In all bacteria so far studied, the nucleotide sequence of these rRNA genes has been found to be highly conserved; that is, during evolution, the nucleotide sequence has changed more slowly than that of the bulk of the DNA molecule. This means that even if two organisms are only distantly related and show no significant DNA homology, there still may be considerable similarity in the nucleotide sequences of their rRNA cistrons. The degree of similarity that exists can therefore be used as a measure of relatedness between organisms, but at a level beyond that of species (at the level of genus, family, order, etc.). RNA homology experiments and RNA oligonucleotide cataloging are two modern methods used to determine the degree of similarity between the rRNA cistrons of different organisms. The techniques are complex and are being used by only a few laboratories.

Classifications based on genetic relatedness come the closest to achieving the taxonomic goals of stability and predictability. Moreover, the data obtained for such classifications allow microbiologists to infer the way in which bacteria have evolved, so that the present-day bacterial genera and species can be arranged in a hierarchy that reflects their ancestral relationships, i.e., in a phylogenetic classification. Much of the work is still fragmentary, but some of the results, especially those obtained by Dr. C. R. Woese of the University of Illinois and his colleagues, have already revolutionized current thinking about how bacteria have evolved and how they are related to one another. In fact, it is now apparent that present-day bacteria evolved by at least two very different major routes from an early ancestral form and that they now comprise two very large groups: the eubacteria (which are the traditional, familiar ones that have received the most study) and the archaeobacteria (consisting of methane-producers, extreme halophiles, and thermoacidophiles). It has been proposed that these two groups be considered as two separate kingdoms of life, and, indeed, they do seem as distantly related to each other as they are to eucaryotic organisms. Although the kingdom question is still debatable, data obtained from rRNA oligonucleotide cataloging nevertheless make it clear that the archaeobacteria are separated from other bacteria by a great phylogenetic gulf (see Fig. 3-3).

Nomenclature

Each species of microorganism has only one officially accepted name, by international agreement. This system provides for precise communication. If an organism were to be called *Escherichia coli* in one country and *Coprobacterium intestinale* in another, chaos would result. It would be difficult to know that the same organism was being studied.

The name of a species is merely a convenient label. It is not necessarily even descriptive, although some names are. For example, *Micrococcus luteus* means "yellow berry" in Latin, and *Proteus vulgaris* is Latin for "common organism of many shapes." Some species are named after persons: for example, *Escherichia coli*—the organism of the colon, named after Theodor Escherich (a German bacteriologist); or *Clostridium barkeri*—the spindle-shaped organism,

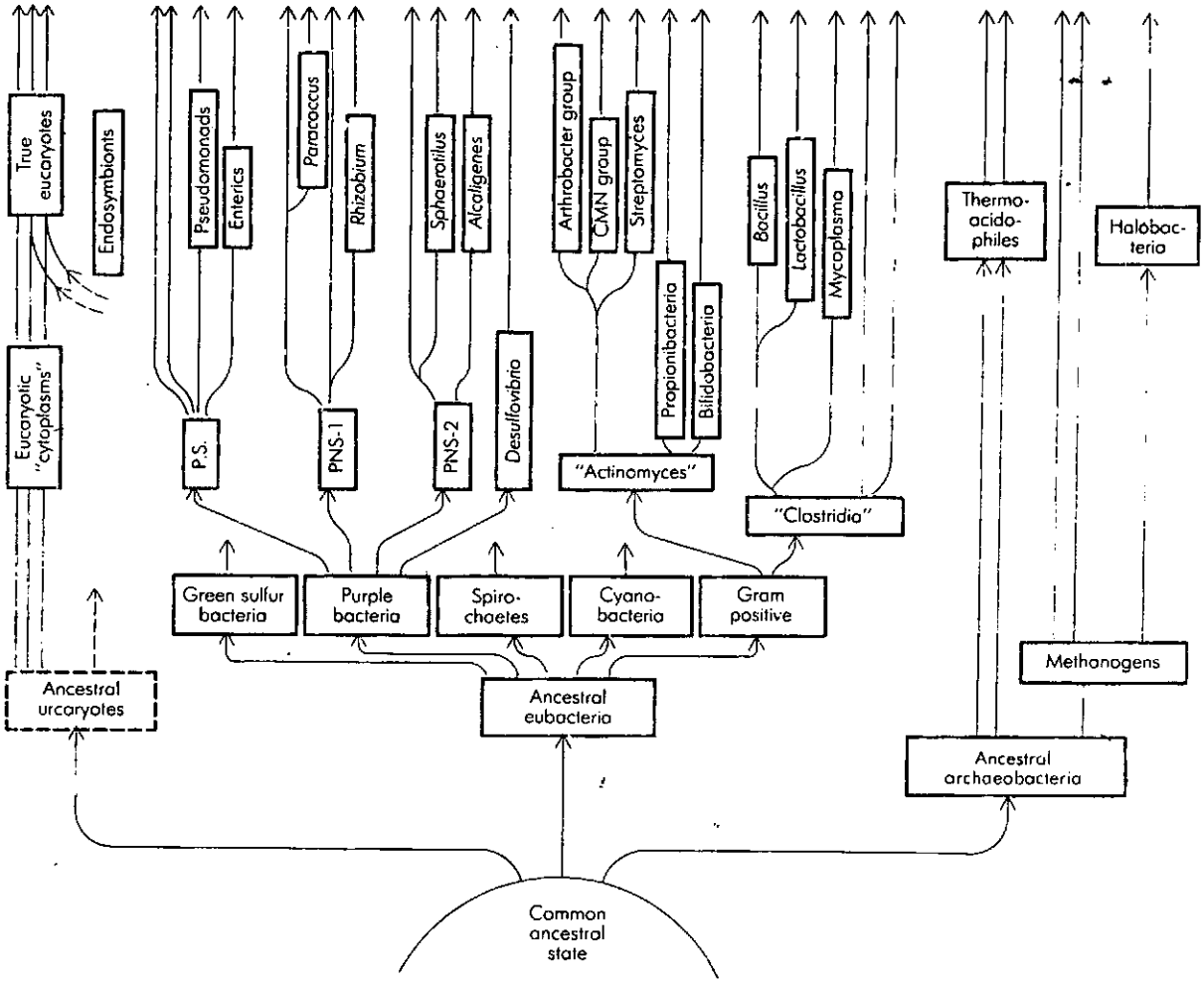

Figure 3-3. Schematic representation of the major lines of procaryotic descent, based on rRNA oligonucleotide cataloging. The archaeobacteria arose from an early ancestral form according to the pathway at the center. (A third line of descent which led to eucaryotic organisms is depicted at the left.) *(Courtesy of G. E. Fox et al., Science* **209**:*457–463, 1980.)*

named after H. A. Barker (an American biochemist). Some names are even nonsensical [e.g., *Runella slithyformis*—"the organism whose shape resembles runes (characters of an ancient alphabet) and which is slithy," the latter term being taken from Lewis Carroll's poem "Jabberwocky" from *Alice in Wonderland*]. The important point is that names are only convenient designations. For example, instead of referring to "the rod-shaped, acid-fast bacterium that is slow-growing, is stimulated by glycerol, causes pulmonary tuberculosis in humans, is spread mainly by airborne droplets, forms buff-colored colonies, synthesizes niacin, reduces nitrate to nitrite, and is pathogenic for guinea pigs," it is much more convenient simply to say "*Mycobacterium tuberculosis*."

Although it might seem that microbial names could be constructed almost at random, the fact is that certain rules must be followed. Bacteria, for example, are named according to rules set down in the *International Code of Nomenclature of Bacteria;* other codes govern the naming of algae, fungi, and viruses.

One rule in bacteriological nomenclature is that a name must be written as a Latin or latinized binomial (two words) and must follow certain rules of Latin grammar. The first word in the binomial is the genus name and is always capitalized. The second word is the specific epithet and is never capitalized. Both the genus name and specific epithet are given in italics (or underlined, which means "italics" to a printer). Bacteria are sometimes referred to by common or colloquial names, which have no official standing in nomenclature and are never italicized (for example, the "colon bacillus," which is *E. coli*, or the "tubercle bacillus," which is *M. tuberculosis*). Such names do not lead to precise communication; for instance, many bacteria occur in the colon besides *E. coli*, and other organisms besides *M. tuberculosis* can cause tuberculosis.

Those bacterial names which have official standing in microbiology were published in the *Approved Lists of Bacterial Names* in January, 1980. Any new or additional names must be published in the *International Journal of Systematic Bacteriology* in order to achieve official recognition.

The International Code of Nomenclature of Bacteria was developed with reference to the much earlier established International Codes of Zoological and Botanical Nomenclature. All of these codes incorporate certain common principles as listed below.

1 Each distinct kind of organism is designated as a species.
2 The species is designated by a Latin binomial to provide a characteristic international label (binomial system of nomenclature).
3 Regulation is established for the application of names.
4 A law of priority ensures the use of the oldest available legitimate name.
5 Designation of categories is required for classification of organisms.
6 Requirements are given for effective publication of new specific names, as well as guidance in coining new names.

Identification

An organism must be classified before it can be identified—that is, given a name. This is true even if the classification is merely the recognition that the organism is different from any known organism. (For example, this occurred with the Legionnaires' disease agent, which caused the famous pneumonia epidemic in 1976 in Philadelphia; this organism was unlike bacteria of any established species; it has now been classified in a new bacterial genus, *Legionella*, and has been assigned the species name *L. pneumophila*.) Once an organism is classified, a few of its characteristics are selected by which it can be identified by other microbiologists. In order to be useful for identification, *the combination of characteristics chosen must occur only in that particular kind of organism* and in no other. The characteristics chosen should also be ones that are *easy to determine*, such as shape, staining reactions, and sugar fermentations. For example, DNA homology experiments, while very useful for classifying an organism, would be quite unsatisfactory for the routine identification of an organism because of the complexity of the procedure.

Many identification schemes are in the form of keys, which give identifying characteristics arranged in a logical fashion. Identification tables are also useful and generally contain more characteristics than do keys, with the information arranged in an easy-to-read, summarized form.

The Past and Present State of Bacterial Taxonomy

The first classification scheme for bacteria was published in 1773, and many more have appeared since. The early schemes were based only on morphological characteristics, but as the science of microbiology developed, other kinds of characteristics became increasingly important for classifications. Each successive classification scheme reflected the level of knowledge available at the time, and this continues to be true. Even present arrangements of bacteria are only provisional, subject to modification or replacement as new information appears.

Many classification schemes presently exist, but most cover only one or a few groups of bacteria. One classification scheme is unique, however, because of its broad scope and wide acceptance: *Bergey's Manual of Determinative Bacteriology*. This international reference work not only provides descriptions of all established genera and species of bacteria, but it also provides a practical arrangement of these taxa that is useful for their identification, together with appropriate keys and tables. Eight editions of *Bergey's Manual* have appeared since 1923, and a new edition is now in preparation, part of which has already been published. The title has been changed to *Bergey's Manual of Systematic Bacteriology* to reflect an increased coverage of bacterial characterization, classification, and taxonomic problems, in addition to the identification aspects. *Bergey's Manual* is written by hundreds of authors from around the world, each an authority on a particular bacterial group.

The arrangement of bacterial taxa in the new edition of *Bergey's Manual* is mainly along traditional, practical lines. Each volume is divided into a number of sections, each bearing a vernacular name such as "The Spirochetes" or "Gram-Negative Anaerobic Cocci" rather than a formal taxonomic name. The emphasis is largely on genera and species and, because of the present incomplete and fragmentary understanding of the real relationships that exist among bacteria, no attempt is made to adhere to any comprehensive, formal taxonomic hierarchy. The present classification scheme has considerable practical value, but the editorial board of *Bergey's Manual* regards it only as an interim arrangement that must eventually give way to a new, general, comprehensive classification scheme based on genetic relatedness. This is expected to provide greater stability and predictability, to lead to improved identification schemes, and to aid our understanding of the origin of present-day genera and species.

QUESTIONS

1 Define the following terms:

Classification	Taxon
Nomenclature	Taxa
Identification	Bacterial strain
Pure culture	Bacterial species
Mol% G + C	%S
Phylogenetic	*Bergey's Manual*

2 Why is it essential to classify microorganisms and what must be done before they can be classified?

3 List and describe briefly the major kinds of microbial characteristics and indicate those that must be determined by examination of individual cells.

4 Why is the type strain the most important strain in a bacterial species?

5 Explain the subjectivity that exists in the definition of a bacterial species.

6 (a) If two microorganisms have an identical mol% G + C value for their DNA, are they necessarily related? Explain.
(b) If two microorganisms have very different mol% G + C values for their DNA, are they necessarily unrelated? Explain.
7 In DNA homology experiments, we directly compare the entire genome (all the DNA) of one organism with that of another organism. What are we comparing when we do rRNA homology experiments or rRNA oligonucleotide cataloging?
8 What advantages do rRNA homology experiments and rRNA oligonucleotide cataloging offer compared to DNA homology experiments?
9 What is the reason that each taxon has only one officially recognized name?
10 What function does the name of a bacterial species serve?
11 Give an example of a bacterial name and write it in its proper form.
12 What makes *Bergey's Manual* unique among microbiological publications?
13 What is the present philosophy of the editorial board of *Bergey's Manual* toward bacterial classification and what sorts of changes may occur in future editions?

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Chapter 4 The Microscopic Examination of Microorganisms

OUTLINE

The **microscope** is the instrument most characteristic of the microbiology laboratory. The **magnification** it provides enables us to see microorganisms and their structures otherwise invisible to the naked eye. The magnifications attainable by microscopes range from X100 to X400,000. In addition, several different kinds of microscopy are available, and many techniques have been developed by which specimens of microorganisms can be prepared for examination. Each type of microscopy and each method of preparing specimens for examination offers advantages for demonstration of specific morphological features. In this chapter we shall describe some of the microbiologists' methods for observing the morphological characteristics of microorganisms. The techniques used to make these examinations are provided in the laboratory manual.

MICROSCOPES AND MICROSCOPY

Microscopes are of two categories, **light (or optical)** and **electron**, depending upon the principle on which magnification is based. **Light microscopy**, in which magnification is obtained by a system of optical lenses using light waves, includes: (1) **bright-field**, (2) **dark-field**, (3) **fluorescence**, and (4) **phase-contrast** microscopy. The **electron microscope**, as the name suggests, uses a beam of electrons in place of light waves to produce the image. Specimens can be examined by either **transmission** or **scanning** electron microscopy.

In a first microbiology course, students perform most of their examinations, if not all, with the bright-field microscope. This is the most widely used instrument for routine microscopic work. The other types of microscopy are used for special purposes or research investigations. However, students should be acquainted with their applications, since each has some unique feature that is useful for demonstrating particular structures of the cell.

Figure 4-1. A stained preparation of bacteria as seen by bright-field microscopy.

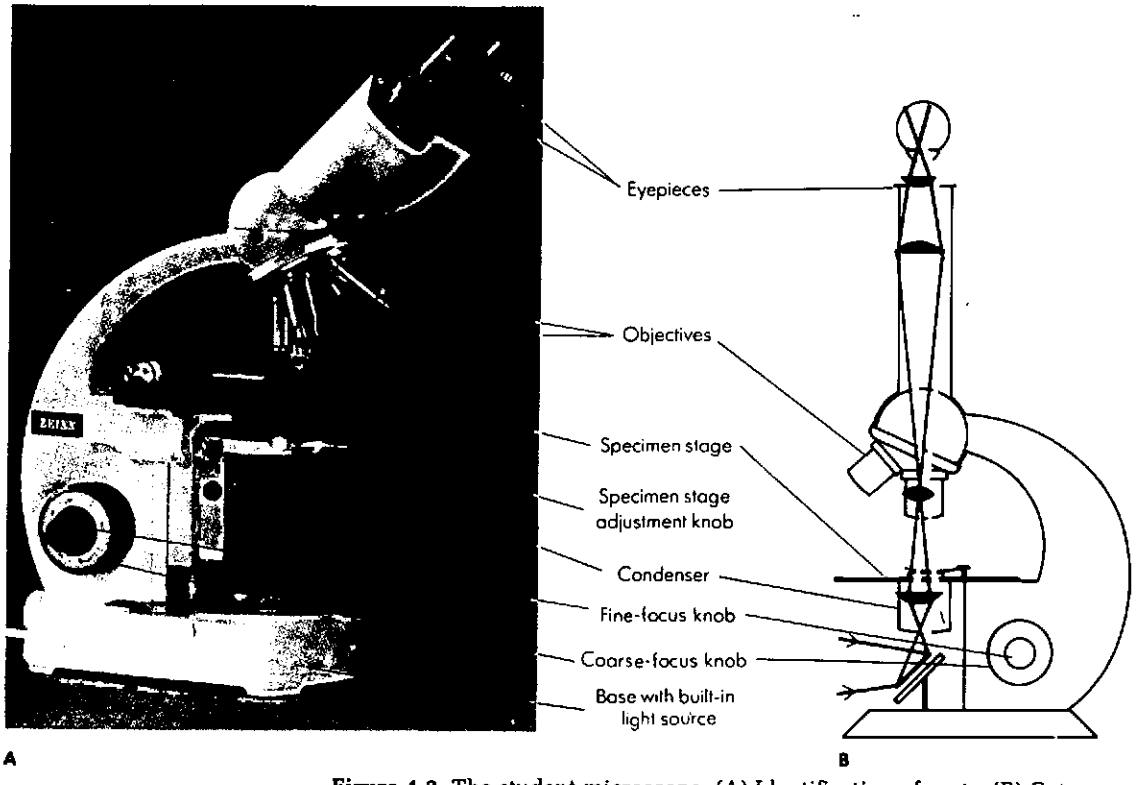


Figure 4-2. The student microscope. (A) Identification of parts. (B) Cutaway sketch of student microscope showing optimal parts and path of light. *(Courtesy of Carl Zeiss, New York.)*

Bright-Field Microscopy

In bright-field microscopy, the microscopic field (the area observed) is brightly lighted and the microorganisms appear dark because they absorb some of the light. Ordinarily, microorganisms do not absorb much light, but staining them with a dye greatly increases their light-absorbing ability (Fig. 4-1), resulting in greater contrast and color differentiation. The optical parts of a typical bright-field microscope and the path the light rays follow to produce enlargement, or magnification, of the object are shown in Fig. 4-2. Generally microscopes of this type produce a useful magnification of about X1,000 to X2,000. At magnifications greater than X2,000 the image becomes fuzzy for reasons we will explain now.

Resolving Power

The basic limitation of the bright-field microscope is one not of magnification but of **resolving power**, the ability to distinguish two adjacent points as distinct and separate. Mere increase in size (greater magnification) without the ability to distinguish structural details (greater resolution) is not beneficial. To state it differently, the largest magnification produced by a microscope may not be the most useful because the image obtained may be unclear or fuzzy. The more lines

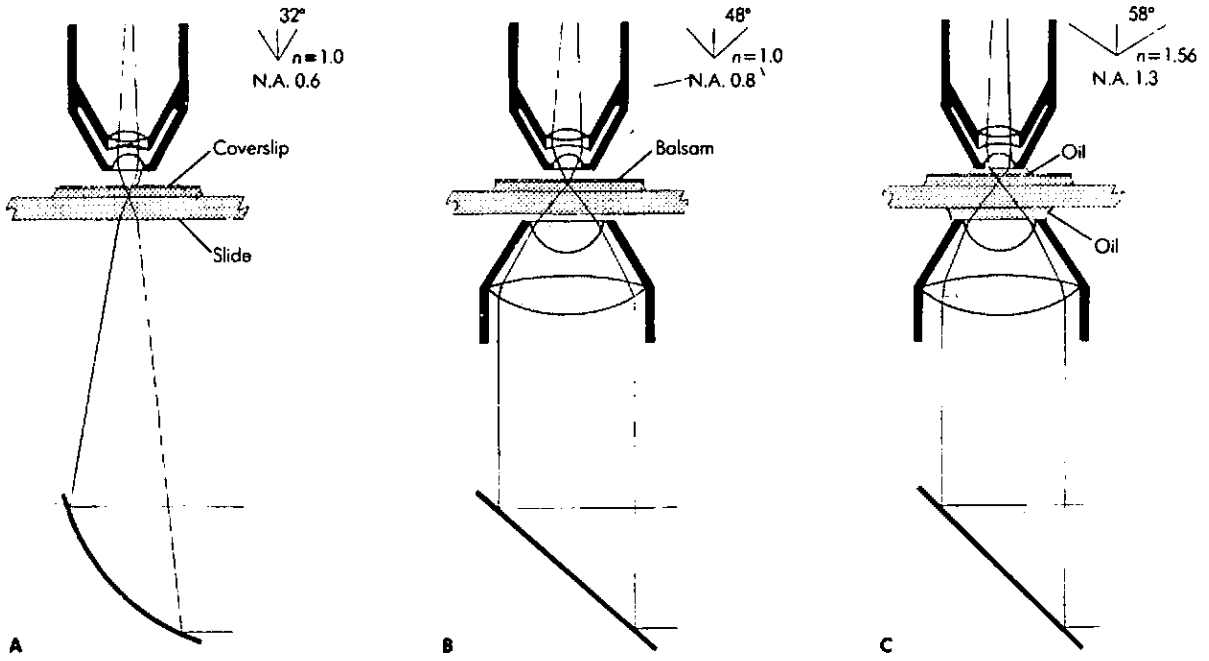


Figure 4-3. The relationship between angular aperture and resolution. (A) A narrow cone of light enters the low-power objective; the total angle is 64°, θ is 32°, and the numerical aperture (N.A.) is 0.6. (B) A substage condenser increases the size of the cone of light to 96°; θ is 48°, N.A. is 0.8. (C) With the oil-immersion objective, the size of the cone of light is increased to 116°; θ is 58°, N.A. is 1.3. The refractive index (*n*) for air is 1.0; for oil, 1.3. The resolution of the lens system, as described in the text, increases as the numerical aperture increases. *(From P. Gray, Handbook of Basic Microtechnique, McGraw-Hill, New York, 1964.)*

or dots per unit area that can be seen distinctly as separate lines or dots, the greater is the resolving power of the microscope system. The resolving power of a microscope is a function of the wavelength of light used and the numerical aperture (NA) of the lens system.

Numerical Aperture

The angle θ subtended by the optical axis and the outermost rays still covered by the objective is the measure of the aperture of the objective; it is the half-aperture angle (Fig. 4-3). The magnitude of this angle is expressed as a sine value. The sine value of the half-aperture angle multiplied by the refractive index *n* of the medium filling the space between the front lens and the cover slip gives the numerical aperture (NA): $NA = n \sin \theta$

With dry objectives the value of *n* is 1, since 1 is the refractive index of air. When immersion oil is used (Fig. 4-3) as the medium, n is 1.56, and if θ is 58°, then

$$NA = n \sin \theta = 1.56 \times \sin 58° = 1.56 \times 0.85 = 1.33$$

The degree to which microscope objectives can be altered to increase the NA

is limited: the maximum NA for a dry objective is less than 1.0, and oil-immersion objectives have an NA value of slightly greater than 1.0 (1.2 to 1.4). The wavelength of light used in optical microscopes is also limited; the visible light range is between 400 nm (blue light) and 700 nm (red light), or 0.4 μm to 0.7 μm. (The abbreviation nm stands for nanometer and is equal to 0.001 μm, or 10^{-9}m.)

Thus it is apparent that the resolving power of the optical microscope is restricted by the limiting values of the NA and the wavelength of visible light.

Limit of Resolution

The limit of resolution is the smallest distance by which two objects can be separated and still be distinguishable as two separate objects. The greatest resolution in light microscopy is obtained with the shortest wavelength of visible light and an objective with the maximum NA. The relationship between NA and resolution can be expressed as follows:

$$d = \frac{\lambda}{2NA}$$

where d = resolution and λ = wavelength of light. Using the values 1.3 for NA and 0.55 μm, the wavelength of green light, for λ, resolution can be calculated as

$$d = \frac{0.55}{2 \times 1.30} = 0.21\ \mu m$$

From these calculations we may conclude that the smallest details that can be seen by the typical light microscope are those having dimensions of approximately 0.2 μm.

Magnification

Magnification beyond the resolving power is of no value since the larger image will be less distinct in detail and fuzzy in appearance. The situation is analogous to that of a movie screen: if we move closer to the screen the image is larger but is also less sharp than when viewed at a distance.

Most laboratory microscopes are equipped with three objectives, each capable of a different degree of magnification. These are referred to as the oil-immersion, high-dry, and low-power objectives. The primary magnification provided by each objective is engraved on its barrel. The total magnification of the system is determined by multiplying the magnifying power of the objective by that of the eyepiece. Generally, an eyepiece having a magnification of X10 is used, although eyepieces of higher or lower magnifications are available.

Dark-Field Microscopy

The effect produced by the dark-field technique is that of a dark background against which objects are brilliantly illuminated. This is accomplished by equipping the light microscope with a special kind of condenser that transmits a hollow cone of light from the source of illumination, as shown in Fig. 4-4. Most of the light directed through the condenser does not enter the objective; the field is essentially dark. However, some of the light rays will be scattered (diffracted) if the transparent medium contains objects such as microbial cells. This diffracted light will enter the objective and reach the eye; thus the object or microbial cell, in this case, will appear bright in an otherwise dark micro-

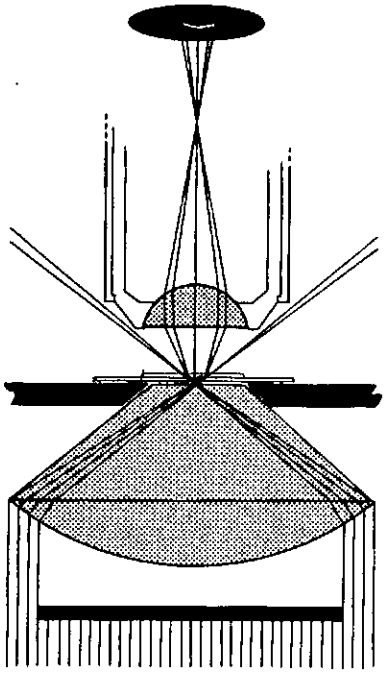


Figure 4-4. Path of light through a dark-field microscope system. Note that only those light waves which strike an object in the microscopic field are "bent" toward the observer's eye. *(Erwin F. Lessel, illustrator.)*

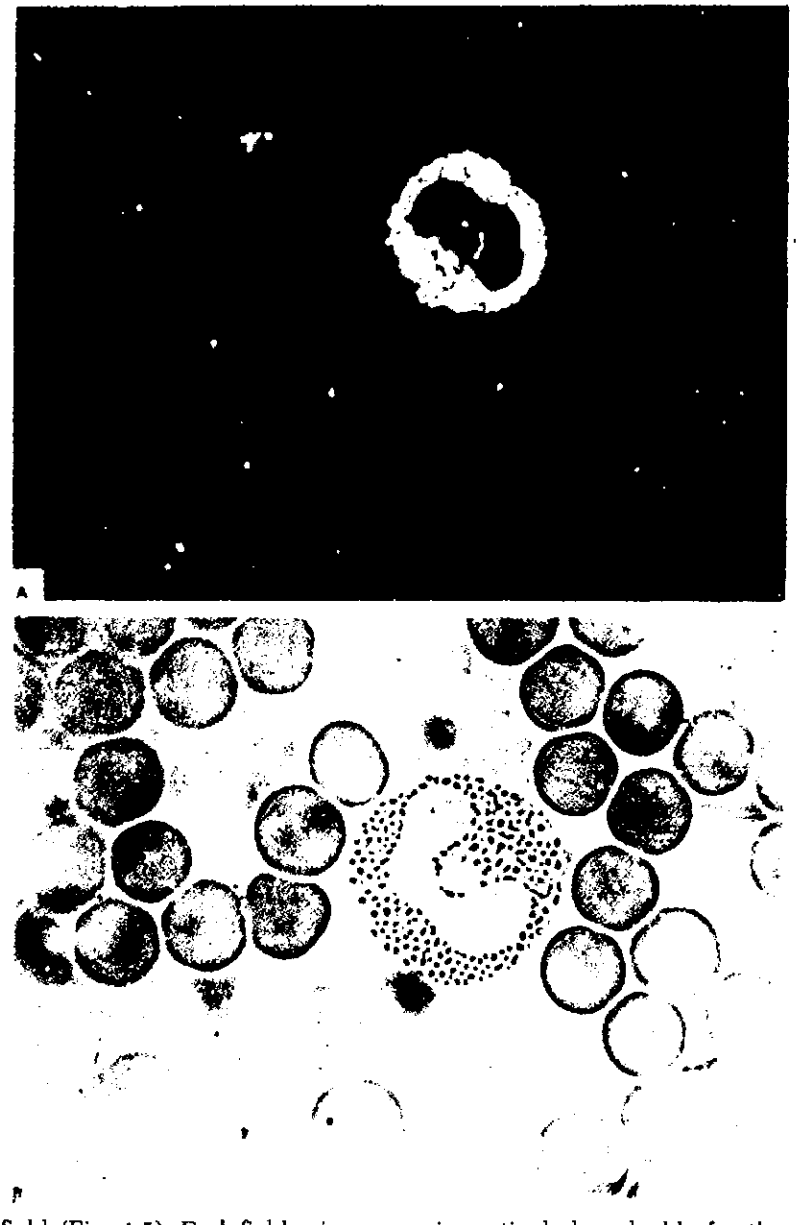


Figure 4-5. Dark-field and bright-field microscopy. The appearance of a white blood cell (eosinophil) surrounded by red blood cells, as viewed by (A) dark-field and (B) bright-field microscopy. *(From Scope, courtesy of The Upjohn Company.)*

scopic field (Fig. 4-5). Dark-field microscopy is particularly valuable for the examination of *unstained microorganisms* suspended in fluid—wet-mount and hanging-drop preparations.

Fluorescence Microscopy

Many chemical substances absorb light. After absorbing light of a particular wavelength and energy, some substances will then emit light of a longer wave-

Microscopic preparations:

a The yeast *Candida albicans* stained by the Gram method, the blue color indicates that it is a Gram-positive organism

b Gram stain of *Streptococcus pyrogenes* shows that this species is Gram-positive

c Gram stain of specimen combining *Neisseria gonorrhoeae*, the causative agent of gonorrhea. This bacterium is Gram-negative; the cells appear red. In this specimen the gonococci appear as small diplococci within a white blood cell

d Flagella stain of *Pseudomonas aeruginosa*. A special staining technique is required to demonstrate with light microscopy the presence of flagella on bacteria

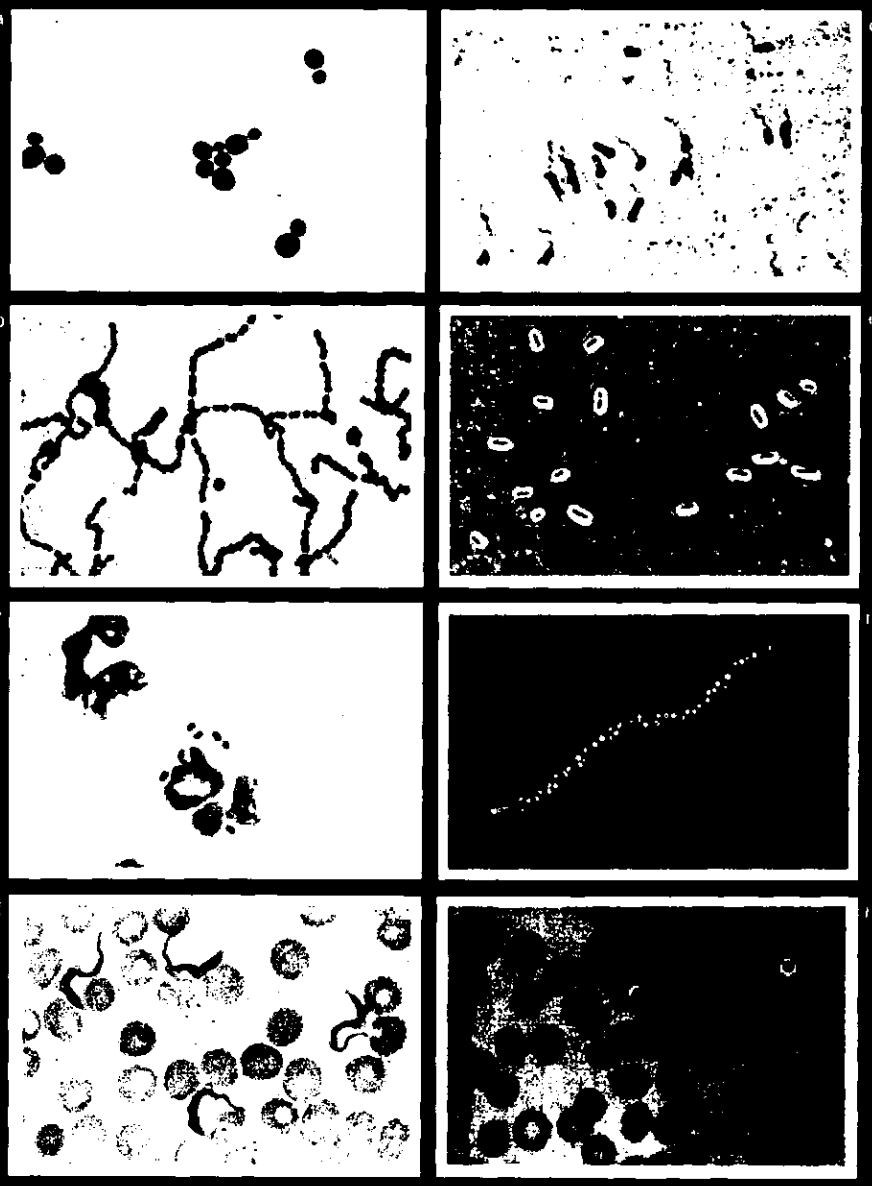
e Capsule stain of *Klebsiella pneumoniae*; the bacterial cells appear dark within a clear envelope.

f *Thiospirillum jenense* with sulfur globules (X1100).

g *Trypanosoma cruzi*, causative agent of Chagas' disease as seen in a stained blood film

h *Plasmodium vivax*, causative agent of malaria. Stain of a blood film showing trophozoite in red blood cell

(Figures a to e courtesy of Microbiology Service, Clinical Pathology Department, National Institutes of Health. Fig. f courtesy of R. L. Gherna, American Type Culture Collection. Figs. g and h courtesy of National Medical Audiovisual Center, Centers for Disease Control, Atlanta, Georgia.)



Fluorescent antibody stains:

a Group A streptococci, the bright clump of cells in the center of field, stained with fluorescein-labeled Group A specific antiserum. The unstained cells in the background are Group C streptococci; they do not react with the labeled Group A antiserum.

b *Neisseria meningitidis* in cerebrospinal fluid.

c Poliomyelitis virus, Mahoney strain, in HeLa cell culture, stained with fluorescein-labeled poliomyelitis antibody. Note the intracytoplasmic accumulation of virus in the cell near the center of the field.

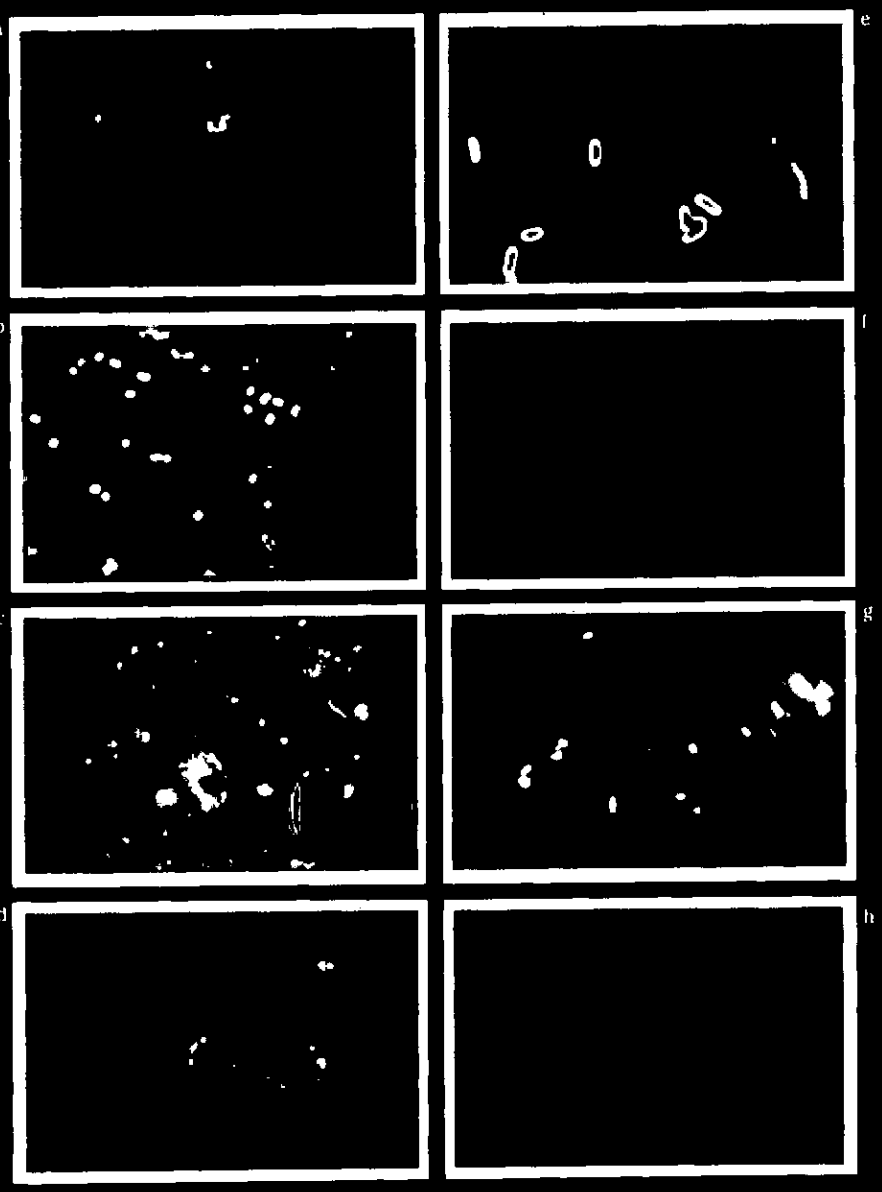
d Negri bodies of rabies virus in mouse brain tissue stained orange. This preparation is stained with rhodamine-labeled antiserum. This technique permits detection of virus aggregates smaller than the Negri bodies observable by light microscopy.

e A mixed smear of *Salmonella typhosa* and *Salmonella virginia* stained with fluorescein-labeled *S. typhosa* antiserum and counterstained with Flazo Orange. The fluorescein-labeled antiserum combines only with the typhoid bacteria.

f A preparation from a pure culture of *Salmonella virginia* stained in the same manner as described in (e). They are stained by Flazo Orange counterstain, which is nonspecific.

g Enteropathogenic *Escherichia coli* in a fecal smear from a case of infant diarrhea.

h *Blastomyces dermatitidis* as seen by direct fluorescent antibody stain.



(Figures a, c, and d courtesy of Baltimore Biological Laboratory. Figs. e and f courtesy of C. T. Hall and P. A. Hansen, Zentralblatt fur Bakteriologie, I. Originale, 184, 1962; Figs. b and g courtesy of W. B. Cherry, Center for Disease Control, DHEW, Atlanta, Georgia. Fig. h courtesy of National Medical Audiovisual Center, Centers for Disease Control, Atlanta, Georgia.)

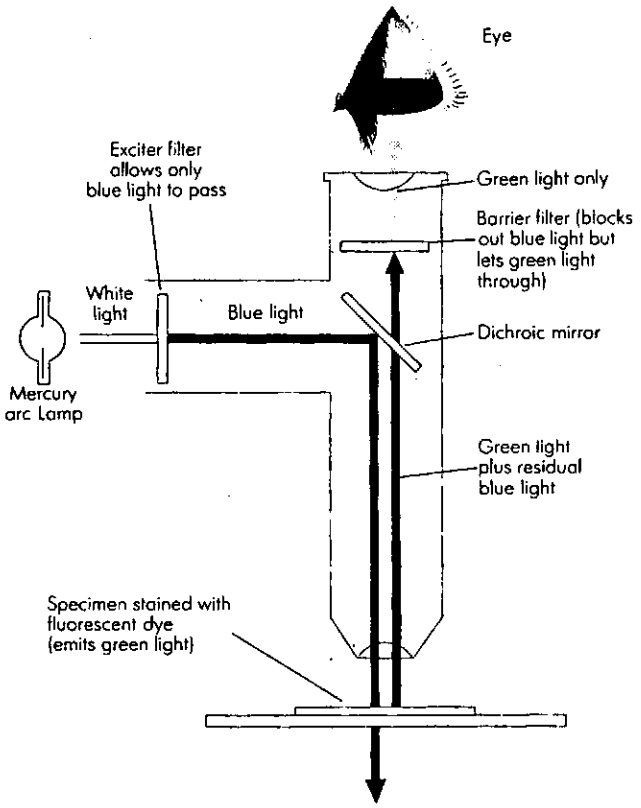


Figure 4-6. The special features of fluorescence microscopy. A high-intensity mercury lamp is used as the light source and emits white light. The exciter filter transmits only blue light to the specimen and blocks out all other colors. The blue light is reflected downward to the specimen by a dichroic mirror (which reflects light of certain colors but transmits light of other colors). The specimen is stained with a fluorescent dye: certain portions of the specimen retain the dye, others do not. The stained portions absorb blue light and emit green light, which passes upward, penetrates the dichroic mirror, and reaches the barrier filter. This filter allows the green light to pass to the eye; however, it blocks out any residual blue light from the specimen which may not have been completely deflected by the dichroic mirror. Thus the eye perceives the stained portions of the specimen as glowing green against a jet black background, whereas the unstained portions of the specimen are invisible. *(Erwin F. Lessel, illustrator.)*

length and a lesser energy content. Such substances are called **fluorescent** and the phenomenon is termed **fluorescence.** Application of this phenomenon is the basis of fluorescence microscopy. In practice, microorganisms are stained with a fluorescent dye and then illuminated with blue light; the blue light is absorbed and green light emitted by the dye.

The special features of fluorescence microscopy with the respect to illumination of the specimen are shown in Fig. 4-6. The function of the **exciter filter** is to remove all but the blue light; the **barrier filter** blocks out blue light and allows green light (or other light emitted by the fluorescing specimen) to pass through and reach the eye. Barrier filters are selected on the basis of the dye used.

An example of direct staining of bacteria with a fluorescent dye is shown in Fig. 4-7.

The Fluorescent Antibody Technique—Immunofluorescence

It is possible to chemically combine fluorescent dyes with antibodies, i.e., substances that combine with specific microorganisms. Antibodies to which a fluorescent dye is attached are referred to as **labeled antibodies.** Thus labeled antibodies can be mixed with a suspension of bacteria and then the preparation examined by fluorescent microscopy. The bacterial cells that have combined

Figure 4-7. *Mycobacterium tuberculosis* in a sputum specimen stained with fluorescent dye shows up a bright bacillus. *(Courtesy Center for Disease Control, Atlanta, Ga.)*

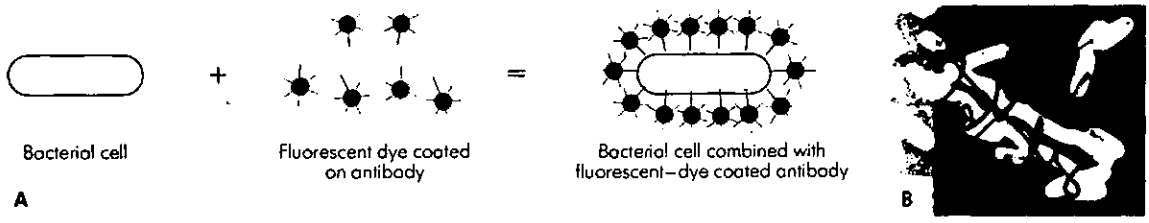


Figure 4-8. Fluorescence staining technique and microscopy. (A) The direct fluorescent antibody staining technique. When a bacterial cell is incubated with specific antibody that is conjugated (combined) with a fluorescent dye, the dye–antibody conjugate will cover the surface of the cell. The technique is performed on a glass slide, the excess fluorescent dye–antibody conjugate is washed off, and the preparation is examined by ultraviolet light microscopy. The bacterial cell will glow brilliantly as a result of fluorescence caused by the ultraviolet illumination of the dye-coated bacterial cell. Any bacterial cells not covered by the dye do not fluoresce and hence are not visible by this technique. (B) Photomicrograph of a fluorescent-stained *Proteus mirabilis* preparation as described above. *(Courtesy of Judith Hoeniger, F. M. Clinits, and E. A. Clinits, J. Bacteriol,* **98**:*226, 1969.)*

with the labeled antibody will be visible in the microscopic preparation (see Fig. 4-8). This procedure is known as the **fluorescent antibody technique**; the phenomenon is termed **immunofluorescence**. Theoretically, it is possible to identify a single microbial cell by this procedure. The application of this test in diagnostic procedures is discussed in Chap. 34.

Phase-Contrast Microscopy

Phase-contrast microscopy is extremely valuable for studying living unstained cells and is widely used in applied and theoretical biological studies. It uses a conventional light microscope fitted with a phase-contrast objective and a phase-contrast condenser. This special optical system makes it possible to distinguish unstained structures within a cell which differ only slightly in their refractive indices or thicknesses.

In principle, this technique is based on the fact that light passing through one material and into another material of a slightly different refractive index and/or thickness will undergo a change in **phase**. These differences in phase, or wavefront irregularities, are translated into variations in brightness of the structures and hence are detectable by the eye.

With phase-contrast microscopy it is possible to reveal differences in cells and their structures not discernible by other microscopic methods. A compari-

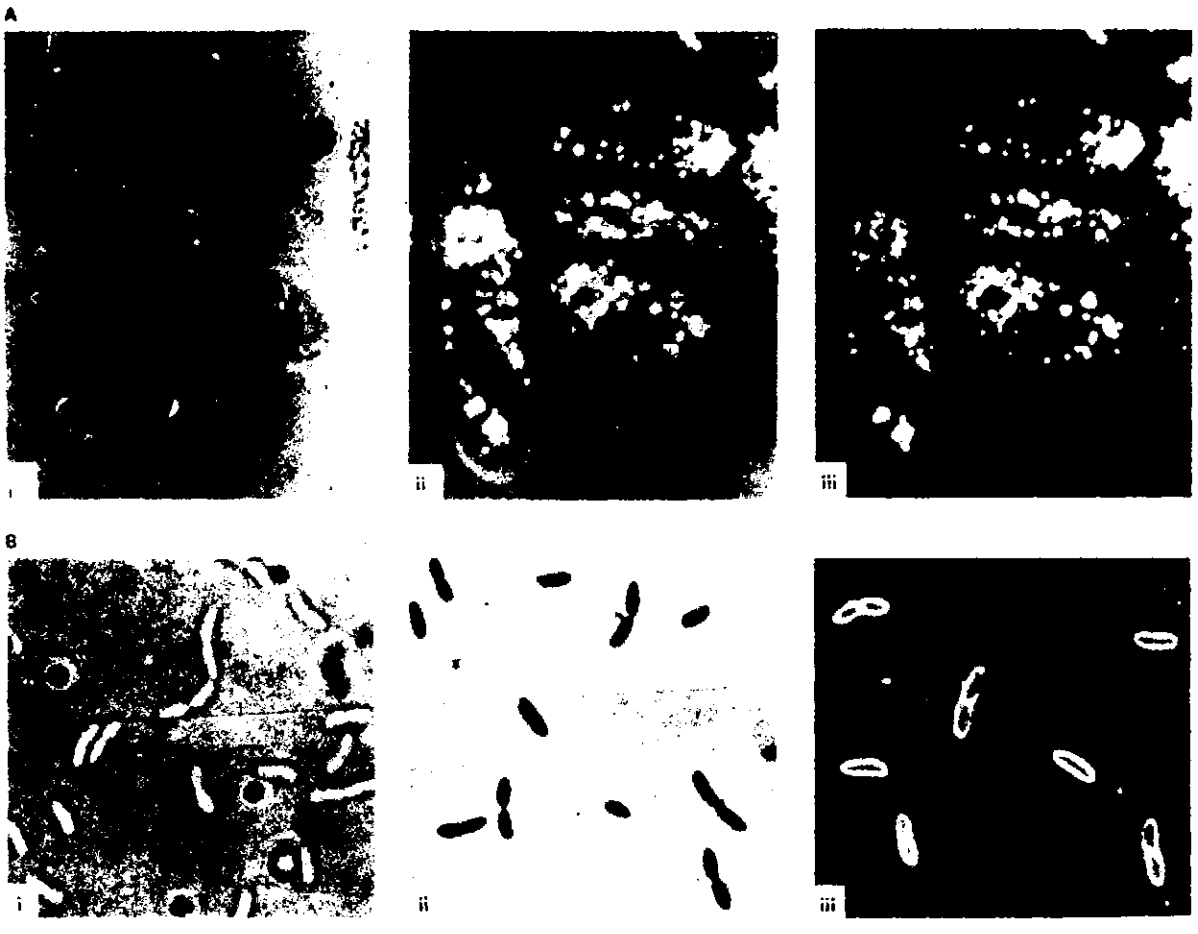


Figure 4-9. (A) Phase-contrast microscopy compared with bright-field and dark-field microscopy. The same specimen of a protozoan as seen by each method: (i) bright-field; (ii) phase-contrast; (iii) dark-field. *(Courtesy of O. W. Richards, Research Department, American Optical Company.)* (B) Photomicrographs of living, unstained, rod-shaped cells of *Pseudomonas fluorescens*. The bacilli are 0.7 to 0.8 μm in width. They can be seen only indistinctly by ordinary bright-field microscopy (i) but are readily visible by phase-contrast (ii) or dark-field microscopy (iii). *(Courtesy N. R. Krieg.)*

son of a specimen viewed by bright-field, dark-field, and phase-contrast microscopy is shown in Fig. 4-9.

Transmission Electron Microscopy

Electron microscopy differs markedly and in many respects from the optical microscopic techniques. The electron microscope provides tremendous useful magnification, because of the much higher resolution obtainable with the ex-

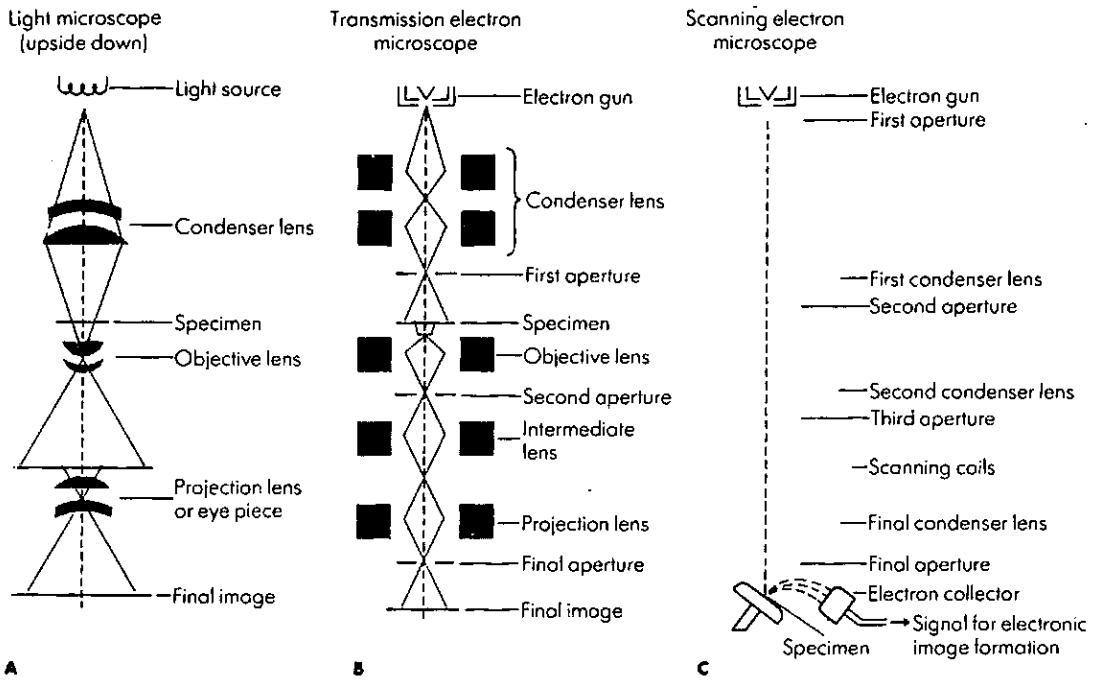


Figure 4-10. Diagrammatic comparison of imaging systems in (A) optical microscope, (B) transmission electron microscope, and (C) scanning electron microscope. *(Courtesy of L. A. Bulla, Jr., G. St. Julian, C. W. Hesseltine, and F. L. Baker, Scanning Electron Microscopy, in Methods in Microbiology, vol. 8, Academic, New York, 1973.)*

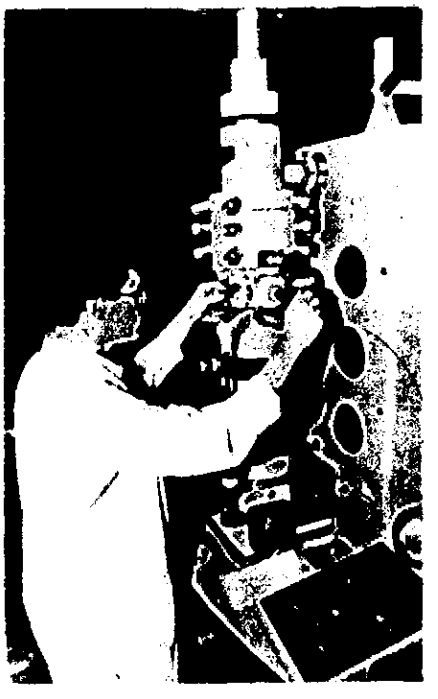


Figure 4-11. A high-resolution electron microscope. *(Courtesy of George Hatjygeorge, Fordham University.)*

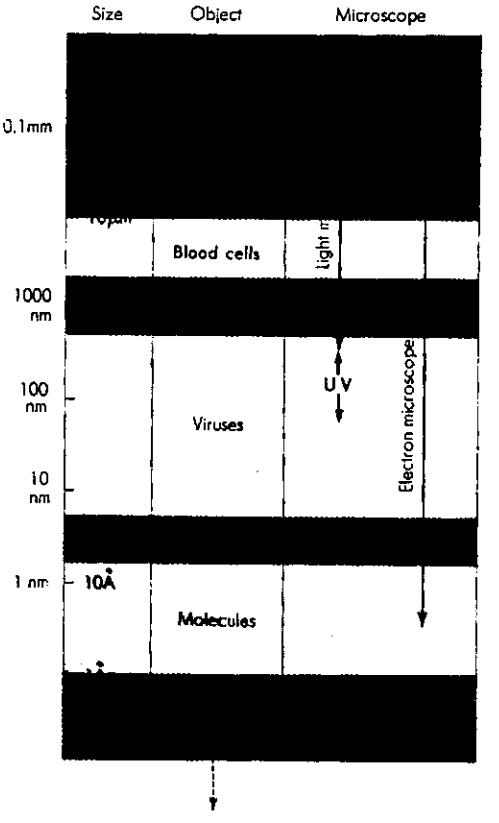


Figure 4-12. Relative size of microbes, molecules, and atoms is depicted here, together with an indication of the useful range of different types of microscopes. *(Courtesy of A. J. Rhodes and C. E. van Rooyen, Textbook of Virology, Williams & Wilkins, Baltimore, 1968.)*

tremely short wavelength of the electron beam used to magnify the specimen As shown in Fig. 4-10, the electron microscope uses electron beams and magnetic fields to produce the image, whereas the light microscope uses light waves and glass lenses.

With an electron microscope (Fig. 4-11) employing 60- to 80-kV electrons the wavelength is only 0.05 Å. Å is the abbreviation for angstrom; 1 Å equals 1/100,000,000 (10^{-8}) cm or 1/10,000 (10^{-4}) μm. (Compare this electron wavelength with the light wavelengths used for optical microscopes.) It is possible to resolve objects as small as 10 Å (Fig. 4-12). The resolving power of the electron microscope is more than *100 times* that of the light microscope, and it produces useful magnification up to ×400,000.

For electron microscopy, the specimen to be examined is prepared as an extremely thin dry film on small screens and is introduced into the instrument at a point between the magnetic condenser and the magnetic objective; this point is comparable to the stage of the light microscope. The magnified image may be viewed on a fluorescent screen through an airtight "window" or recorded on a photographic plate by a camera built into the instrument.

Numerous techniques are available for use with electron microscopy which extend its usefulness in characterizing cellular structure. Some of these are described below.

Shadow-casting

This technique involves depositing an extremely thin layer of metal (e.g., platinum) at an oblique angle on the organism so that the organism produces a shadow on the uncoated side. The shadowing technique produces a topographical representation of the surface of the specimen (see Fig. 4-13).

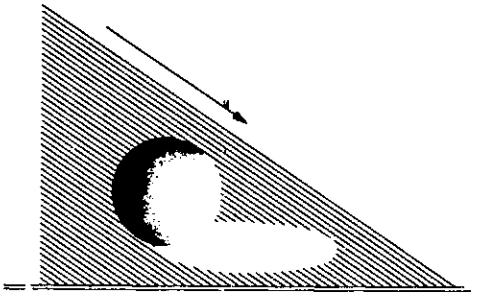


Figure 4-13. Shadow-casting technique. The specimen is dried on a special grid which is placed in a vacuum jar. Atoms of a heavy metal such as platinum are projected (from a highly heated filament) at an angle that produces a "shadow" behind the particles being examined. Examination of the shadowed image provides information as to the shape of the specimen particles. *(Erwin F. Lessel, illustrator.)*

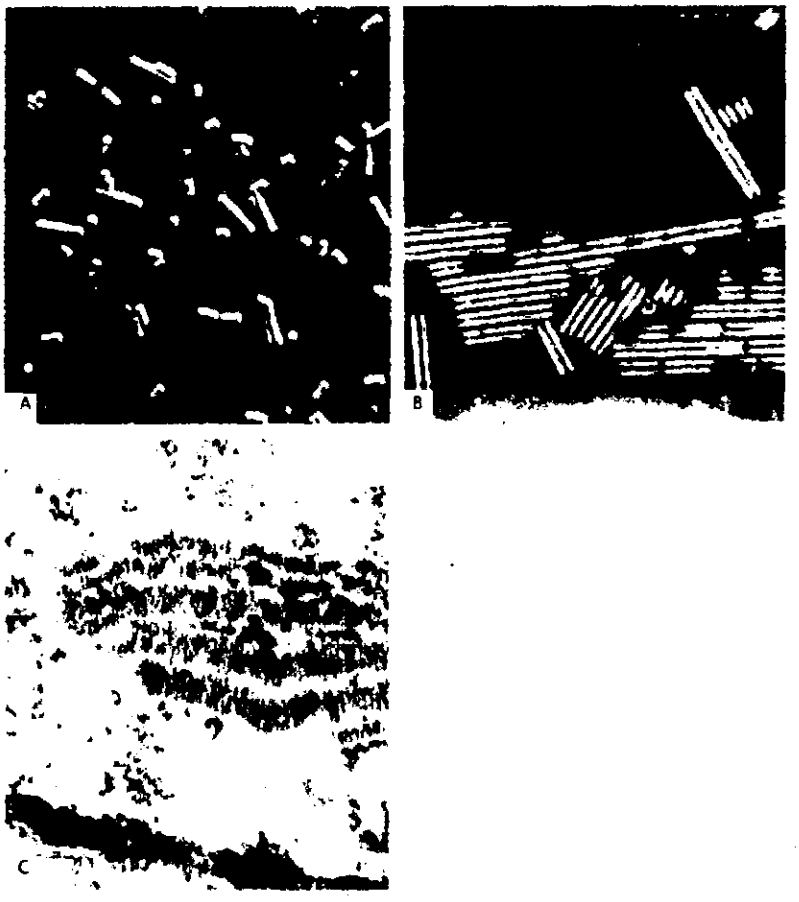


Figure 4-14. Electron micrographs of tobacco rattle virus as seen in three different preparations (A, B, and C). This virus characteristically appears as particles of two different sizes; the larger particle measures 184 by 25 nm and the smaller particle measures 74 by 25 nm. (A) Shadow-cast preparation using chromium. (B) Negative-stain preparation using potassium phosphotungstate. (C) Ultrathin section of infected leaf showing intracellular virus crystals, stained with uranyl acetate and lead citrate. *(Courtesy of M. Kenneth Corbett.)*

Figure 4-15. Electron micrograph of freeze-etched preparation of *Neisseria gonorrheae*. This bacterium measures approximately 0.6 to 1.0 μm in diameter. *(Courtesy of Ivan L. Roth.)*

Negative Staining

An electron-dense material such as phosphotungstic acid can be used as a "stain" to outline the object. The electron-opaque phosphotungstate does not penetrate structures but forms thick deposits in crevices (see Fig. 4-14). Fine detail of objects such as viruses or bacterial flagella can be seen by this technique.

Ultrathin Sectioning

In order to make observations of intracellular structures, the material for examination must be extremely thin. An intact microbial cell is too thick to allow distinct visualization of its internal fine structure by electron microscopy. However, techniques are available for sectioning (slicing) a bacterial cell; for example, bacterial cells can be embedded in a plastic material and then this "block" can be cut into ultrathin slices, as thin as 60 nm. These slices are then prepared for microscopic examination. As you might expect, the slices will reveal cells sliced at different levels and at different angles. Improvement in contrast of structures is possible through use of special electron-microscope stains such as uranium and lanthanum salts.

Freeze-Etching

Freeze-etching was developed to prepare sections of the specimen without resorting to the chemical treatment of the fixation process, which can produce artifacts. The specimen is sectioned while contained in a frozen block. Carbon replicas of these exposed surfaces are then prepared which reveal internal structures of the cell (see Fig. 4-15).

Localization of Cell Constituents

Special techniques have been developed making it possible to locate chemical constituents of the cell. For example, thin sections of a cell can be treated with

. ferritin-labeled antibody. Ferritin is an iron-containing substance of high density that markedly affects passage of the electron beam. The combination of this ferritin-labeled antibody with antigen in the cell produces a complex which manifests a higher contrast in the electron-microscope image.

Localization of Enzymes in Thin Sections

Electron-microscope techniques have been developed to locate the position of enzymes within cells. The intracellular localization of the enzyme isocitrate dehydrogenase of *Escherichia coli* is shown in Fig. 4-16. This was accomplished by first preparing ultrathin sections of the bacterial cells, followed by an immunochemical technique by which colloidal gold is affixed specifically to the isocitrate dehydrogenase enzyme.

Autoradiography

Autoradiography is a cytochemical method in which the location of a particular chemical constituent in a specimen is determined by observing the site at which radioactive material becomes positioned. The cells are first exposed to the radioactive substance to permit its uptake. In practice, the specimen prepared for microscopic examination is covered with a layer of photographic emulsion and stored in the dark for a period of time. The ionizing radiation emitted during the decay of the radioactive substance produces latent images in the emulsion, and, after photographic processing, the developed image is seen as grains of silver in the preparation.

Scanning Electron Microscopy

In scanning electron microscopy the specimen is subjected to a narrow electron beam which rapidly moves over (scans) the surface of the specimen. This causes the release of a shower of secondary electrons and other types of radiation from

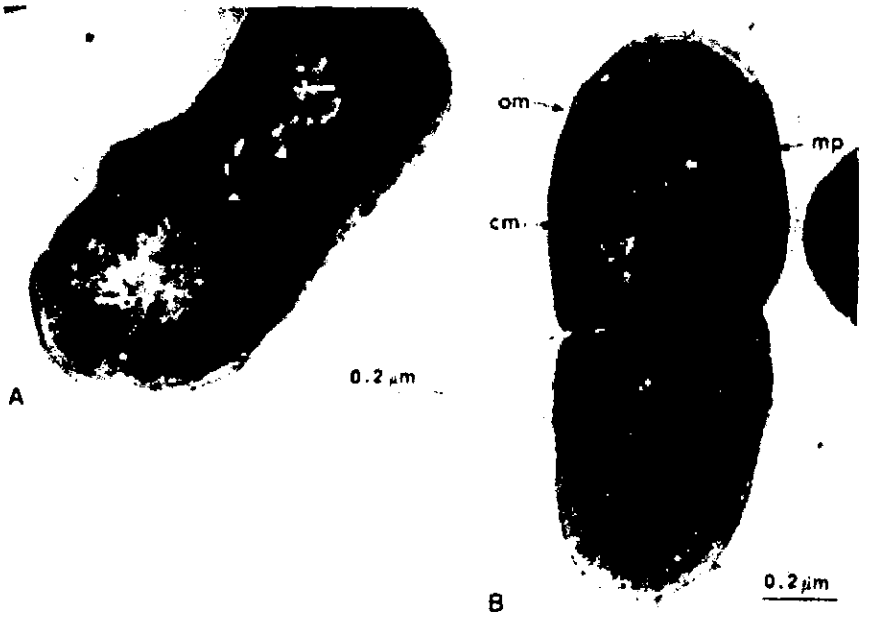


Figure 4-16. Intracellular localization of isocitrate dehydrogenase in *Escherichia coli* as seen by electron microscopy. (A) Ultrathin section of *E. coli* treated with a specific fraction of antiserum to the enzyme and then with a protein–gold particle complex which reacts with the antiserum. Location of gold particles and hence the enzyme is shown by arrows. (B) Control section treated with preimmune serum and protein–gold, showing outer membrane (om), peptidoglycan (mp), and cytoplasmic membrane (cm). *(Courtesy of J. R. Swafford et al., Science* 121:295, *1983.)*

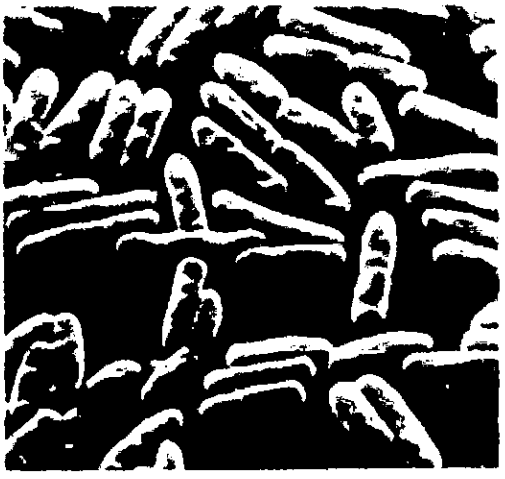


Figure 4-17. Scanning electron micrograph of cells of *Pseudomonas aeruginosa*. The average size of this bacterium is 0.5 to 1.0 μm by 1.5 to 4 μm. (*Courtesy of David Greenwood.*)

the specimen surface. The intensity of these secondary electrons depends on the shape and the chemical composition of the irradiated object. The secondary electrons are collected by a detector which generates an electronic signal. These signals are then scanned in the manner of a television system to produce an image on a cathode ray tube. The image system for the scanning electron microscope is shown in Fig. 4-10.

The scanning electron microscope lacks the resolving power obtainable with the transmission electron microscope but has the advantage of revealing a striking three-dimensional picture. The surface topography of a specimen can be revealed with a clarity and a depth of field not possible by any other method. An example of a scanning electron microscope picture (micrograph) is shown in Fig. 4-17.

LIMITATIONS OF ELECTRON MICROSCOPY

Despite the great advantage of tremendous resolution and magnification, there are several limitations to electron microscopy. For example, the specimen being examined is in a chamber that is under a very high vacuum. Thus cells cannot be examined in a living state. In addition, the drying process may alter some morphological characteristics. Another limitation of the technique is the low penetration power of the electron beam, necessitating the use of thin sections to reveal the internal structures of the cell.

The real problem confronting the researcher who attempts to unravel the fine intracellular structure of the microbial cell is identification of intracellular material. Frequently it is necessary to correlate results obtained with the same organism viewed by different microscopic techniques, e.g., phase-contrast, bright-field (stained preparations), and electron microscopy. Each method contributes different kinds of information. Interpretation of this information, particularly comparison of what is revealed by each technique, makes it possible to identify cellular structures. But considerable experience in microscopy is required before a researcher can correctly interpret the results.

Table 4-1. A Comparison of different Types of Microscopy

Type of Microscopy	Maximum Useful Magnification	Appearance of Specimen	Useful Applications
Bright-field	1,000–2,000	Specimens stained or unstained; bacteria generally stained and appear color of stain	For gross morphological features of bacteria, yeasts, molds, algae, and protozoa
Dark-field	1,000–2,000	Generally unstained; appears bright or "lighted" in an otherwise dark field	For microorganisms that exhibit some characteristic morphological feature in the living state and in fluid suspension, e.g., spirochetes
Fluorescence	1,000–2,000	Bright and colored; color of the fluorescent dye	Diagnostic techniques where fluorescent dye fixed to organism reveals the organism's identity
Phase-contrast	1,000–2,000	Varying degrees of "darkness"	For examination of cellular structures in living cells of the larger microorganisms, e.g., yeasts, algae, protozoa, and some bacteria
Electron	200,000–400,000	Viewed on fluorescent screen	Examination of viruses and the ultrastructure of microbial cells

Some of the major features of the several kinds of microscopy are summarized in Table 4-1.

PREPARATIONS FOR LIGHT-MICROSCOPE EXAMINATIONS

Two general techniques are used to prepare specimens for light-microscope examination. One is to suspend organisms in a liquid (the wet-mount or the hanging-drop techniques), and the other is to dry, fix, and stain films or smears of the specimen.

The Wet-Mount and Hanging-Drop Techniques

Wet preparations permit examination of organisms in a normal living condition. A wet mount is made by placing a drop of fluid containing the organisms onto a glass slide and covering the drop with a cover slip. To reduce the rate of evaporation and exclude the effect of air currents, the drop may be ringed with petroleum jelly or a similar material to provide a seal between the slide and cover slip. A special slide with a circular concave depression is sometimes used for examination of wet preparations. A suspension of microbial specimen is placed on a cover slip, then inverted over the concave depression to produce a "hanging drop" of the specimen.

Examination of microorganisms in wet preparation is desirable in the following instances:

1 The morphology of spiral bacteria is greatly distorted when these bacteria are dried and stained; they should be examined in living condition. For example, in the examination of serous exudates suspected of containing the spirochete that causes syphilis, the wet preparations are examined by dark-field microscopy. This provides a sharp contrast between the organisms and the dark background. The normal arrangement of cells can also be better determined in a wet preparation.
2 The observation of bacteria to determine whether or not they are motile obviously requires that they be suspended in a liquid medium, free to move about.
3 To observe cytological changes occurring during cell division and to determine the rate at which the division occurs, the organisms must be examined in the living state (i.e., wet mount). Spore formation and germination must also be observed in living cells.
4 Some cell inclusion bodies, e.g., vacuoles and lipid material, can be observed readily by this method.

When wet preparations are examined by bright-field microscopy, it is extremely important to control the light source. The reason is that the lack of a stain makes the cells less distinctly visible; adjustment of the intensity of the light source can enhance their visibility. Partially closing the substage condenser diaphragm helps to increase contrast; however, some resolving power is lost. Dark-field and phase-contrast microscopy offer the distinct advantage of providing both high contrast and high resolving power for examination of unstained preparations.

Fixed, Stained Smears

Fixed, stained preparations are most frequently used for the observation or the morphological characteristics of bacteria. The advantages of this procedure are that (1) the cells are made more clearly visible after they are colored, and (2) differences between cells of different species and within the same species can be demonstrated by use of appropriate staining solutions (differential or selective staining).

The essential steps in the preparation of a fixed, stained smear are (1) preparation of the film or smear, (2) fixation, and (3) application of one or more staining solutions.

Microbiological Stains

A large number of colored organic compounds (dyes) are available for staining microorganisms. These compounds are generally rather complex in terms of molecular structure. On this basis they may be classified into groups such as **triphenylmethane dyes, oxazine dyes,** and **thiazine dyes.**

A more practical classification for the cytologist is one based on the chemical behavior of the dye; namely, **acid, basic,** or **neutral.** An acid (or anionic) dye is one in which the charge on the dye ion is negative; a basic (or cationic) dye is one in which the charge carried by the dye ion is positive. A neutral dye is a complex salt of a dye acid with a dye base, e.g., eosinate of methylene blue. Acid dyes generally stain basic cell components, and basic dyes generally stain acidic cell components.

The process of staining may involve ion-exchange reactions between the stain and active sites at the surface of or within the cell. For example, the colored ions of the dye may replace other ions on cellular components. Certain chemical groupings of cell proteins or nucleic acids may be involved in salt formation with positively charged ions such as Na^+ or K^+. Thus we might view these peripheral areas of the cell as carrying a negative charge in combination with positively charged ions; for example,

$$(\text{Bacterial cell}^-)(Na^+)$$

In a basic dye like methylene blue, the colored ion is positively charged (a cation), and if we represent this ion by the symbol MB, the dye, which is actually methylene blue chloride, may be represented as

$$MB^+Cl^-$$

The ionic exchange which takes place during staining can be represented by the following equation, in which the MB^+ cation replaces the Na^+ cation in the cell:

$$(\text{Bacterial cell}^-)(Na^+) + (MB^+)(Cl^-) \rightarrow (\text{bacterial cell}^-)(MB^+) + (Na^+Cl^-)$$

Simple Staining. The coloration of bacteria by applying a single solution of stain to a fixed smear is termed simple staining. The fixed smear is flooded with a dye solution for a specified period of time, after which this solution is washed off with water and the slide blotted dry. The cells usually stain uniformly. However, with some organisms, particularly when methylene blue is used, some granules in the interior of the cell may appear more deeply stained than the rest of the cell, indicating a different type of chemical substance.

Differential Staining. Staining procedures that make visible the differences between bacterial cells or parts of a bacterial cell are termed differential staining techniques. They are slightly more elaborate than the simple staining technique in that the cells may be exposed to more than one dye solution or staining reagent.

Gram Staining. One of the most important and widely used differential staining techniques in microbiology is Gram staining. This technique was introduced by Christian Gram in 1884. In this process the fixed bacterial smear is subjected to the following staining reagents in the order listed: crystal violet, iodine solution, alcohol (decolorizing agent), and safranin or some other suitable counterstain. Bacteria stained by the Gram method fall into two groups: Gram-positive bacteria, which retain the crystal violet and hence appear deep violet in color; and Gram-negative bacteria, which lose the crystal violet, are counterstained by the safranin, and hence appear red in color. Why does this procedure stain some bacteria purple-violet and others red?

The most plausible explanations for this phenomenon are associated with the structure and composition of the cell wall. (See Chap. 5 for a discussion of the relative differences between the cell walls of Gram-negative and Gram-positive bacteria.) Differences in the thickness of cell walls between these two groups

may be important; the cell walls of Gram-negative bacteria are generally thinner than those of Gram-positive bacteria. Gram-negative bacteria contain a higher percentage of lipid than do Gram-positive bacteria. Experimental evidence suggests that during staining of Gram-negative bacteria the alcohol treatment extracts the lipid, which results in increased porosity or permeability of the cell wall. Thus the crystal violet–iodine (CV-I) complex can be extracted and the Gram-negative organism is decolorized. These cells subsequently take on the color of the safranin counterstain. The cell walls of Gram-positive bacteria, because of their different composition (lower lipid content), become dehydrated during treatment with alcohol. The pore size decreases, permeability is reduced, and the CV-I complex cannot be extracted. Therefore these cells remain purple-violet.

Another explanation, somewhat similar, is also based on permeability differences between the two groups of bacteria. In Gram-positive bacteria, the CV-I complex is trapped in the wall following ethanol treatment, which presumably causes a diminution in the diameter of the pores in the cell-wall peptidoglycan. Walls of Gram-negative bacteria have a very much smaller amount of peptidoglycan, which is less extensively cross-linked than that in the walls of Gram-positive bacteria. The pores in the peptidoglycan of Gram-negative bacteria remain sufficiently large even after ethanol treatment to allow the CV-I complex to be extracted. These two explanations are not mutually exclusive, and it is likely that both may contribute to the explanation of the mechanism of the Gram stain. Furthermore, if Gram-positive cells are treated with lysozyme (an enzyme) to remove the cell wall, the resulting structures, called **protoplasts** (cells lacking walls), will be stained by the CV-I complex. However, they are easily decolorized by alcohol. All this evidence points to the cell-wall structure of Gram-positive bacteria as the site of retention of the primary stain.

Although Gram-negative organisms consistently fail to retain the primary crystal violet stain, Gram-positive organisms may sometimes show variations in this respect, i.e., a **Gram-variable reaction**. For example, old cultures of Gram-positive bacteria lose the ability to retain the crystal violet and hence will be stained by the safranin. A similar effect may sometimes be due to changes in the environment of the organism or a slight modification in staining technique.

Within some groups of bacteria, such as the archaeobacteria (see Chap. 5), some are Gram-positive and others Gram-negative; yet the cell wall structure and chemical composition of these bacteria is very different from that of other groups of Gram-positive and Gram-negative bacteria.

Gram-positive bacteria differ from Gram-negative bacteria in other characteristics besides staining reaction. Gram-positive bacteria are usually more susceptible to penicillin and less susceptible to disintegration by mechanical treatment or exposure to some enzymes than Gram-negative bacteria. Gram-negative bacteria as a group are more susceptible to other antibiotics such as streptomycin. There are other differences between these two groups of bacteria.

The Gram stain has its greatest use in characterizing bacteria. This staining technique is not generally applicable for other groups of microorganisms such as protozoa and fungi; however, yeasts consistently stain Gram-positive.

Other Differential Stains. There are numerous other staining techniques de-

signed to identify some particular feature of cell structure or composition. These techniques are summarized here. Detailed descriptions of these procedures appear in the laboratory manual.

NAME OF STAINING TECHNIQUE	APPLICATION
Acid-fast stain	Distinguishes acid-fast bacteria such as *Mycobacterium* spp. from non-acid-fast bacteria
Endospore stain	Demonstrates spore structure in bacteria as well as free spores
Capsule stain	Demonstrates presence of capsules surrounding cells
Flagella stain	Demonstrates presence and arrangement of flagella
Cytoplasmic inclusion stains	Identifies intracellular deposits of starch, glycogen, polyphosphates, hydroxybutyrate, and other substances
Giemsa stain	Particularly applicable for staining rickettsias and some protozoa

QUESTIONS

1 Define the following terms:

Resolving power	Fluorescence
Limit of resolution, d	Autoradiography
NA (numerical aperture)	Anionic dye
Angle θ	Cationic dye

2 What are the usual magnifications obtainable with light microscopy? What determines its useful limit?

3 Assume that a yeast cell is examined by (*a*) bright-field, (*b*) phase-contrast, and (*c*) dark-field microscopy. Describe the likely differences in the appearance of the cell when viewed by these methods.

4 Why are microorganisms stained?

5 What is the function of oil when used with the oil-immersion objective?

6 Name several different staining techniques and describe their particular applications.

7 Compare the kind of image obtained with scanning electron microscopy with that obtained using transmission electron microscopy.

8 Compare the resolving power of the electron microscope with that of the light microscope.

9 Name two limitations of electron microscopy.

10 What are some major differences between Gram-positive and Gram-negative bacteria?

11 Why is the Gram stain one of the most important and widely used stains in bacteriology?

12 Compare the appearance of microorganisms as seen by dark-field and by phase-contrast microscopy.

13 Describe two special applications of fluorescence microscopy.

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PART TWO
MICROORGANISMS–
BACTERIA

Some elegant experiments with bacterial flagella

It had been known for many years that bacterial flagella—the helically shaped filaments projecting from bacterial cells—are somehow responsible for the ability of bacterial cells to swim. Bacteria lacking flagella, and mutants that have lost the ability to make flagella, are unable to swim; moreover, mutants that make only straight flagella are unable to swim, indicating that a helical flagellar shape is required for swimming. Prior to 1973, however, it was not clear just how flagella operated. One theory was that if the flagella could rotate as on a bearing, this could cause the flagella to screw through the medium, just as a rotating corkscrew can penetrate a piece of cork. The discovery of disk-shaped structures at the base of bacterial flagella suggested that there might indeed be a "flagellar motor" that could cause flagella to spin. Unfortunately, individual bacterial flagella were so thin that they could not be observed microscopically "in action" on a living bacterium.

In 1973, Michael Silverman and Melvin Simon at the University of California, San Diego, performed experiments that indicated unequivocally that bacterial flagella do actually rotate. They realized that a bacterial flagellum might be analogous to the shaft of an electric motor: if the motor housing is bolted to a table, then the shaft will rotate. However, if the motor is not bolted to a table but rather is grasped by the shaft and held up in the air, *then the shaft will be stationary and the motor housing will rotate*. Using this analogy, Silverman and Simon devised experiments whereby they could prevent a bacterial flagellum from rotating. The consequence of this would be that the *bacterial cell would rotate instead*—and this was something that could be easily seen with an ordinary microscope.

Silverman and Simon chose a mutant bacterium that had a single straight flagellum on one side of the cell. This mutant could not swim because the flagellum was not helical; nevertheless, if the rotational hypothesis were correct, the flagellum should still be able to spin. They prepared antibodies against the flagellum and then added a mixture of the bacteria and the antibodies to a slide. The slide became coated with the antibodies, and the flagella adhered to the antibodies. Thus, each cell became "tethered" by its flagellum to the slide. In this condition the flagella of these cells could not rotate; however, each tethered cell began to spin like a pinwheel!

It was difficult to explain how such behavior could occur unless, in a free-swimming cell, the flagella spun—probably by means of a rotary motor at their base. Additional experiments on untethered cells also supported the idea of flagellar rotation. Silverman and Simon found that small latex beads could be attached to a bacterial flagellum by means of antibodies; such beads, which were easily visible with a microscope, were observed to rotate rapidly about an invisible axis (the straight flagellum)!

It is now generally accepted that bacterial flagella do rotate as on a bearing—a type of motion that may be unique among living organisms.

Preceding page. Cell walls of *Mycobacterium tuberculosis* obtained from cells exposed to extremely high pressures under special conditions. Note the shape maintained by the "hollow" cell fragments which is indicative of the rigid structure of the cell wall. ×41,500 *(Courtesy of E. Ribi.)*

Chapter 5 The Morphology and Fine Structure of Bacteria

OUTLINE

Among the major characteristics of bacterial cells are their size, shape, structure and arrangement. These characteristics constitute the **morphology** of the cell. Depending on the species, individual cells are spherical, rodlike, or helical, although many variations of these three basic shapes occur. Furthermore, in certain species of bacteria the cells are arranged in groups, the most common of which are pairs, clusters, chains, trichomes, and filaments. It is important to recognize these patterns of shape and arrangement, since they are often characteristic of a taxonomic group, e.g., a genus. Some bacteria also possess appendages, which can be made visible by special staining techniques or by electron microscopy. All of these morphological features are regared as the gross morphological characteristics of bacterial cells.

The bacterial cell possesses a detailed internal structure. The discovery of this internal structure was made possible by the development of electron-microscope techniques and of instruments for slicing a bacterial cell into extremely thin sections. The terms **microbial cytology** and **bacterial anatomy** have become commonplace in microbiological literature.

The various structures of a bacterial cell differ from one another not only in their physical features but also in their chemical characteristics and in their functions. Thus biologists today seek to integrate the structural, chemical, and

functional properties of the bacterial cell. This area of research studied by biologists is sometimes referred to as **biochemical cytology**.

THE SIZE, SHAPE, AND ARRANGEMENT OF BACTERIAL CELLS

Size

Bacteria are very small, most being approximately 0.5 to 1.0 μm in diameter. An important consequence of the small size of microorganisms is that the **surface area/volume ratio** of bacteria is exceedingly high compared to the same ratio for larger organisms of similar shape (Table 5-1). A relatively large surface through which nutrients can enter (or waste products leave) compared to a small volume of cell substance to be nourished accounts for the unusually high rate of growth and metabolism of bacteria. Moreover, because of the high surface area volume ratio, the mass of cell substance to be nourished is very close to the surface; therefore, no circulatory mechanism is needed to distribute the nutrients that are taken in, and there is thought to be little or no cytoplasmic movement within a bacterial cell. Despite these advantages, a high surface area/volume ratio limits the size of bacteria to microscopic dimensions.

Shape and Arrangement

The shape of a bacterium is governed by its rigid cell wall; however, exactly what attribute of this rigid material determines that a cell will have a particular shape is not yet understood. Typical bacterial cells are spherical (**cocci**; singular, **coccus**); straight rods (**bacilli**; singular, **bacillus**); or rods that are helically curved (**spirilla**; singular, **spirillum**) as illustrated in Fig. 5-1. Although most bacterial species have cells that are of a fairly constant and characteristic shape, some have cells that are **pleomorphic**, i.e., that can exhibit a variety of shapes (Fig. 5-2).

Table 5-1. Comparison of the Surface Area/Volume Ratio of Spherical Organisms of Different Sizes*

Diameter of Sphere, μm	Surface Area, μm^2 ($4\pi r^2$)	Volume, μm^3 ($4\pi r^3/3$)	$\frac{\text{Surface Area}}{\text{Volume}}$, μm^{-1} ($3/r$)
1 μm	3.1	0.52	6
1,000 μm	3.1×10^6	5.2×10^8	0.006
1,000,000 μm	3.1×10^{12}	5.2×10^{17}	0.000006

* For a given volume, the geometrical shape that has the smallest surface area/volume ratio is a sphere; i.e., if two organisms have the same volume, one being spherical and the other cylindrical, the cylindrical organism has the greater surface area/volume ratio.

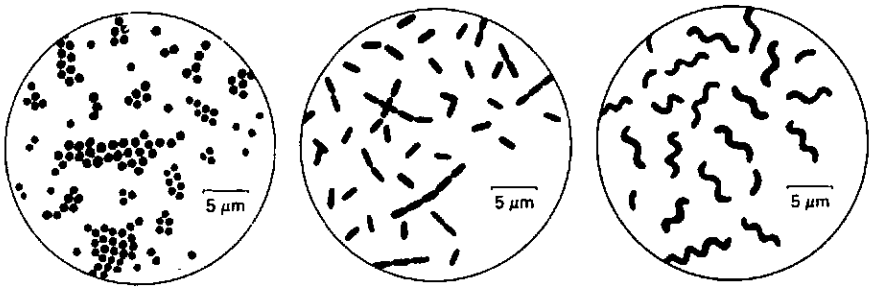

Figure 5-1. Bacteria are generally either (A) spherical (cocci); (B) rodlike (rods or bacilli); or (C) helical (spirilla). However, there are many modifications of these three basic forms. *(Erwin F. Lessel, illustrator.)*

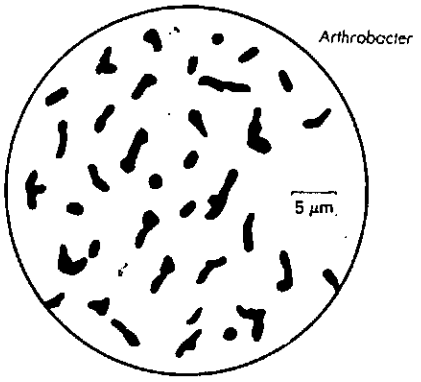

Figure 5-2. Drawing of pleomorphic cells of the genus *Arthrobacter*. *(Erwin F. Lessel, illustrator.)*

Figure 5-4. Photomicrograph of the trichomes of *Saprospira grandis*, composed of individual cylindrical cells that are 1 to 5 μm long and closely attached to one another (X1,650). *(Courtesy of G. J. Hageage, Jr.)*

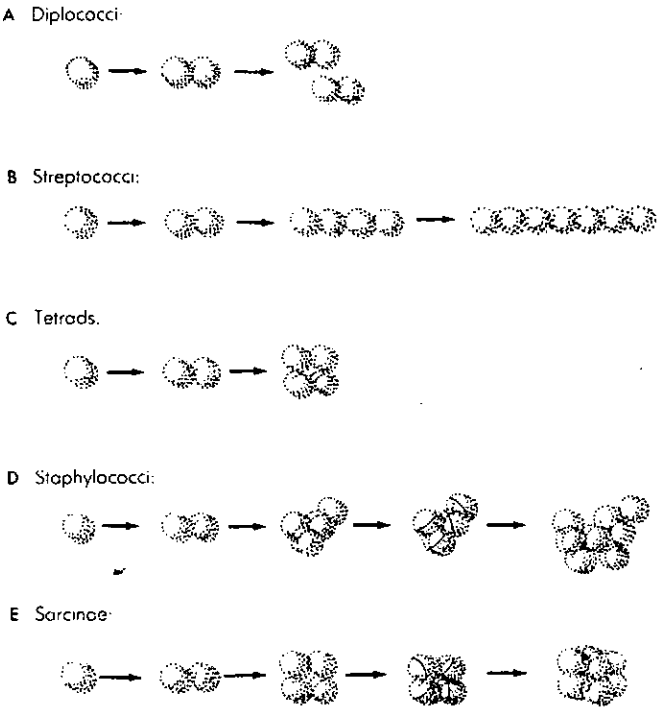

Figure 5-3. Characteristic arrangements of cocci, with schematic illustrations of patterns of multiplication. (A) Diplococci: cells divide in one plane and remain attached predominantly in pairs. (B) Streptococci: cells divide in one plane and remain attached to form chains. (C) Tetracocci: cells divide in two planes and characteristically form groups of four cells. (D) Staphylococci: cells divide in three planes, in an irregular pattern, producing "bunches" of cocci. (E) Sarcinae: cells divide in three planes, in a regular pattern, producing a cuboidal arrangement of cells.

Bacterial cells are usually arranged in a manner characteristic of their particular species. Although it is rare that all the cells of a species are arranged in the same manner, it is the predominant arrangement that is the important feature.

Cocci appear in several characteristic arrangements, depending on the plane of cellular division and whether the daughter cells stay together following division (Fig. 5-3). Bacilli are not arranged in patterns as complex as those of cocci, and most occur singly or in pairs **(diplobacilli)**.But some species, such as *Bacillus subtilis*, form chains **(streptobacilli)**;others, such as *Beggiatoa* and *Saprospira* species, form **trichomes**,which are similar to chains but have a much larger area of contact between the adjacent cells (Fig. 5-4). In other bacillus species, such as *Corynebacterium diphtheriae*, the cells are lined side by side like matchsticks **(palisade arrangement)**and at angles to one another (Fig. 5-5).

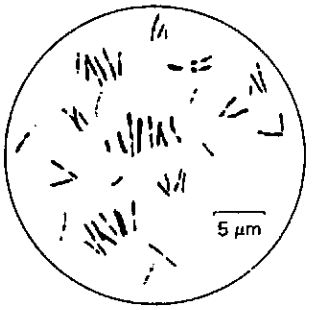

Figure 5-5. Drawing of the cells of *Corynebacterium diphtheriae* showing palisade arrangements. *(Erwin F. Lessel, illustrator.)*

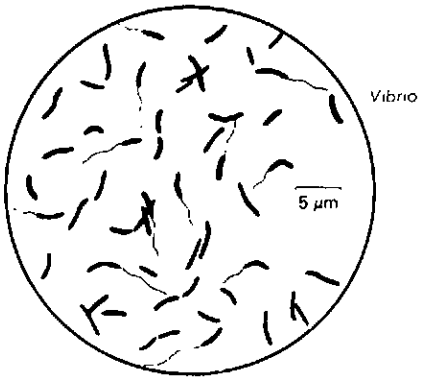

Figure 5-7. Drawing of cells of the genus *Vibrio*, showing the characteristic curved shape and the polar flagella. The flagella are not visible by ordinary staining procedures. *(Erwin F. Lessel, illustrator.)*

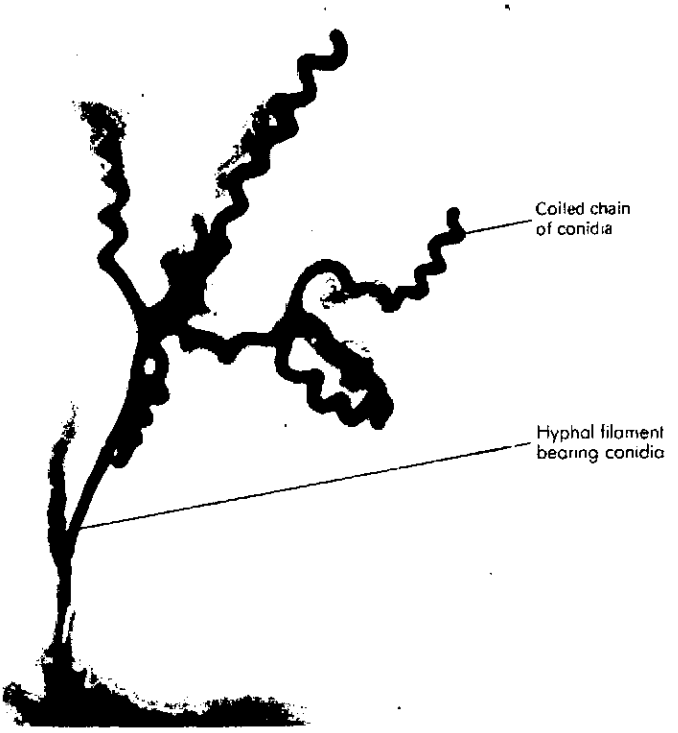

Figure 5-6. Photomicrograph of *Streptomyces viridochromogenes*. This bacterium produces coiled chains of spores (called conidia) which develop at the ends of vegetative filaments called hyphae. *(Courtesy of Mary P. Lechevalier.)*

Still others, such as *Streptomyces* species, form long, branched, multinucleate filaments called hyphae (singular, hypha) which collectively form a mycelium (Fig. 5-6). (Note that the terms *hyphae* and *mycelium* are also commonly applied to the filaments formed by fungi, described in Chap. 17).

Curved bacteria are usually curved with a twist. Bacteria with less than one complete twist or turn have a vibrioid shape (Fig. 5-7), whereas those with one or more complete turns have a helical shape. Spirilla are rigid helical bacteria, whereas spirochetes are highly flexible (Fig. 5-8).

In addition to the common bacterial shapes, many others also occur: pear-shaped cells (e.g., *Pasteuria*); lobed spheres (e.g., *Sulfolobus*); rods with squared rather than the usual hemispherical ends (e.g., *Bacillus anthracis*); disks arranged like stacks of coins (e.g., *Caryophanon*); rods with helically sculptured surfaces (e.g., *Seliberia*); and many others.

BACTERIAL STRUCTURES

Examination of a bacterial cell reveals various component structures. Some of these are external to the cell wall (Fig. 5-9); others are internal to the cell wall

(Fig. 5-10). Some structures are present in only certain species; some are more characteristic of certain species than of others; and still other cellular parts, such as the cell wall, are naturally common to almost all bacteria. The following are brief descriptions of the readily evident structures of bacteria.

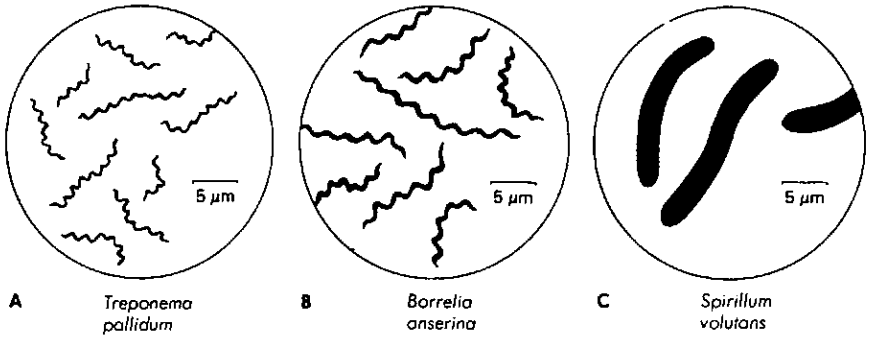

Figure 5-8. Drawings of spirochetes (A and B) and spirilla (C). Spirochetes are flexible and can twist and contort their shape, whereas spirilla are relatively rigid. *(Erwin F. Lessel, illustrator.)*

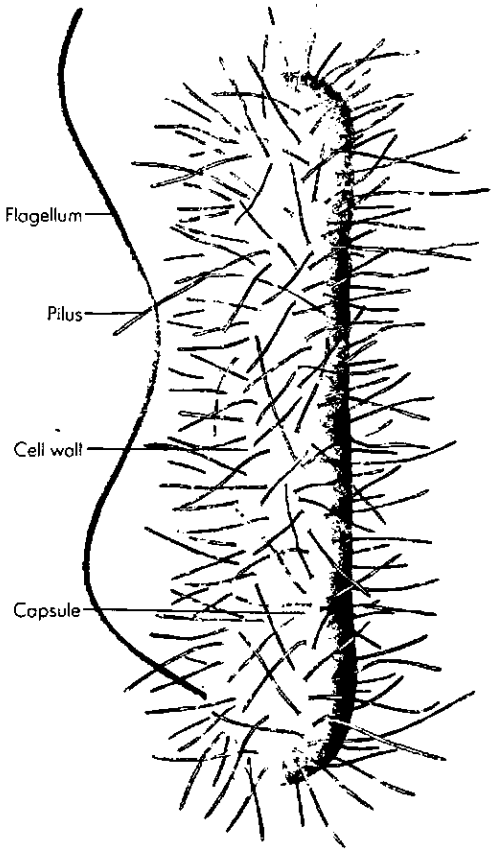

Figure 5-9. Drawing of the major structures external to the bacterial cell wall. Certain structures, e.g., capsules, flagella, and pili, are not common to all bacterial cells. *(Erwin F. Lessel, illustrator.)*

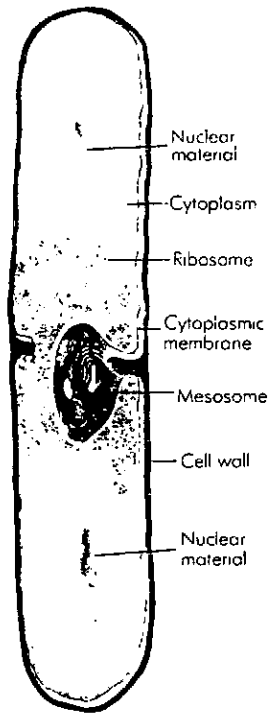

Figure 5-10. Drawing of the major structures which occur within the bacterial cell wall. Certain structures, e.g., mesosomes, are not common to all bacterial cells. *(Erwin F. Lessel, illustrator.)*

STRUCTURES EXTERNAL TO THE CELL WALL

Flagella and Motility

Bacterial **flagella** (singular, **flagellum**) are hairlike, helical appendages that protrude through the cell wall and are responsible for swimming motility. They are much thinner than the flagella or cilia of eucaryotes, being 0.01 to 0.02 μm in diameter, and they are also much simpler in structure. Their location on the cell varies depending on the bacterial species and may be **polar** (at one or both ends of the bacterium) or **lateral** (along the sides of the bacterium). Some arrangements of bacterial flagella are shown in Fig. 5-11. A flagellum is composed of three parts (Fig. 5-12): a **basal body** associated with the cytoplasmic membrane and cell wall, a short **hook**, and a helical **filament** which is usually several times as long as the cell. Some Gram-negative bacteria have a sheath surrounding the flagellum; this sheath is continuous with the outer membrane of the Gram-negative cell wall. The chemical composition of the basal body is unknown, but the hook and filament are composed of protein subunits (monomers) arranged in a helical fashion. The protein of the filament is known as **flagellin**.

Unlike a hair, a flagellum grows at its tip rather than at the base. Flagellin monomers synthesized within the cell are believed to pass along the hollow center of the flagellum and are added to the distal end of the filament.

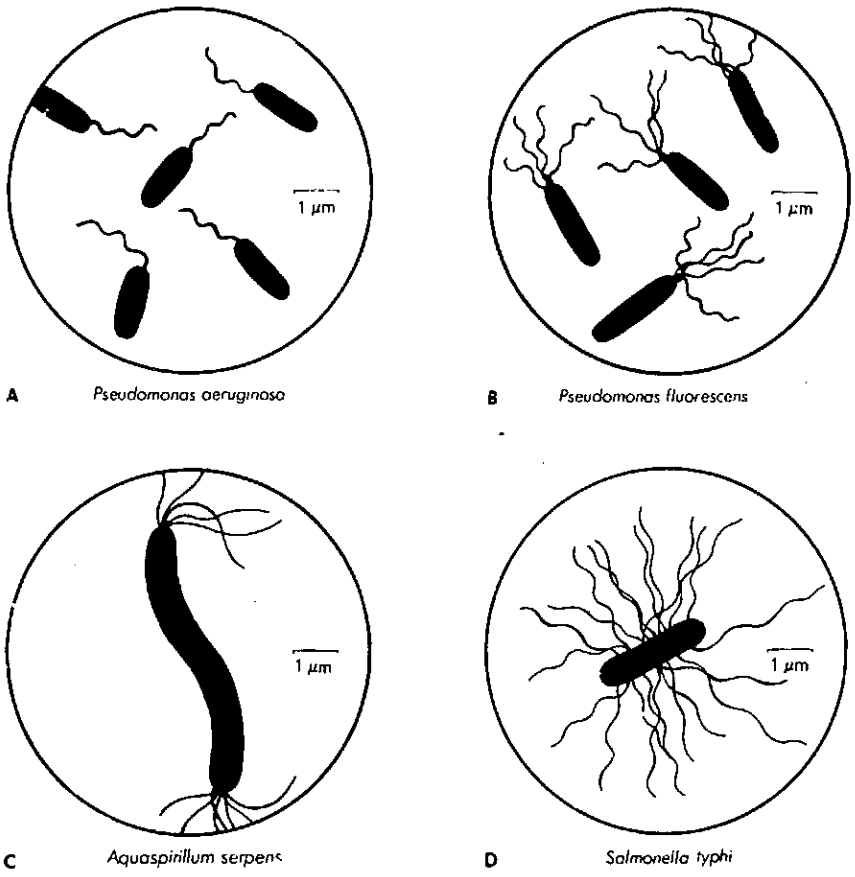

Figure 5-11. Drawings of various arrangements of bacterial flagella. (A) Monotrichous; a single polar flagellum. (B) Lophotrichous; a cluster of polar flagella. (C) Amphitrichous; flagella, either single or clusters, at both cell poles. (D) Peritrichous; surrounded by lateral flagella. *(Erwin F. Lessel, illustrator.)*

Hydrodynamics of Flagella

Large motile bodies such as boats and fish make use of the inertia of water for their propulsion. When pushed against with, for example, an oar, a propeller blade, or fins, the water temporarily acts as a solid, thereby enabling the boat or fish to generate a forward propulsive force. However, the small size of bacteria prohibits their use of the inertia of water to gain propulsive force, because the drag forces due to the viscosity of water become thousands of times greater than any forces that can be generated from inertia. The difficulty would be similar to what we would encounter if we attempted to row a boat on a lake filled with thick molasses. However, bacteria can swim many times their own length per second under analogous conditions!

Bacteria propel themselves by rotating their helical flagella. The principle involved can be illustrated by imagining the penetration of a piece of cork by a corkscrew. If one tries to ram the corkscrew directly through the cork, great force will probably be needed. On the other hand if one merely rotates the corkscrew, the cork can be easily penetrated. In the case of bacteria, the cork is analogous to the viscous medium and the corkscrew to the helical flagellum. It is apparent from this analogy that a mutant bacterium having straight rather than helical flagella would be unable to swim. The nature of the rotary motor that spins each corkscrew-shaped flagellum is still not understood, but the rings found in the basal body (Fig. 5-12) are probably involved. It is known that the flagellar motor is driven by the protonmotive force, i.e., the force derived from the electrical potential and the hydrogen-ion gradient across the cytoplasmic membrane (see Chap. 10). Moreover, recent studies suggest that the concentration of cGMP (guanosine 3',5'-cyclic phosphoric acid) within the cell governs the direction in which the rotation occurs.

Bacteria having polar flagella swim in a back-and-forth fashion; they reverse their direction of swimming by reversing the direction of flagellar rotation. Bacteria having lateral flagella swim in a more complicated manner. Their flagella operate in synchrony to form a bundle that extends behind the cell (Fig.

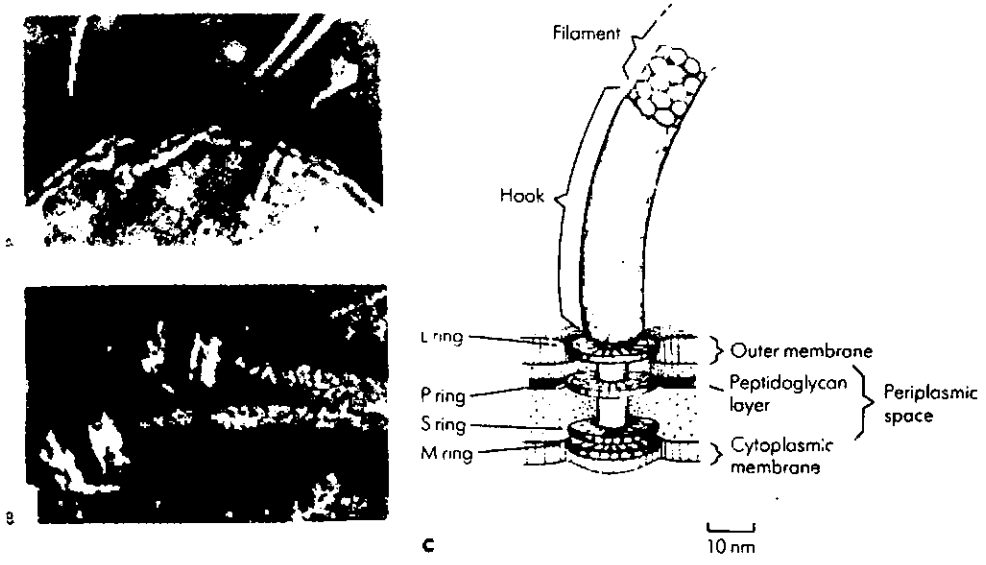

Figure 5-12. The mechanism of attachment of flagella to a Gram-negative bacterial cell (*Pseudomonas aeruginosa*). (A) Prior to electron-microscope examination, the cells were partially lysed and then negatively stained to make the point of flagellar attachment (basal body) more visible (X80,000 approx.). (B) Isolated flagella showing basal body at one end. (C) Model of basal body illustrating its structure and attachment to a Gram-negative bacterium. The flagella of Gram-positive bacteria have only two basal rings. (*Courtesy of T. Iino, University of Tokyo.*)

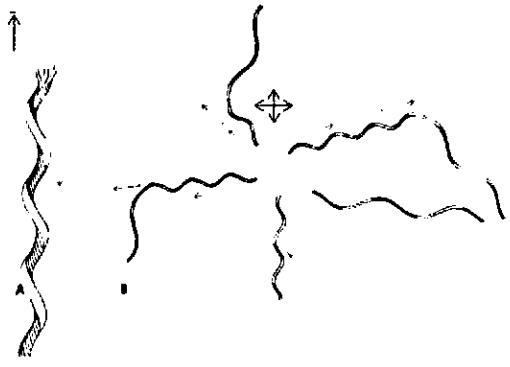

Figure 5-13. Diagram of the configuration and arrangement of peritrichous flagella during swimming and tumbling. The small arrows indicate the direction of propagation of helical waves along the flagella. (A) During swimming the flagella are in the form of left-handed helices and rotate counterclockwise in synchrony to form a bundle. The large arrow indicates the direction of swimming. (B) During tumbling the flagella reverse their rotation, portions of the flagella acquire a short wavelength and right-handed configuration, and the bundle flies apart. The cell cannot swim under these conditions and instead exhibits a chaotic motion, as symbolized by the large crossed arrows. *(Courtesy of R. M. MacNab and M. K. Ornston, J Mol Biol* **112**:*1, 1977.)*

5-13). However, when the flagellar motors reverse, conformational changes occur along the flagella, the bundle flies apart, and the cell tumbles wildly. Finally, the flagellar motors resume their normal direction, the flagellar bundle again forms, and the cell begins to swim—but now in a different direction. This sequence of events occurs repeatedly, so that the motility becomes a series of periods of swimming (runs) punctuated by periods of tumbling (twiddles), with a change in direction after each tumble.

Swimming Motility Without Flagella

Certain helical bacteria (spirochetes) exhibit swimming motility, particularly in highly viscous media, yet they lack external flagella. However, they have flagellalike structures located within the cell, just beneath the outer cell envelope (see Fig. 13-1). These are called **periplasmic flagella**; they have also been termed **axial fibrils** or **endoflagella**. They are responsible for the motility of spirochetes, but how they accomplish this is not yet clear. Other helical bacteria called **spiroplasmas** are able to swim in viscous media, yet lack any apparent organelles for motility, even periplasmic flagella. The mechanism for their motility is completely unknown.

Gliding Motility

Some bacteria, e.g., *Cytophaga* species, are motile only when they are in contact with a solid surface. As they glide they exhibit a sinuous, flexing motion. This kind of movement is comparatively slow, only a few μm per second. The mechanism of gliding motility is unknown; no organelles responsible for motility have been observed.

Bacterial Chemotaxis

Many, perhaps most, motile bacteria are capable of directed swimming toward or away from various chemical compounds—a phenomenon called **bacterial chemotaxis**. Swimming toward a chemical is termed **positive chemotaxis**; swimming away is **negative chemotaxis**. Although chemicals may act as attractants or repellents, the stimulus is in fact not the chemical itself but rather a change in the concentration of the chemical with time, i.e., a **temporal gradient**. Such gradients are sensed by means of protein **chemoreceptors** which are located on the cytoplasmic membrane and are specific for various attractants and repellents.

By means of its chemoreceptors, a bacterium continually compares its immediate environment with the environment it had experienced a few moments

earlier. To illustrate this, suppose we are observing the behavior of a bacterium that has peritrichous flagella and for which glucose is an attractant. If the cell is placed in a homogeneous glucose broth, the glucose concentration remains constant regardless of the direction of the bacterium's swimming, and the glucose-specific chemoreceptors can sense no change in glucose concentration. Consequently, the cell exhibits a normal swimming pattern—periods of swimming with intermittent periods of tumbling. Suppose that the cell is now placed in a long capillary tube with a higher concentration of glucose at one end than at the other. If the cell happens to swim toward the higher concentration of glucose (i.e., in the "right" direction), the chemoreceptors sense that the glucose concentration is increasing with time. This results in suppression of normal tumbling, causing the cell to swim smoothly ahead for a long period before it tumbles. On the other hand, if the cell happens to swim toward the end of the tube where there is less glucose (i.e., in the "wrong" direction), the chemoreceptors sense that the glucose concentration is decreasing with time, and no suppression of tumbling occurs. Therefore, the cell soon tumbles, changes direction, and tries again until finally the "right" direction is achieved. (In a gradient of a repellent compound, the right direction would be *down* the gradient, i.e., toward a *decreasing* concentration, and the wrong direction would be up the gradient.)

Tactic responses are not limited to chemical gradients. For instance, phototrophic bacteria exhibit positive phototaxis toward increasing light intensities and are repelled by decreasing light intensities. Still another type of taxis is exhibited by *Aquaspirillum magnetotacticum*; this organism exhibits directed swimming in response to the earth's magnetic field or to local magnetic fields (magnets placed near the culture). This is attributed to a chain of magnetite inclusions (magnetosomes) within the cell, which allows the cell to become oriented as a magnetic dipole (Fig. 5-14). Because of the downward inclination of the Earth's magnetic field in the regions where these bacteria have been found, magnetotaxis may serve to direct the cells downward in aquatic environments toward oxygen-deficient areas more favorable for growth.

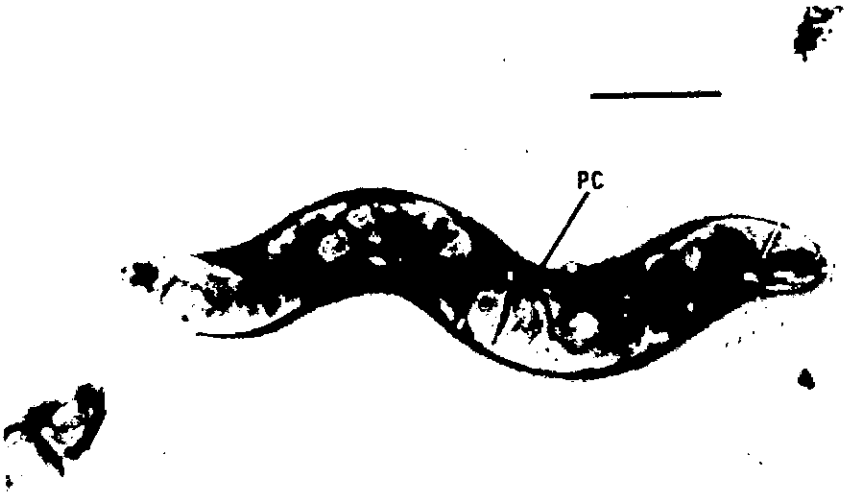

Figure 5-14. Negatively stained cell of *Aquaspirillum magnetotacticum* showing a particle chain (PC) of highly electron-dense magnetite inclusions (magnetosomes) within the cell. The bar represents 1 μm. (*Courtesy of D. L. Balkwill, D. Maratea and R. P. Blakemore, J. Bacteriol* **141**:1399, 1980.)

Pili (Fimbriae)

Pili (singular, **pilus**) are hollow, nonhelical, filamentous appendages that are thinner, shorter, and more numerous than flagella (Fig. 5-15). They do not function in motility, since they are found on nonmotile as well as motile species. There are, however, several functions associated with different types of pili. One type, known as the F pilus (or sex pilus), serves as the port of entry of genetic material during bacterial mating (see Chap. 12). Some pili play a major role in human infection by allowing pathogenic bacteria to attach to epithelial cells lining the respiratory, intestinal, or genitourinary tracts. This attachment prevents the bacteria from being washed away by the flow of mucous or body fluids and permits the infection to be established.

Capsules

Some bacterial cells are surrounded by a viscous substance forming a covering layer or envelope around the cell wall. If this layer can be visualized by light microscopy using special staining methods, it is termed a **capsule.** If the layer is too thin to be seen by light microscopy it is termed a **microcapsule;** if it is so abundant that many cells are embedded in a common matrix, the material is called **slime.**

By light microscopy, capsules appear to be amorphous gelatinous areas surrounding a cell (Fig. 5-16A); however, special techniques designed to preserve delicate structures for observation by electron microscopy have revealed that capsules consist of a mesh or network of fine strands (Fig. 5-16B).

In many instances capsular material is not highly water-soluble and therefore does not readily diffuse away from the cells that produce it. In other instances

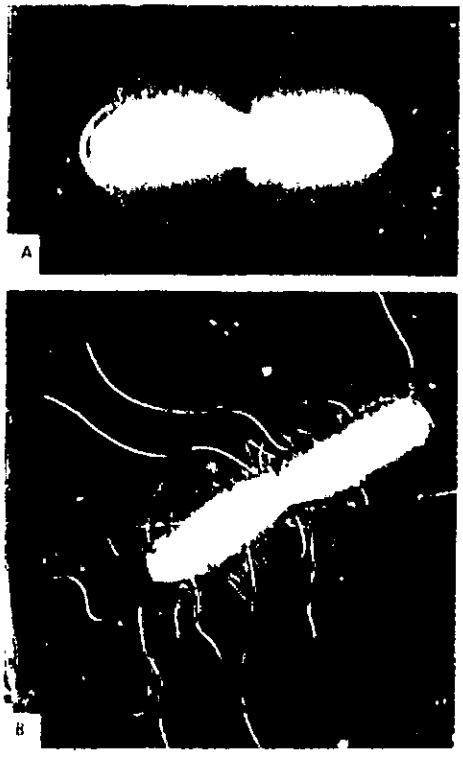

Figure 5-15. Fimbriated bacteria. (A) *Shigella flexneri:* dividing bacilli with numerous fimbriae surrounding the cells (X20,000). (B) *Salmonella typhi:* dividing bacilli with numerous fimbriae and a few flagella (the very long appendages) (X12,500). *(Courtesy of J. P. Duguid and J. F. Wilkinson and The Society of General Microbiology: Symposium XI. 1961.)*

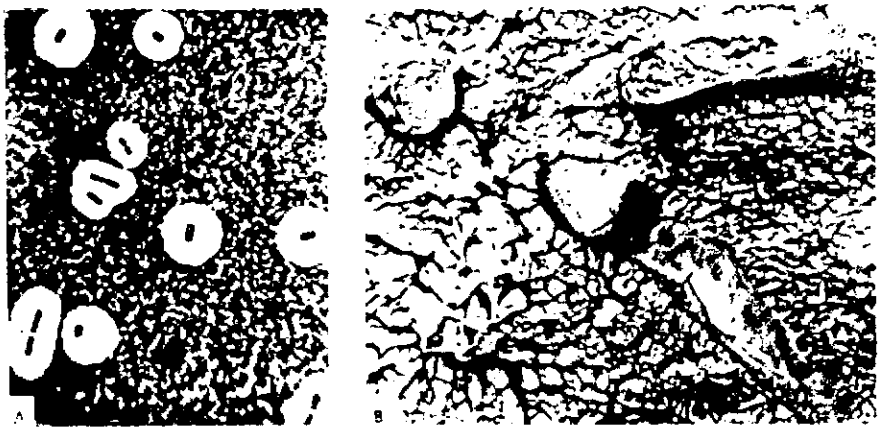

Figure 5-16. Bacterial capsules as seen by light microscopy (A) and electron microscopy (B). (A) India-ink preparation of a capsulated bacterium isolated from a paper-mill operation. The particles of carbon in the ink cannot penetrate the capsules (white areas around the cells). *Courtesy of P. M. Borick, Wallace and Tiernan, Inc.)* (B) Freeze-etch preparation of Gram-positive rod-shaped bacteria isolated from acid mine water, showing a fibrillar polymer network surrounding the cells. The freeze-fracture process has also revealed various internal and surface structures of the cells. *(Courtesy of P. R. Dugan, C. B. MacMillan, and R. M. Pfister, J. Bacteriol* **101**:*982, 1970.)*

the material is highly water-soluble and dissolves in the medium, sometimes dramatically increasing the viscosity of the broth in which the organisms are cultured.

Capsules can serve a number of functions, depending on the bacterial species (1) They may provide protection against temporary drying by binding water molecules. (2) They may block attachment of bacteriophages. (3) They may be antiphagocytic; i.e., they inhibit the engulfment of pathogenic bacteria by white blood cells and thus contribute to invasive or infective ability (virulence). (4) They may promote attachment of bacteria to surfaces; for example, *Streptococcus mutans*, a bacterium associated with producing dental caries, firmly adheres to the smooth surfaces of teeth because of its secretion of a water-insoluble capsular glucan. (5) If capsules are composed of compounds having an electrical charge, such as sugar–uronic acids, they may promote the stability of bacterial suspension by preventing the cells from aggregating and settling out, because cells bearing similarly charged surfaces tend to repel one another.

Most bacterial capsules are composed of polysaccharides. Capsules composed of a single kind of sugar are termed **homopolysaccharides;** are usually synthesized outside the cell from disaccharides by exocellular enzymes. The synthesis of glucan (a polymer of glucose) from sucrose by *S. mutans* is an example. Other capsules are composed of several kinds of sugars and are termed **heteropolysaccharides;** these are usually synthesized from sugar precursors that are activated (energized) within the cell, attached to a lipid carrier molecule, transported across the cytoplasmic membrane, and polymerized outside the cell. The capsule of *Klebsiella pneumoniae* is an example.

A few capsules are polypeptides. For example, the capsule of the anthrax organism, *B. anthracis*, is composed entirely of a polymer of glutamic acid. Moreover, this peptide is an unusual one because the glutamic acid is the rare D optical isomer rather than the usual L isomer commonly found in nature.

Sheaths

Some species of bacteria, particularly those from freshwater and marine environments, form chains or trichomes that are enclosed by a hollow tube called a This structure is most readily visualized when some of the cells have

Figure 5-17. Sheathed bacteria. Sheath and cells of *Sphaerotilus natans* stained with nigrosin. Dimensions of individual cells are 1 μm by 2 to 6 μm, and the sheaths may reach a length of several millimeters. *(Courtesy of J. L. Stokes, J Bacteriol 67:279, 1954.)*

migrated from it (Fig. 5-17). Sheaths may sometimes become impregnated with ferric or manganese hydroxides, which strengthen them.

Prosthecae and Stalks

Prosthecae (singular, prostheca) are semirigid extensions of the cell wall and cytoplasmic membrane and have a diameter that is always less than that of the cell. They are characteristic of a number of aerobic bacteria from freshwater and marine environments. Some bacterial genera such as *Caulobacter* have a single prostheca; others such as *Stella* and *Ancalomicrobium* have several (Fig. 5-18). Prosthecae increase the surface area of the cells for nutrient absorption, which is advantageous in dilute environments. Some prosthecate bacteria may form a new cell (bud) at the end of a prostheca; others have an adhesive substance at the end of a prostheca that aids in attachment to surfaces.

Although the term stalk is sometimes used interchangeably with the terms *prostheca* or *hypha*, it is perhaps better to restrict its use to certain nonliving ribbonlike or tubular appendages that are excreted by the cell, such as those found in the genera *Gallionella* or *Planctomyces* (see Chap. 15). These stalks aid in attachment of the cells to surfaces.

THE CELL WALL

Beneath such external structures as capsules, sheaths, and flagella and external to the cytoplasmic membrane is the cell wall, a very rigid structure that gives shape to the cell. Its main function is to prevent the cell from expanding and eventually bursting because of uptake of water, since most bacteria live in hypotonic environments (i.e., environments having a lower osmotic pressure than exists within the bacterial cells). The rigidity of the wall can be readily demonstrated by subjecting bacteria to very high pressures or other severe physical conditions: most bacterial cells retain their original shapes during and after such treatments. To obtain isolated cell walls for analysis, bacteria usually must be mechanically disintegrated by drastic means, as by sonic or ultrasonic treatment or by exposure to extremely high pressures with subsequent sudden

Figure 5-18. *Ancalomicrobium adetum*, a budding bacterium with several prosthecae per cell. Electron micrograph of whole cell, negatively stained. The bar represents 1.0 μm. *(Courtesy of J. T. Staley, J Bacteriol* **95**:1921, 1968.)

release of pressure. The broken cell walls are then separated from the rest of the components of the disintegrated cells by differential centrifugation. Isolated cell walls, devoid of other cellular constituents, retain the original contour of the cells from which they were derived.

Among the ordinary or typical bacteria (which are sometimes called eubacteria to distinguish them from the phylogenetically distinct group known as the archaeobacteria, discussed in Chap. 3), the walls of Gram-negative species are generally thinner (10 to 15 nm) than those of Gram-positive species (20 to 25 nm). The walls of Gram-negative archaeobacteria are also thinner than those of Gram-positive archaeobacteria. Since the chemical composition of the walls of archaeobacteria is quite different from that of eubacteria, wall thickness rather than chemical composition may be the major factor in the Gram reaction.

The cell wall constitutes a significant portion of the dry weight of the cell; depending on the species and culture conditions, it may account for as much as 10 to 40 percent. Bacterial cell walls are usually essential for bacterial growth and division. Cells whose walls have been completely removed (i.e., protoplasts) are incapable of normal growth and division.

Structure and Chemical Composition

For eubacteria, the shape-determining part of the cell wall is largely peptidoglycan (sometimes called murein), an insoluble, porous, cross-linked polymer of enormous strength and rigidity. Peptidoglycan is found only in procaryotes; it occurs in the form of a "bag-shaped macromolecule" surrounding the cytoplasmic membrane. Peptidoglycan differs somewhat in composition and structure from one species to another, but it is basically a polymer of *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-alanine, D-glutamate, and a diamino acid (LL- or meso-diaminopimelic acid, L-lysine, L-ornithine, or L-diaminobutyric acid). The structure of this polymer is depicted in Figs. 11-6 and 11-7. It is important to realize that as tough as peptidoglycan is, it is also in a dynamic state. That is, in order for the cell to grow and divide, portions of the peptidoglycan must continually be degraded by wall-associated hydrolytic enzymes so that new polymer can be added.

Peptidoglycan

Walls of Archaeobacteria

Although most archaeobacteria possess cell walls, these do not contain peptidoglycan, and their cell-wall fine structure and chemical composition is very different from that of eubacteria. Their walls are usually composed of proteins, glycoproteins, or polysaccharides. A few genera, such as *Methanobacterium*, have walls composed of pseudomurein, a polymer whose structure superficially resembles eubacterial peptidoglycan but which differs markedly in chemical composition (see Chap. 15).

Walls of Gram-Positive Eubacteria

Gram-positive bacteria usually have a much greater amount of peptidoglycan in their cell walls than do Gram-negative bacteria; it may account for 50 percent or more of the dry weight of the wall of some Gram-positive species, but only

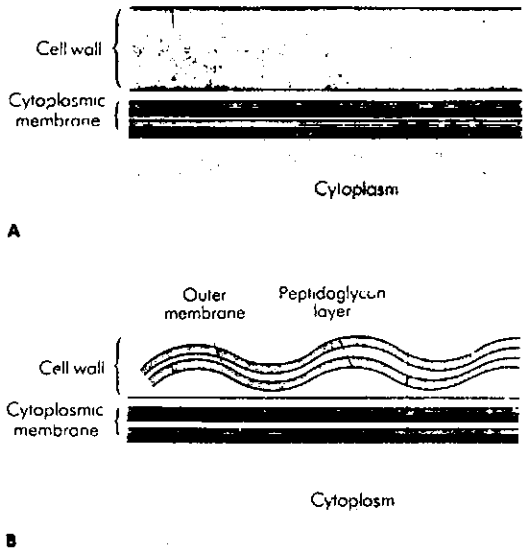

Figure 5-19. Schematic interpretation of cell walls of eubacteria from electron-microscope observations. (A) Gram-positive bacteria, showing thick wall consisting mainly of peptidoglycan. Although the wall is often homogeneous in appearance, in some bacteria it may consist of several layers. (B) Gram-negative bacteria, showing outer membrane and thin peptidoglycan layer. *(Courtesy of A. I. Laskin and H. A. Lechevalier (eds.), Handbook of Microbiology, CRC Press, Inc., Cleveland, 1974.)*

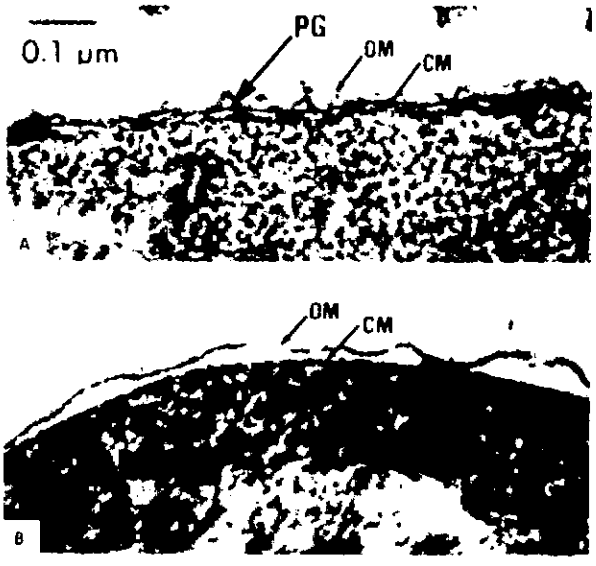

Figure 5-20. (A) Thin section of *Aquaspirillum serpens* showing the wavy outer membrane (OM), the peptidoglycan layer (PG), and the cytoplasmic membrane (CM). (B) Companion preparation of a spheroplast formed by treatment of the cells with a chelating agent and lysozyme. The peptidoglycan layer is missing. *(From R. G. E. Murray, P. Steed and H. E. Elson, Can J Microbiol* 11:547, 1965.)

about 10 percent of the wall of Gram-negative bacteria. Other substances may occur in addition to peptidoglycan. For instance, the walls of *Streptococcus pyogenes* contain **polysaccharides** that are covalently linked to the peptidoglycan and which can be extracted with hot dilute hydrochloric acid. The walls of *Staphylococcus aureus* and *Streptococcus faecalis* contain **teichoic acids**—acidic polymers of ribitol phosphate or glycerol phosphate—which are covalently linked to peptidoglycan and which can be extracted with cold dilute acid. Teichoic acids bind magnesium ions, and there is some evidence that they help to protect bacteria from thermal injury by providing an accessible pool of these cations for stabilization of the cytoplasmic membrane. The walls of most Gram-positive bacteria contain very little lipid, but those of *Mycobacterium, Corynebacterium*, and certain other genera are exceptions, being rich in lipids called These compounds have the following general structure:

$$R_1\text{—}\underset{\displaystyle OH}{\underset{|}{CH}}\text{—}\underset{\displaystyle R_2}{\underset{|}{CH}}\text{—}COOH.$$

where R_1 and R_2 are long hydrocarbon chains. The ability of mycobacteria to exhibit acid-fast staining (i.e., when stained, the cells cannot be decolorized

easily despite treatment with dilute acids) is correlated with the presence of cell wall mycolic acids. A mycolic acid derivative called **cord factor** (trehalose dimycolate) is toxic and plays an important role in the diseases caused by *C. diphtheriae* and *M. tuberculosis*, described in Chap. 36.

Walls of Gram-Negative Eubacteria

The walls of Gram-negative bacteria are more complex than those of Gram-positive bacteria. The most interesting difference is the presence of an **outer membrane** that surrounds a thin underlying layer of peptidoglycan (Figs. 5-19 and 5-20). Because of this membrane, the walls of Gram-negative bacteria are rich in lipids (11 to 22 percent of the dry weight of the wall), in contrast to those of Gram-positive bacteria. This outer membrane serves as an impermeable barrier to prevent the escape of important enzymes, such as those involved in cell wall growth, from the space between the cytoplasmic membrane and the outer membrane (**periplasmic space**). The outer membrane also serves as a barrier to various external chemicals and enzymes that could damage the cell. For example, the walls of many Gram-positive bacteria can be easily destroyed by treatment with an enzyme called **lysozyme**, which selectively dissolves peptidoglycan; however, Gram-negative bacteria are refractory to this enzyme because large protein molecules cannot penetrate the outer membrane. Only if the outer membrane is first damaged, as by removal of stabilizing magnesium ions by a chelating agent, can the enzyme penetrate and attack the underlying peptidoglycan layer (see Fig. 5-20B).

The outer membrane of the Gram-negative cell wall is anchored to the underlying peptidoglycan by means of **Braun's lipoprotein** (Fig. 5-21). The membrane is a bilayered structure consisting mainly of **phospholipids, proteins,** and **lipopolysaccharide (LPS).** The LPS has toxic properties and is also known as **endotoxin.** It occurs only in the outer layer of the membrane (Fig. 5-21) and is composed of three covalently linked parts: (1) **Lipid A**, firmly embedded in the membrane; (2) **core polysaccharide**, located at the membrane surface; and (3) polysaccharide **O antigens**, which extend like whiskers from the membrane surface into the surrounding medium (Fig. 5-21). Many of the serological prop-

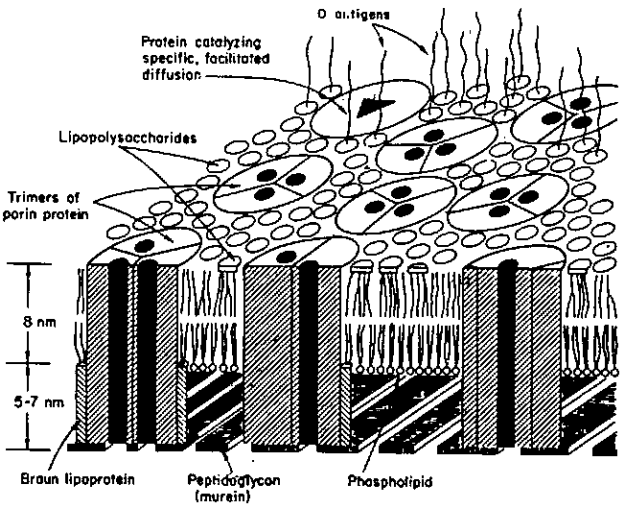

Figure 5-21. Tentative model of the cell wall of a Gram-negative bacterium like *Escherichia coli* or *Salmonella typhimurium*. Not shown is the cytoplasmic membrane, which is located below the peptidoglycan layer. The 8-nm-thick outer membrane of the cell wall is separated from the peptidoglycan layer by a 5 to 7 nm space. Molecules of Braun's lipoprotein extend across this space and anchor the outer membrane to the peptidoglycan. Porins extend from the external surface of the outer membrane down to the peptidoglycan layer. *(Courtesy of H. Nikaido and T. Nakae, Adv Microbial Physiol* **20**:163, 1979.)

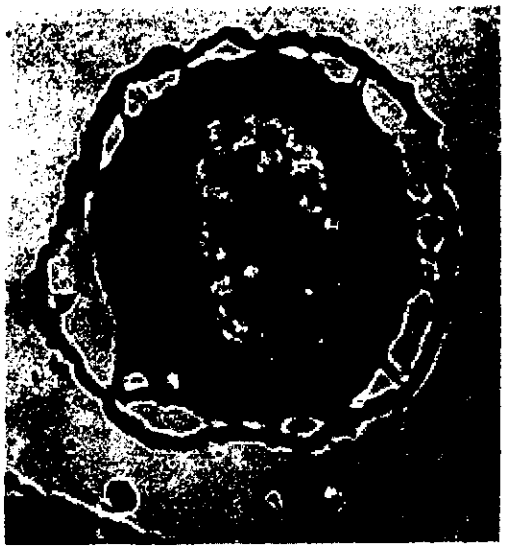

Figure 5-22. Thin section of an *Escherichia coli* cell that was plasmolyzed in a 20% sucrose solution, causing the protoplast to contract. Numerous adhesions are evident between the cytoplasmic membrane and the outer membrane of the cell wall. The light, fibrillar area in the center of the cell is the nuclear material. The bar represents 0.1 μm. *(Courtesy of M. E. Bayer, J Gen Microbiol* **53**:*395, 1968.)*

erties of Gram-negative bacteria are attributable to O antigens; they can also serve as receptors for bacteriophage attachment.

Although impermeable to large molecules such as proteins, the outer membrane can allow smaller molecules, such as nucleosides, oligosaccharides, monosaccharides, peptides, and amino acids, to pass across. This is accomplished by means of channels in special proteins called **porins**, which span the membrane (Fig. 5-21). The various porins are specific for different kinds or classes of small molecules, and some can even allow certain essential large molecules to penetrate, such as vitamin B_{12}. Many porins also serve as receptors for attachment of bacteriophages and bacteriocins.

One of the questions posed by the structure of Gram-negative cell walls is: How can water-insoluble, lipophilic substances such as LPS pass from their place of synthesis within the cytoplasm and cytoplasmic membrane across a watery periplasmic space to be inserted into the outer membrane? A likely explanation has been provided by the discovery of numerous **adhesions**, or points of direct contact between the two membranes (Fig. 5-22). These adhesions seem to be the export sites for newly synthesized LPS and porins, and they are also the sites at which pili and flagella are made.

Macromolecular Surface Arrays

The cell walls of some bacteria, both Gram-negative and Gram-positive, are covered by a mosaic layer of protein subunits (Fig. 5-23). The functions of these mosaic layers are not well understood, but at least one function is to protect Gram-negative bacteria against attack and penetration by other small, predatory bacteria known as bdellovibrios.

STRUCTURES INTERNAL TO THE CELL WALL

Immediately beneath the cell wall is the **cytoplasmic membrane**. This structure is approximately 7.5 nm (0.0075 μm) thick and is composed primarily of phos-

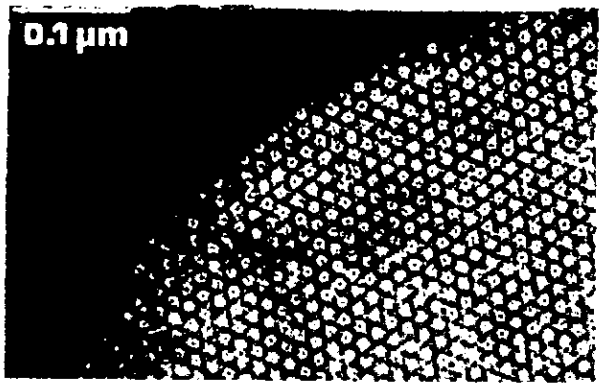

Figure 5-23. Electron micrograph showing a macromolecular surface array of protein subunits of the outer surface of a cell-wall fragment from *Aquaspirillum serpens*. *(Courtesy of R. G. E. Murray. From N. R. Krieg, Bacteriol Rev 40:55, 1976.)*

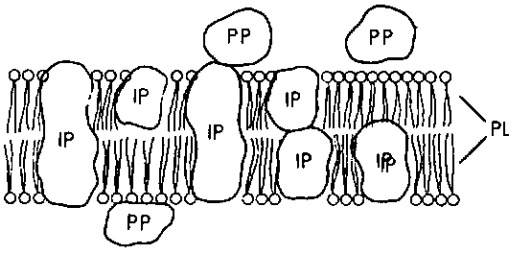

Figure 5-24. Schematic interpretation of the structure of the cytoplasmic membrane. Phospholipids (PL) are arranged in a bilayer such that the polar portions (circles) face outward and the nonpolar portions (filaments) face inward. IP = integral protein; PP = peripheral protein. Note that some integral proteins, such as transport proteins, are believed to span the membrane.

The Cytoplasmic Membrane

pholipids (about 20 to 30 percent) and proteins (about 60 to 70 percent). The phospholipids form a bilayer in which most of the proteins are tenaciously held (integral proteins) (Fig. 5-24); these proteins can be removed only by destruction of the membrane, as with treatment by detergents. Other proteins are only loosely attached (peripheral proteins) and can be removed by mild treatments such as osmotic shock. The lipid matrix of the membrane has fluidity, allowing the components to move around laterally. This fluidity appears to be essential for various membrane functions and is dependent on factors such as temperature and on the proportion of unsaturated fatty acids to saturated fatty acids present in the phospholipids.

A significant difference exists between the phospholipids of eubacteria and those of archaeobacteria. In eubacteria the phospholipids are phosphoglycerides, in which straight-chain fatty acids are ester-linked to glycerol (Fig. 5-25). In archaeobacteria, the lipids are polyisoprenoid branched-chain lipids, in which long-chain branched alcohols (phytanols) are ether-linked to glycerol (Fig. 5-25).

The cytoplasmic membrane is a hydrophobic barrier to penetration by most water-soluble molecules. However, specific proteins in the membrane allow, indeed facilitate, the passage of small molecules (i.e., nutrients and waste products) across the membrane; these transport systems are discussed in Chap. 11. The cytoplasmic membrane also contains various enzymes involved in respiratory metabolism and in synthesis of capsular and cell-wall components; moreover, because of its impermeability to protons (hydrogen ions), the cytoplasmic membrane is the site of generation of the protonmotive force—the force that drives ATP synthesis in many organisms, certain nutrient transport systems, and flagellar motility (see Chaps. 10 and 11). Consequently the cytoplasmic

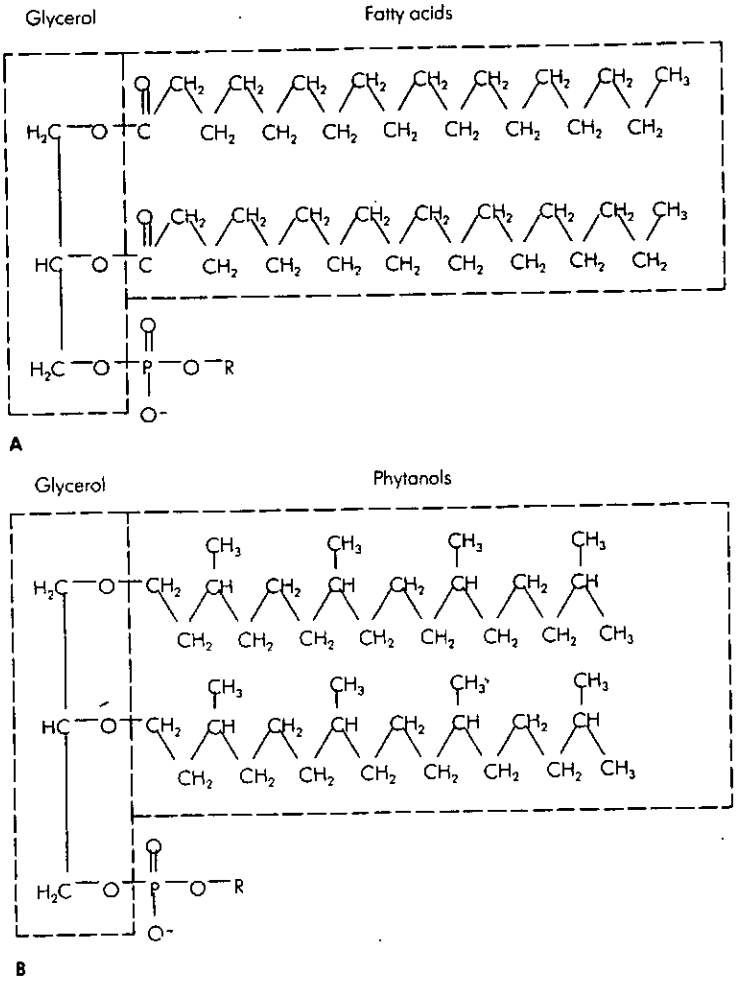

Figure 5-25. (A) Example of a eubacterial phospholipid, showing two unbranched, long-chain fatty acids ester-linked to glycerol. (B) Example of an archaeobacterial phospholipid, showing two branched phytanol chains that are ether-linked to glycerol. (R is any of several compounds such as ethanolamine, choline, serine, inositol, or glycerol.)

membrane is an extremely important functional structure, and damage to it by physical or chemical agents can result in the death of the cell.

Proteins are synthesized within the cell, but some can pass across the cytoplasmic membrane barrier to the outside; examples of such exported molecules are the protein components of cell walls (e.g., porins or lipoproteins) or the exocellular enzymes that are secreted by many bacteria into their culture medium, such as penicillinases, proteinases and amylases. Other proteins made within the cell may pass into the cytoplasmic membrane and remain there (e.g., enzymes such as cytochromes and membrane-bound dehydrogenases). The mechanism by which transport of these proteins occurs into or across the cytoplasmic membrane is unknown. A related question is: How does a cell "know" which of the many kinds of proteins within the cell to transport out of the cell? This question has been partially answered: The genes that code for these proteins carry a message that results in the addition of a sequence of about 20 extra amino acids (the **signal peptide**) to the proteins during their synthesis

within the cell. Unlike ordinary proteins, proteins carrying a signal peptide are destined to be transported into or across the cytoplasmic membrane. According to one hypothesis, special membrane proteins might bind the signal peptide at the inner surface of the cytoplasmic membrane and form a channel by which the protein can traverse the membrane. Whatever its function, the signal peptide is subsequently removed by a proteolytic enzyme and does not appear in the final, transported protein.

Protoplasts, Spheroplasts

Protoplasts

A **protoplast** is that portion of a bacterial cell consisting of the cytoplasmic membrane and the cell material bounded by it. Protoplasts can be prepared from Gram-positive bacteria by treating the cells with an enzyme such as lysozyme, which selectively dissolves the cell wall, or by culturing the bacteria in the presence of an antibiotic such as penicillin, which prevents the formation of the cell wall. In either case, the osmotic pressure of the medium must be sufficiently high to protect the organisms from bursting. Bacteria normally occur in hypotonic environments (i.e., environments having a lower osmotic pressure than that within the bacterial cells) and they continually take up water by osmosis; thus, they tend to expand, pressing the cytoplasmic membrane tightly against the rigid cell wall. In the absence of a rigid cell wall, there is nothing to prevent the continued expansion and eventual bursting of a protoplast. This bursting can be prevented by preparing protoplasts in an **isotonic** medium, i.e., in a medium that has an osmotic pressure similar to that of the protoplast. Such osmotically protected protoplasts are soft and fragile and are spherical, regardless of the original shape of the cell.

Spheroplasts

Round, osmotically fragile forms of Gram-negative bacteria can be prepared by procedures similar to those used for the protoplasts of Gram-positive bacteria. However, the cell walls of Gram-negative bacteria differ from those of Gram-positive bacteria by possessing an outer membrane. Although the peptidoglycan of the cell wall may be destroyed by lysozyme or its synthesis inhibited by antibiotics, the flexible outer membrane of the cell wall remains (Fig. 5-20B). Because the treated cell has two membranes, the cytoplasmic membrane of the protoplast plus the outer membrane of the cell wall, the cell is called a **spheroplast** rather than a protoplast.

Some bacteria, the mycoplasmas, never have cell walls and are bounded by only a cytoplasmic membrane; therefore, they have many of the properties of protoplasts, yet they manage to thrive nonetheless. Most mycoplasmas are parasites of animals, plants, or arthropods, and therefore live in osmotically favorable or isotonic environments. Some are able to attain a degree of rigidity by incorporating cholesterol into their cytoplasmic membranes. Most mycoplasmas have a more or less spherical shape, but one genus, *Spiroplasma*, consists of helical cells. How such cells are able to maintain this shape in the absence of a cell wall is unknown.

Membranous Intrusions and Intracellular Membrane Systems

Bacterial cells do not contain membrane-enclosed organelles corresponding to the mitochondria and chloroplasts of eucaryotic cells. However, bacteria may have specialized invaginations of the cytoplasmic membrane that can increase their surface area for certain functions.

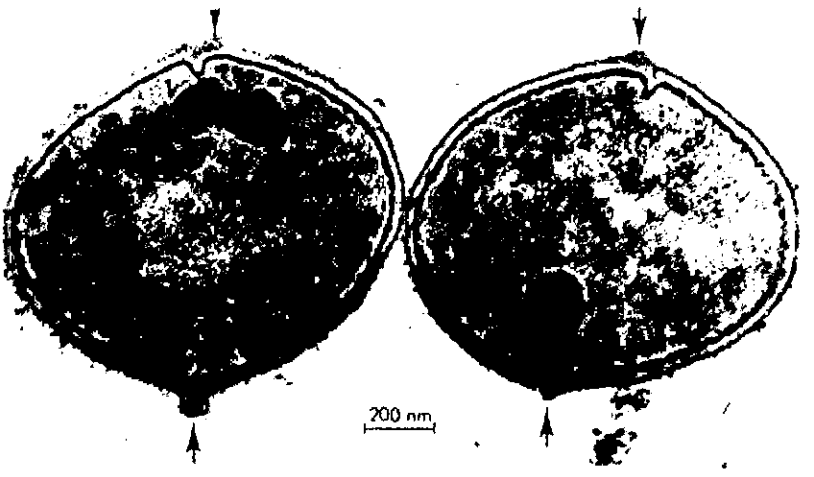

Figure 5-26. Thin section of the Gram-positive bacterium *Streptococcus faecalis*, showing the beginning stages of cell division occurring beneath a thickened equatorial ridge of the cell wall (arrows). A central mesosome (m) is present in each cell and is seen to be a complex invagination of the cytoplasmic membrane. Nuclear material (n) appears as a light, fibrillar area. *(Courtesy of J. M. Garland, A. R. Archibald and J. M. Baddiley, J Gen Microbiol* **89**:*73, 1975.)*

Many bacteria, especially Gram-positive bacteria, possess membrane invaginations in the form of systems of convoluted tubules and vesicles termed **mesosomes**. Those known as **central mesosomes** penetrate deeply into the cytoplasm, are located near the middle of the cell, and seem to be attached to the cell's nuclear material; they are thought to be involved in DNA replication and cell division (Fig. 5-26). In contrast, **peripheral mesosomes** show only a shallow penetration into the cytoplasm, are not restricted to a central location, and are not associated with nuclear material; they seem to be involved in export of exocellular enzymes such as penicillinase.

Extensive intracellular membrane systems occur in methane-oxidizing bacteria, in certain chemoautotrophic bacteria (Fig. 5-27), and in nearly all phototrophic bacteria. They serve to increase surface area for various metabolic activities. For example, in phototrophic bacteria they are the site of the photosynthetic apparatus of the cell; the infoldings provide a large surface area to accommodate a high content of light-absorbing pigments. In the phototrophs known as cyanobacteria, special intracellular membranes(thylakoids) occur that seem to be separate from the cytoplasmic membrane.

The Cytoplasm

The cell material bounded by the cytoplasmic membrane may be divided into (1) the cytoplasmic area, granular in appearance and rich in the macromolecular RNA-protein bodies known as **ribosomes**, on which proteins are synthesized; (2) the chromatinic area, rich in DNA; and (3) the fluid portion with dissolved substances. Unlike animal or plant cells, there is no endoplasmic reticulum to which ribosomes are bound; some ribosomes are free in the cytoplasm, and others, especially those involved in the synthesis of proteins to be transported out of the cell, are associated with the inner surface of the cytoplasmic membrane. When the ribosomes of procaryotes undergo sedimentation in a centrifuge, they have a sedimentation coefficient of 70 Svedberg units (70S) and are composed of two subunits, a 50S and a 30S subunit. This is in contrast to the

ribosomes of eucaryotic organisms, which have a sedimentation coefficient of 80S and are composed of a 60S and a 40S subunit.

Cytoplasmic Inclusions and Vacuoles

Concentrated deposits of certain substances are detectable in the cytoplasm of some bacteria. Volutin granules, also known as metachromatic granules, are composed of polyphosphate. They stain an intense reddish-purple color with dilute methylene blue and can be observed by light microscopy. By electron microscopy they appear as round, dark areas (Fig. 5-28). Volutin serves as a reserve source of phosphate. Another polymer often found in aerobic bacteria, especially under high-carbon, low-nitrogen culture conditions, is a chloroform-soluble, lipidlike material, poly-β-hydroxybutyrate, (PHB), which can serve as a reserve carbon and energy source. PHB granules can be stained with lipid-soluble dyes such as Nile blue. By electron microscopy they appear as clear round areas (Fig. 5-28). Polysaccharide granules, i.e., glycogen, can be stained brown with iodine. By electron microscopy they appear as dark granules (Fig. 5-28). Another type of inclusion is represented by the intracellular globules of elemental sulfur that may accumulate in certain bacteria growing in environments rich in hydrogen sulfide.

Some bacteria that live in aquatic habitats form gas vacuoles that provide buoyancy. By light microscopy these are bright, refractile bodies; by electron microscopy they are seen to have a regular shape: hollow, rigid cylinders with more or less conical ends and having a striated protein boundary. This boundary is impermeable to water, but the various dissolved gases in the culture medium can penetrate it to fill the cavity. The identifying feature of gas vacuoles is that they can be made to collapse under pressure and thereby lose their refractility.

Nuclear Material

In contrast to eucaryotic cells, bacterial cells contain neither a distinct membrane-enclosed nucleus nor a mitotic apparatus. However, they do contain an

Figure 5-27. Electron micrograph of a thin section of a chemoautotrophic bacterium, *Nitrosococcus oceanus*, showing an extensive intracellular membrane system. *(Courtesy of S. W. Watson.)*

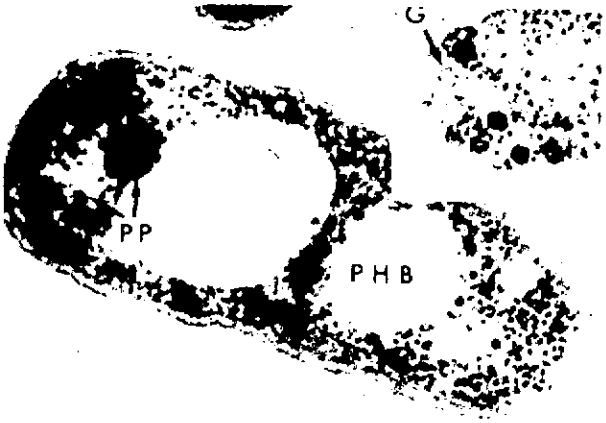

Figure 5-28. Thin section of *Pseudomonas pseudoflava* showing polyphosphate (volutin) granules (PP), poly-β-hydroxybutyrate granules (PHB), and glycogenlike granules (G). *(Courtesy of G. Auling, M. Reh and H. G. Schlegel, Int J Syst bacteriol* **28**:82, 1978.)

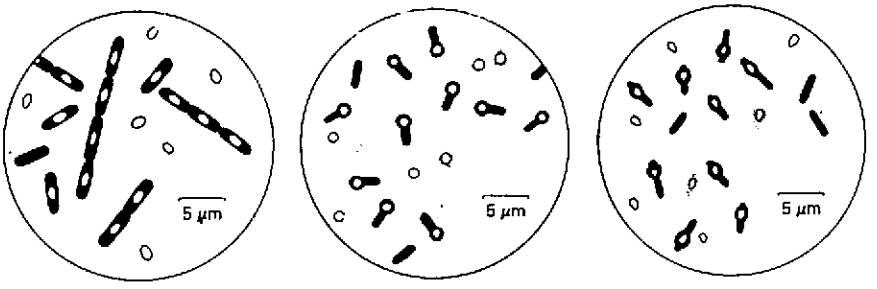

Figure 5-29. Drawings showing the location, size, and shape of endospores in cells of various species of *Bacillus* and *Clostridium*. *(Erwin F. Lessel, illustrator.)*

area near the center of the cell that is regarded as a nuclear structure, and the DNA of the cell is confined to this area. Because it is not a discrete nucleus, this nebulous structure has been designated by such terms as the **nucleoid**; the **chromatin body**; the **nuclear equivalent**; and even the **bacterial chromosome**, since it consists of a single, circular DNA molecule in which all the genes are linked. The nucleoid can be made visible under the light microscope by Feulgen staining, which is specific for DNA. By electron microscopy it appears as a light area with a delicate fibrillar structure (for example, see Figs. 5-22 and 5-26). The behavior of the nucleoid in growing, dividing bacteria has been observed by use of phase-contrast microscopy with a medium having a high refractive index.

SPORES AND CYSTS

Certain species of bacteria produce spores, either within the cell (**endospores**) or external to the cell (**exospores**). The spore is a metabolically dormant form which, under appropriate conditions, can undergo germination and outgrowth to form a vegetative cell.

Endospores

These structures are unique to bacteria. They are thick-walled, highly refractile bodies that are produced (one per cell) by *Bacillus*, *Clostridium*, *Sporosarcina*, *Thermoactinomyces*, and a few other genera. The shapes of endospores and also their location within the vegetative cell vary depending on the species (Fig. 5-29). The structural changes that occur during the development of endospores have been extensively studied in *Bacillus* and *Clostridium* species (Fig. 5-30). Endospores are usually produced by cells growing in rich media but which are approaching the end of active growth. Various factors such as aging or heat treatment are needed to activate the dormant spores (i.e., permit them to be able to undergo germination and outgrowth when they are placed in a suitable medium).

Endospores are extremely resistant to desiccation, staining, disinfecting chemicals, radiation, and heat. For example, the endospores of *Clostridium botulinum* type A have been reported to resist boiling for several hours. The degree of heat resistance of endospores varies with the bacterial species, but most can resist treatment at 80°C for at least 10 minutes. What causes this heat resistance has

been a subject of intense study, but the explanation is still not clear. During sporulation, a dehydration process occurs in which most of the water in the developing spore is expelled; the resulting dehydrated state may be an important factor for heat resistance.

All endospores contain large amounts of **dipicolinic acid (DPA)**, a unique compound that is undetectable in the vegetative cells yet can account for 10 to 15 percent of the spore's dry weight. It occurs in combination with large amounts of calcium and is probably located in the core, i.e., in the central part of the spore. The calcium-DPA complex may possibly play a role in the heat resistance of endospores. Synthesis of DPA and the uptake of calcium occur during advanced stages of sporulation.

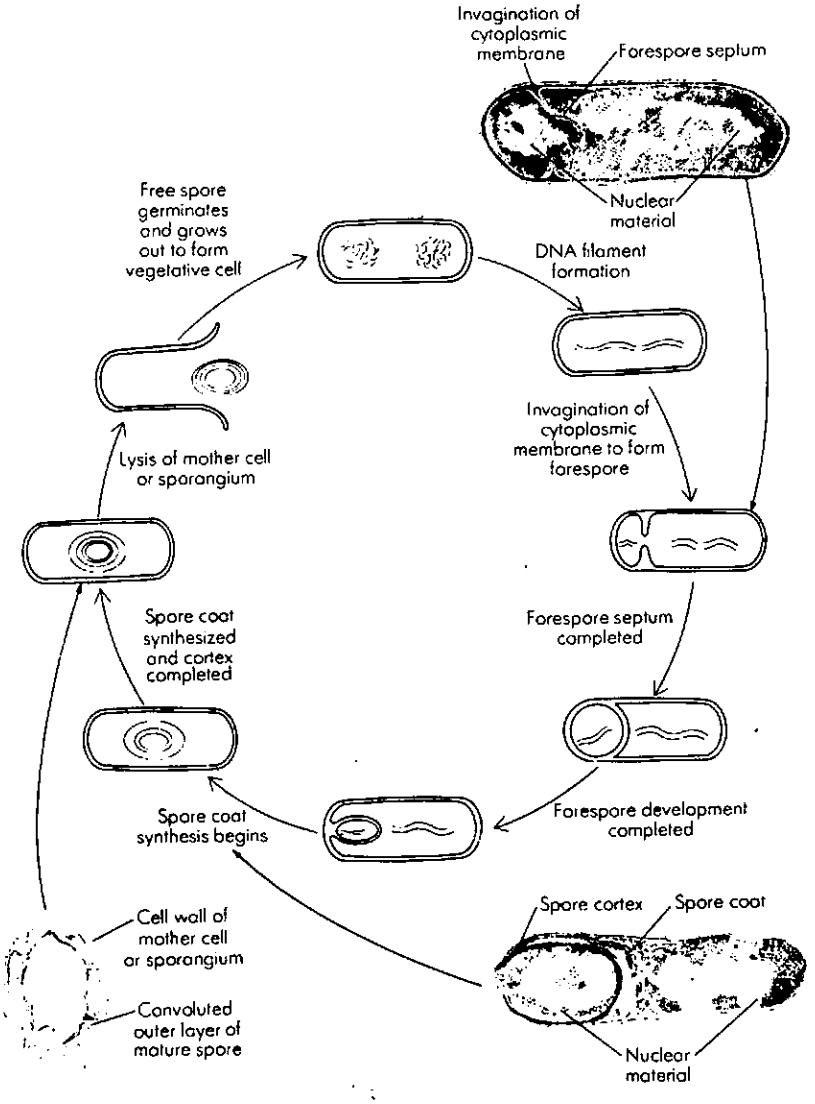

Figure 5-30. Structural changes in the bacterial cell during sporulation. *(Erwin F. Lessel, illustrator; redrawn, with modifications from L. E. Hawker and A. H. Linton, Microorganisms—Function, Form and Environment, 2d ed., University Park Press, Baltimore, 1979.)*

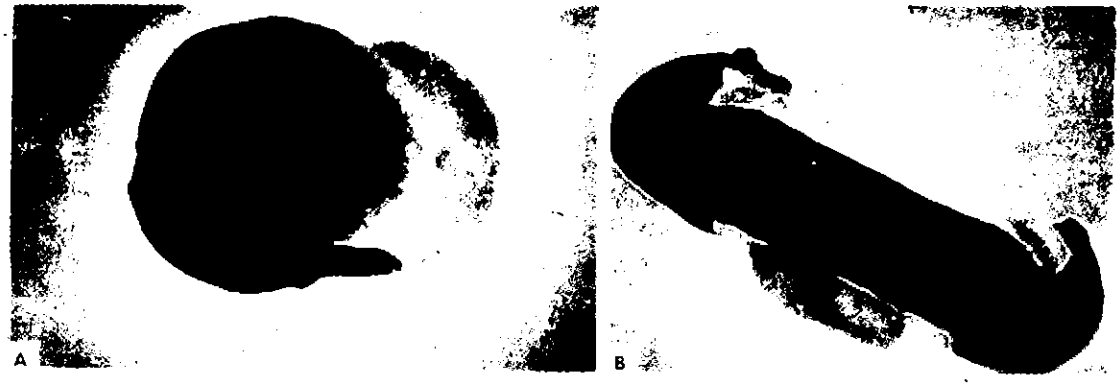

Figure 5-31. Outgrowth of spores from cultures of *Bacillus mycoides:* (A) grown 2 h at 35°C (X44,000), (B) grown 1¾ h at 35°C (X46,000). The two halves of the severed spore coat appear at the ends of the vegetative cell. *(SAB photos LS 203 and 204 courtesy of G. Knaysi, R. F. Baker, and J. Hillier, J Bacteriol,* **53**:525, 1947.)

Figure 5-32. Outgrowth of cysts from cultures of an *Azotobacter* strain. Vegetative cells are also evident. [*Courtesy of Y.-T. Tchan and P. B. New, from N. R. Krieg (ed.), Bergey's Manual of Systematic Bacteriology, vol. 1, Williams & Wilkins, Baltimore, 1984.*]

During germination, endospores lose their resistance to heat and staining. Subsequent outgrowth occurs, characterized by synthesis of new cell material and development of the organism into a growing cell (Fig. 5-31).

Exospores

Cells of the methane-oxidizing genus *Methylosinus* form exospores, i.e., spores external to the vegetative cell, by budding at one end of the cell. These are desiccation- and heat-resistant, but unlike endospores they do not contain DPA.

Conidiospores and Sporangiospores

The large group of bacteria known as the actinomycetes form branching hyphae; spores develop, singly or in chains, from the tips of these hyphae by crosswall formation (septation). If the spores are contained in an enclosing sac (sporangium), they are termed sporangiospores; if not, they are called conidiospores (or conidia) (Fig. 5-6). The spores do not have the high heat resistance of endospores, but they can survive long periods of drying.

Cysts

Cysts are dormant, thick-walled, desiccation-resistant forms that develop by differentiation of a vegetative cell and which can later germinate under suitable conditions (Fig. 5-32). In some ways cysts resemble endospores; however, their structure and chemical composition are different and they do not have the high heat resistance of endospores. The classic example of a cyst is the structurally

Figure 5-33. Fine structure of an *Azotobacter* cyst. The exosporium (Ex) and the two layers of exine (CC_1 and CC_2) are visible. A nuclear region (Nr) and a cytoplasmic region containing ribosomes are observable within the central body. [*Courtesy of Y.-T. Tchan and P. B. New, from N. R. Krieg (ed.), Bergey's Manual of Systematic Bacteriology, vol. 1, Williams & Wilkins, Baltimore, 1984.*]

complex type produced by the genus *Azotobacter* (Fig. 5-33). Several other bacteria can differentiate into cystlike forms, but these seem to lack the degree of structural complexity characteristic of *Azotobacter* cysts.

QUESTIONS

1 How does the cell's surface area/volume ratio compare with that of larger organisms? What advantages does a high surface area/volume ratio offer? What constraints does it place on a cell?
2 What bacterial cell structures may help to increase the cell's surface area/volume ratio?
3 If you performed a microscopic examination of an appropriately stained preparation of *Staphylococcus aureus*, would you expect all the cells to be arranged in clusters? Explain.
4 Explain why some species of cocci appear as chains but others appear in a cuboidal arrangement.
5 Draw a typical bacterial cell and identify all parts.
6 Contrast propulsion by a bacterial flagellum with that by a screw propeller on a submarine.
7 What functions might chemotaxis, phototaxis, and magnetotaxis have for bacteria in their natural habitats?
8 What problems associated with the shape and motility of spiroplasmas still remain to be solved?
9 What function might a capsule serve for the following bacteria?
   (a) a pathogenic bacterium
   (b) a soil bacterium where the soil is periodically subjected to drought conditions
   (c) a bacterium living in a flowing stream
10 Why are Gram-negative eubacteria usually much easier to disrupt by sonic oscillation than Gram-positive eubacteria?
11 Compare the structure and chemistry of the cell walls of Gram-positive eubacteria versus those of Gram-negative eubacteria. List some major differences between the cell walls of archaeobacteria versus those of eubacteria.
12 What function do the porins of the outer membrane of a Gram-negative eubacterial cell wall serve? What functions do cytoplasmic membrane/outer membrane adhesions serve?

13 In what kinds of bacteria and in what kinds of bacterial cell structures would we be most likely to find the following compounds: (*a*) peptidoglycan, (*b*) teichoic acids, (*c*) calcium dipicolinate, (*d*) cholesterol, (*e*) lipopolysaccharide, (*f*) phytanols ether-linked to glycerol?

14 Is spore formation in bacteria a method of reproduction or a means of multiplication? Explain.

15 What are the similarities and differences between protoplasts and spheroplasts?

16 Is it proper to refer to bacterial cells as containing a typical nucleus? Explain.

17 Name several cytoplasmic inclusions or substances. What function might be associated with each of these?

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