Introduction and Historical Perspective

The First Microscopes and the Discovery of Microorganisms

In 1590 a Dutch craftsman named Zacharias Janssen who specialized in making spectacles produced the first compound microscope. At first such microscopes had lenses of poor quality and could not be used to see microbial life. By 1630, however, single-lens microscopes came into existence. The oldest records in English referring to microscopic examinations are those of Nathaniel Highmore in 1651, who described his observations on the development of the chicken embryo with the help of “Glasses” and “this Glasse,” probably referring to a compound microscope and a single-lens instrument, respectively (Bardell, 1982).

The first English book completely devoted to microscopy was entitled Experimental Philosophy in Three Books: Containing New Experiments—Microscopical, Mercurial, Magnetical (Power, 1664). One year later, Robert Hooke published his famous book Micrographia (Hooke, 1665) in which he described various discoveries made with both the compound microscope and “small single Magnifying Glasses.” His discoveries included the presence of “cells” in cork and the presence in white sand of “grains exactly shap'd and wreath'd like a Shell” (fossilized shells of a foraminiferan), which he realized were the remains of a microscopically small animal. Hooke also was the first to describe some other eukaryotic microorganisms, such as fungi (under Mucorales) growing on leather and rust fungi growing on the surface of a rose leaf (Bardell, 1988). Yet, Hooke never recorded observations of bacteria, most likely because he only used dry inanimate preparations.

The Discovery of Bacteria

Antonie Van Leeuwenhoek (1632–1723), a draper and amateur lens maker living in Delft, The Netherlands, discovered bacteria using his homemade single-lens microscopes (Dobell, 1932; Porter, 1976). These microscopes were unique instruments containing an almost spherical lens mounted between two small metal plates. The magnification that could be obtained, which was a property of the lens, ranged from 50 to 500 times (O'Mara, 1979). The reasons for the success of Van Leeuwenhoek were manifold: his skill as an instrument maker and the high quality of his lenses, his ability to handle instruments, his way of mounting and illuminating specimens with a kind of dark-field microscopy, his enormous curiosity, his long and productive life, and the fact that, as a well-to-do merchant in textiles, he had the means and the time to work on his avocation. Most important was the fact that he communicated his work to the Royal Society of London through his friend Reinier de Graaff, a Dutch member of the Society. For almost 50 years until his death in 1723, Van Leeuwenhoek described all the main types of microorganisms from a variety of (mostly wet) sources: bacteria, yeasts, protozoa, and algae. In one of his letters published in 1676 he gave the first descriptions of some of his “kleine diertjes” (little animals), descriptions that proved that he had seen bacteria. In
his letter of 17 September 1683, he provided the first picture of bacteria, in his now-famous drawings of the types of bacteria he had found in scrapings from his teeth. These drawings demonstrated that he had seen all the major morphological forms of bacteria: coccus, rod, vibrio, and spirillum.

On Spontaneous Generation

For one and a half centuries after Van Leeuwenhoek's discoveries, virtually no further advances were made in the knowledge of microorganisms (Clarke, 1985). During this time the scientific world was caught in a paralyzing debate on spontaneous generation, due to the fact that no clear distinction was being made between the living and nonliving world. The view persisted that mice, worms, frogs, and “lower” forms of life could develop spontaneously in decaying organic matter and mud. This view was challenged by L. Spallanzani (1729–1799) who tried to deal with the extremely controversial and confused issue of spontaneous generation in a systematic way. Earlier experiments by F. Redi (1621–1697) and L. Joblot (1645–1723) provided the models for the work of Spallanzani. Redi had shown that insects did not arise spontaneously from decaying meat, and Joblot had demonstrated that hay infusions boiled for 15 minutes and subsequently covered with parchment did not produce “living insects” or “infusoria.” Experiments by Spallanzani published in his book Opuscoli de Fisica, Animale e Vegetabile (1776) included numerous microscopic observations made on infusions of vegetables, seeds, and other materials after applying various physical or chemical treatments (Doetsch, 1976). He concluded that small forms of life could be killed by various treatments and that these animalcula did not arise de novo. Spallanzani was a preformationist who believed that all life was already present from the day of the creation either as eggs, seeds, or in preorganized corpuscles. His opponents claimed that air contained some heat-labile element essential for the operation of the vegetative power responsible for spontaneous generation. Although Spallanzani did not devise the decisive experiment, he was instrumental in suggesting the type of experiment to be done. In 1861 the French chemist Louis Pasteur shattered the theory of spontaneous generation.

Louis Pasteur: the Beginning of Scientific Microbiology

Starting as a chemist, Pasteur had discovered stereoisomerism around 1853. This was followed by the finding that the isomeric forms of tartaric acid differed in their susceptibility to microbial attack. Pasteur thereafter came to the conclusion that only living agents could produce optically active, asymmetrical compounds and that a study of molecular asymmetry would shed light on the origin of life. This thought became the link between his past work in chemistry and his subsequent work in biology (Dubos, 1988). At that time, the common view was that processes such as those involved in wine and beer fermentations were merely the result of spontaneous chemical changes in decaying matter. This view was vigorously defended by the most eminent chemists of that time: Woehler, Berzelius, and Von Liebig. In a short paper titled: “Memoire sur la fermentation appelée lactique” Pasteur (1862) described the real microbial nature of the fermentation in milk. This work, regarded as the beginning of scientific microbiology, contained the first notion of microbial fermentation. It also touched upon subjects like the specificity of substrate use, regulation of life processes by environmental factors, anaerobic life, antibiotic activity of certain substances, competition between microorganisms, and microbial growth. The study of the souring of milk was followed by numerous other experiments that revealed that fermentation of sugar to alcohol, wine to vinegar, the putrefaction of meat, and other changes in organic matter were all caused by microorganisms. Pasteur showed that each particular kind of fermentation was accompanied by the development of a specific type of microorganism, each favored by specific environmental conditions.

The next question was where did these microorganisms come from? In a series of ingenious experiments, Pasteur was able to demonstrate that microorganisms did not arise from solutions or air after they had been adequately sterilized. Among the experiments designed to rule out spontaneous generation, the one with the swan-necked flasks became the best known. Not only did Pasteur's work lead to a greatly enlarged understanding of the distribution of microorganisms in the environment and to the discovery that blood and urine of normal humans and animals were free of microbes, the fundamental techniques of aseptic manipulation and sterilization were also worked out in the course of these experiments as a necessity in order to deal with the theory of spontaneous generation. Furthermore, these studies provided a new understanding of the ancient practice of canning as developed by Nicholas Appert (1752–1841). Rapid improvements of applied processes in the fermentation industry were the result of Pasteur's work.

Another view held by Pasteur against the spirit of the time, that of anaerobic life, was published in 1861. Further achievements included the elucidation of the
role of microorganisms in the spoilage of wine (1863) and beer (1871) and the description of a microbial cause for a disease among silkworms (1865). The first demonstration that microbes can cause disease dates from 1845 when it was shown by M. J. Berkeley that the fungus *Phytophthora infestans* was the cause of the Irish potato blight. Later discoveries by I. Semmelweis and J. Lister also provided evidence for the importance of microorganisms in causing human diseases. In 1877 Pasteur demonstrated that a microbe was involved in anthrax, an important disease affecting man and animals. While Pasteur was studying the cause of anthrax, its microbial origin was also being studied by the German physician Robert Koch. At that time Pasteur had just developed a regimen for heat attenuation of the causative agent of chicken cholera, and he used a similar approach in his studies of virulent anthrax bacilli. In historic field trials at Pouilly-le-Fort in 1881, Pasteur showed that these heat-attenuated organisms were capable of producing immunity against a later challenge with virulent strains of the bacillus in animals, and he therefore was given credit for developing the first vaccine effective in the prevention of anthrax (Mikesell et al., 1983).

**Koch and the Beginning of Medical Bacteriology**

Robert Koch (1843–1910) is considered to be the founder of medical bacteriology (Brock, 1988). In 1876 Koch had isolated the anthrax bacillus from diseased animals (cattle) and conclusively proved that there was a causal relationship between the large nonmotile bacilli and the disease. In his experimental work, Koch identified a series of criteria or rules-of-proof for establishing causality:

1. Occurrence of the microorganisms must be demonstrated in all cases of the disease.
2. The distribution of the microorganism must correlate with and explain the course of the disease and the disease symptoms.
3. The microorganism must be cultivated outside the diseased animal and healthy animals, subsequently infected with these cultivated microorganisms must then display the same symptoms as the original diseased animal.

These criteria are known as the Koch-Henle postulates. Henle was one of Koch's teachers who may have anticipated these criteria. It now seems more likely that Koch's ideas came from Edwin Klebs, who delivered an important speech in 1877 (cited by Koch in 1878) in which he argued that diseases should be associated with causal agents (Codell Carter, 1984).

In 1882 Koch announced his discovery of the tubercle bacillus as the causative agent of tuberculosis at a time when this disease had many victims in Europe (Groeschel, 1982). Two years later he published the discovery of the cholera bacillus. Koch played an extremely important role in the development of public health and hygiene and was involved in studies of drinking water supply, sewage disposal, and air quality. He studied a host of tropical diseases in various continents and became a driving force in the development of microbiology. His discoveries led directly to the development of vaccines and other immunological procedures. Originally put forward by Koch as a remedy against tuberculosis, tuberculin became an important tool in the diagnosis of this disease. Also, Koch's studies of the reactions of infected animals and humans to tuberculin or to reinfection (Koch'scher Grundversuch) became the basis for Pirquet's theory of allergy and eventually for Calmette and Guerin's work on tuberculous vaccination. The techniques developed by Koch (sterilization, pure culture, staining reactions) have served over the years as major tools in the field of bacteriology and medical microbiology.


Control of host immunity by the use of an attenuated vaccine had been introduced by Jenner for smallpox as early as 1798, but remained for nearly a century an isolated phenomenon, which no one thought of applying to other diseases. The history of research on bacterial toxins is described by Alouf (1987).
Methods of Pure Culture

To Pasteur, who always used liquid cultures, it had been less important whether his cultures were completely pure than that their activities could be shown to be biological and not chemical and that the microorganisms involved could be shown to be derived from the air and not by spontaneous generation. Pasteur was also concerned with the question of whether bacteria and other microorganisms were able to carry out various specific chemical transformations.

Pure culture techniques were originally developed by De Bary and Brefeld for the study of fungi (Bull and Slater, 1982). They succeeded in isolating single cells and in cultivating fungi on solid media, but the techniques were not suited for use with bacteria. It was Robert Koch, together with some of his students and colleagues, who deserves the credit for developing many of the basic techniques, media, and equipment necessary for handling and studying prokaryotes. In Koch’s laboratory agar-solidified media (Mrs. Hesse), covered culture dishes (Petri), and staining techniques (Koch and Ehrlich) were developed. These findings allowed other scientists to purify and study bacteria and other microorganisms. Potatoes and gelatin were used as the only solid growth media in the early days of microbiology. Gelatin has the disadvantages that it is hydrolyzed by many proteolytic enzymes and that it liquefies at 37°C. The use of agar as the solidifying agent in bacteriology was introduced by Fanny Eilshemius, a German-American housewife married to Walther Hesse, a physician in Germany who experimented in air bacteriology. Hesse communicated the discovery to Robert Koch around 1881, and Koch used agar in his famous study on the tubercle bacillus (Groeschel, 1982).

It is likely that the development of Koch’s methods—although vital for the growth of medical microbiology as a science—may have hindered the understanding of the microbial interactions which occur in natural microbial communities. By focussing on the pure culture and laboratory study, Koch’s methods did not permit an understanding of how microbes interacted in such complex natural habitats as soil (Winogradsky, 1949).

Preservation of Food

In 1877 the British physicist John Tyndall showed that media contaminated with heat-resistant bacteria could not be sterilized by simple boiling. Around this time the German botanist Ferdinand Cohn showed that endospores conferred bacterial heat-resistance. Therefore, Tyndall developed a method for sterilizing media by alternate boiling and cooling. Cooling allowed the germination of endospores into growing cells which were sensitive to subsequent boiling. Today, this process, called tyndallization, is occasionally used to sterilize certain heat-sensitive media, but heat sterilization at 121°C for 15–25 min is generally used to sterilize media. Pasteur established the practice of heating fluids under pressure, thus introducing autoclaving into laboratory practice. By reference is not an exact match Pasteur had also shown that many organisms were killed by heating at about 60°C, but the process of pasteurization was not used until later, e.g., by the dairy industry beginning in England in 1890.

Bacteria in Nature and the Enrichment Culture

Under the influence of the work of Koch, the study of microorganisms as agents of infectious disease became the main theme of microbiology during the last decades of the 19th century. However, another approach, initiated by Pasteur through his investigations on the role of microorganisms in fermentation, focused on the role of microorganisms as agents of geochemical changes. This introduced quite another view of microbiology.

Ferdinand Cohn (1872) was the first to realize the role of microorganisms in the transformation of organic matter and inorganic substances on earth with subsequent recycling of the elements to sustain other forms of life. One of the early studies on the nature and activities of a specialized mixed microbial community was that of Ward in 1892 (Clarke, 1985). The most significant contributions to the knowledge of the various types of microorganisms responsible for specific chemical transformations in nature (especially the cycles of nitrogen and sulfur) came largely from the laboratories of reference is not an exact match Sergius Winogradsky (1856–1953) and Martinus Willem Beijerinck (1851–1931). During the 1880s, Winogradsky advanced the concept of chemosynthesis on the basis of his studies of the colorless sulfur bacteria, Thiotrix and Beggiatoa (Winogradsky, 1949). He concluded that these aerobic bacteria obtain their energy for autotrophic growth by oxidizing reduced sulfur compounds like sulfides and sulfur to sulfate. For the first time it was shown
that, in addition to the energy from incident light, energy released during the oxidation of a reduced inorganic compound could drive autotrophic fixation of carbon dioxide.

Another microbiologist at the time was Beijerinck, a keen observer and a pioneer in opening up new fields for others to exploit with new but simple and effective methods (Waksman, 1931). With contributions from Winogradsky, Beijerinck developed the useful and adaptable technique of enrichment culture. Tailoring culture conditions to favor microbes with a particular metabolic activity usually leads to a rapid enrichment of the desired organism even if its original numbers are very low in the sample (Van Iterson et al., 1940). One of the early examples of the application of this principle was in Beijerinck's paper on sulfate reduction, which led to the discovery and isolation of Spirillum (Desulfovibrio) desulfuricans, described in a preliminary paper in 1894. Results of enrichment studies included the discovery of important groups of bacteria with differing mechanisms of energy generation such as the phototrophic, chemolithotrophic, and chemoorganotrophic bacteria, an awareness of the ubiquitous distribution of bacteria, and an insight in the astonishing variety and variability of microbes and their metabolism.

In 1890 Winogradsky provided the first instance of a conscious application of the principle of enrichment cultures in his work on nitrifying organisms. Winogradsky had applied enrichment or elective cultures for his research on sulfur bacteria and nitrifying bacteria (1893, 1894) and also had used this technique to identify nitrogen-fixing bacteria in soil. Culture media had to be as free from nitrogen compounds as possible, but should contain all other necessary elements and glucose as a source of carbon and energy. The medium was poured in a thin layer (8–9 mm) in conical flat-bottomed flasks, and after the medium had been inoculated with some soil, a stream of purified air was passed over the cultures. A strictly anaerobic sporeforming bacterium named Clostridium pasteurianum, which performed a typical butyric acid fermentation, was isolated. This isolation was possible because oxygen was removed by aerobic organisms present in the inoculum, quite an unexpected event. It was not until 1901 in a paper on urea-hydrolyzing bacteria that Beijerinck, working in Delft, The Netherlands, insisted on the great significance of this principle. In a footnote he made the noteworthy remark that “its importance should be judged not only from the scientific, but also from the didactic point of view.” However, Beijerinck never wrote a paper on the technique itself and the principles behind it, but his student F. Stockhausen published a number of essays on microbial ecology in a brewery journal which also appeared as a booklet (Stockhausen, 1907) and which included a description of the enrichment technique.

Beijerinck contributed materially to our understanding of the role of microorganisms in natural processes and indicated numerous applications of the activities of these microscopic forms of life to agriculture and industry. “No doubt that the science of general microbiology began in Delft” (Van Niel, 1949). In the years to come after Beijerinck, the success of the batch enrichment culture technique was demonstrated over and over again in the work of Kluyver, Van Niel, Stanier, and their students and associates (Van Niel, 1967; Veldkamp, 1965).

At a time when the discovery of a bewildering variety of microbial forms and activities reached its height, Kluvyer and Donker (1926) published their well-known paper on unity in biochemistry. This synthetic paper was based on an integration of the ideas of Neuberg, Wieland, Warburg, and Harden. The concept was that, in addition to a number of hydrolases, cells contained various oxidoreductases catalyzing reactions that could be considered as chains of step reactions, each of which represented a simple mechanism in which hydrogen is transferred from one molecule, the hydrogen donor, to another, the H acceptor. Hydrogen acceptors other than oxygen might also be used (e.g., sulfate). The first report on the view that photosynthesis can be considered as a light-dependent reaction in which different substrates, specific for the different kinds of photosynthetic organisms, serve as H donors for the reduction of carbon dioxide came from Van Niel (1930). Phototaxis by purple bacteria had been observed before by Engelmann who believed that these bacteria were true phototrophic organisms despite the lack of oxygen evolution when these organisms were exposed to light. Van Niel described photosynthesis in a general formula (see Van Niel, 1967, for a historical account), calling it the light-dependent transfer of hydrogen from an appropriate donor, H₂A, to CO₂:

\[2H₂A + CO₂ → 2A + (CH₂O) + H₂O\]

Van Niel's work was extended greatly by N. Pfennig and his students, who grew many of the anaerobic photosynthetic bacteria in pure culture.
The World of Strictly Anaerobic Bacteria

Early developments and attempts at anaerobic culture were initiated in the 1880s and included the use of shake cultures gassed with hydrogen, the use of alkaline pyrogallol to remove oxygen, sealed tubes, and the picking of colonies with a curved capillary pipette. Yet, even by 1940 the only anaerobes known were sporeformers and several nonsporeforming bacteria of clinical importance, while most of the anaerobes in sediments, soils, the gastrointestinal tract, and other anaerobic habitats were still unknown. This was in part due to the fact that techniques were insufficient to reach the necessary anaerobic conditions but also to the fact that the media used were not habitat-simulating. The investigations on anaerobic bacteria were also hampered by the limited availability of rubber stoppers that were sufficiently impermeable to oxygen. Hungate (1950), the first U.S. student of Van Niel, developed an anaerobic roll tube technique based on various approaches from the older literature and used habitat-simulating media in the isolation of cellulolytic rumen bacteria. More detailed descriptions of theory and general methodology are given in Hungate (1966, 1969, 1985). The Hungate technique has been very successful and has been widely adapted by microbiologists from various other fields (dentistry, sewage, and sediment microbiology).

Further Readings in the History of Microbiology and Bacteriology

Important developments in techniques for the isolation and cultivation of bacteria have been discussed in the introductory chapters of many old (e.g., Topley and Wilson, 1936; Stephenson, 1938; Thimann, 1963; Stanier et al., 1957; Brock, 1979) and recent textbook editions (e.g., Brock and Madigan, 1991; Van Demark and Batzing, 1987; Stanier et al., 1986; Cano and Colome, 1986). Much general information can also be found in Leadbetter and Poindexter (1985). Bulloch (1938) wrote a monograph on the history of bacteriology, and several monographs, and reviews describe the life and work of famous microbiologists, e.g., Van Leeuwenhoek (Dobell, 1932; Porter, 1976), Pasteur (Dubos, 1988; Porter, 1961), Koch (Brock, 1988), Beijerinck (Van Iterson et al., 1940), and Kluyver (Kamp et al., 1959). Brock (1961) translated and commented on a series of articles that could considered as milestones in the development of microbiology. Several microbiology reviews that can be used as sources for the history of microbiology were listed by Poupard and Farzanfar (1985).

A Center for the History of Microbiology was established by the American Society for Microbiology (ASM) in 1985 at the University of Maryland, where the ASM archives collection has been kept since 1977. The center coordinates research into the history of microbiology and into surveys of sources (ASM News 52:539–540, 1986). The address is: ASM Archives, University of Maryland, Catonsville, MD 21228, USA.

General Aspects of Nutrition and Growth

Enrichment, isolation, and cultivation of bacteria are directly connected with, and largely dependent on, appropriate growth conditions. For this reason, the basic principles of energy transduction encountered among prokaryotes, the nutritional types of bacteria, the nature of cultivation media, the general procedures of cultivation, and the environmental factors influencing growth are discussed in this chapter. Since many textbooks, manuals, monographs, and periodicals deal with these topics, the citation of literature has been kept to a minimum.

Principles of Energy Transduction

All forms of life require a source of energy for growth and maintenance. In principle, two types of energy are available, namely light energy and chemical energy; organisms that rely on the first type are called phototrophs, whereas those that use chemical redox reactions for energy generation are called chemotrophs (Table 1). The basic difference between the two is that, in the case of light, electrons required for metabolic energy transduction are released by way of photooxidation of a cell-bound pigment, whereas, in the case of chemical energy, external organic or inorganic energy substrates serve as electron donors. As a consequence, energy generation from light does not depend on the availability of an external electron acceptor because electrons that are released by photooxidation may flow back to the oxidized pigment. In contrast, chemotrophs rely on the presence of an external electron acceptor (e.g.,
oxygen, nitrate, or sulfate) or one formed internally (fermentation). In either case, biochemical energy is generated during subsequent metabolic reactions and is made available to the cell in one of two forms, namely: 1) an electrochemical transmembrane gradient; and 2) chemical energy contained in, for example, the phospho-anhydride bond of ATP. Metabolically, these two forms of energy are interconvertible and can be used to drive a number of processes (Figure 1).

Table 1. Classification of microbial metabolism on the basis of energy source and electron donor requirement.

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Energy source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inorganic</td>
</tr>
<tr>
<td>Chemical (chemotrophs)</td>
<td>Chemolithotrophic</td>
</tr>
<tr>
<td>Light (phototrophs)</td>
<td>Photolithotrophic</td>
</tr>
</tbody>
</table>

Fig. 1. Simplified scheme of the generation and utilization of metabolic energy.

Electron transfer

\[ \text{Energized membrane} \]

Motility

Mg, Ca

ATPase

ATP

Biomass formation
Transhydrogenation

When bacterial growth is considered as a chemical process in which nutrients are converted into biomass, it appears from the chemical composition of the biomass that in most cases it is more reduced than the nutrients from which it is derived. This is most apparent when carbon dioxide is the major source of carbon, but it is by no means restricted to this carbon source. As a consequence, reducing equivalents must be available to carry out the necessary reductive processes. In microbial metabolism, electron donors therefore not only serve to generate energy but also to yield reducing power for the cells to carry out a variety of reductive processes. This must be taken into account when the classification of prokaryotes on the basis of energy source and electron donor requirements is considered (see Table 1).

The prokaryotes developed over a period of billions of years before the atmosphere became aerobic and before carbohydrates became abundant products of oxygenic phototrophic biomass production. During their early evolution, the prokaryotes learned to exploit a multitude of sources of energy and cell carbon that are no longer among the predominant nutrient sources. This has led to a truly bewildering versatility in their ability to utilize substrates, their peripheral metabolic pathways, and their modes of energy transduction. Although microbes cannot disregard the laws of thermodynamics or transgress the rules of chemistry, within these boundaries their ability to catalyze chemical reactions and derive energy therefrom is unique in the living world. The most outstanding metabolic properties, which are either restricted to only a few eukaryotes or completely lacking among eukaryotes, are the use of inorganic electron donors for energy generation, anaerobic growth, and the fixation of molecular nitrogen.

Principles of Bacterial Nutrition

All bacteria, like all other organisms, require nutrients for growth and multiplication. Of the more than 100 elements that appear in the periodic table, some 35–40 are considered essential. Although the majority of these are metals, the composition of bacterial cells, which is a reflection of their major elemental requirements for growth, demonstrates that six nonmetals (C, O, H, N, S, and P) and two metals (K and Mg) comprise an average of 98% of the dry weight of prokaryotes. These elements are usually collectively called macroelements because they are needed in relatively high concentration in the growth medium. Their functions are indicated in Table 2. In addition to these, a variety of microelements is required by most bacteria; among these, Fe, Ca, Mn, Co, Cu, Mo, Zn, Ni, V, Cl, Na, and B are most often included in so-called trace element solutions, although certain organisms, strict anaerobes in particular, may also require more exotic elements such as selenium and tungsten. In many instances the microelements are present in adequate amounts as contaminants of the mineral salts used in media or as contaminants of glassware and water. Only occasionally must they be added separately to growth media. Most bacteria do not require sodium; however, many marine bacteria, certain phototrophs, and a number of strict anaerobes require this element.

Table 2. Macroelements and their physiological functions.

<table>
<thead>
<tr>
<th>Element</th>
<th>Physiological functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>Main constituent of organic cellular material</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Constituent of proteins, nucleic acids, and coenzymes</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>Organic material and water</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Organic material and water</td>
</tr>
<tr>
<td>Sulfur</td>
<td>Constituent of proteins and coenzymes</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>Constituent of nucleic acids, phospholipids, nucleotides, and coenzymes</td>
</tr>
<tr>
<td>Potassium</td>
<td>Principal inorganic cation in the cell and cofactor for some enzymes</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Cofactor for many enzymes and present in cell walls and membranes</td>
</tr>
</tbody>
</table>

Carbon Requirement.

The biochemistry of this planet is based on carbon. This element constitutes approximately 50% of the dry weight of most prokaryotes, and thus carbon-containing compounds are therefore the most important nutrients for their growth. The range of carbon compounds that can be utilized is vast. This is illustrated by the fact that all carbonaceous material synthesized by biological processes can be degraded by microbes. Traditionally, bacteria have been divided into two major groups with respect to their carbon requirement, namely autotrophs and heterotrophs (organotrophs).

The autotrophs encompass those bacteria that use carbon dioxide as the principal source of cellular carbon. These organisms rely on light and/or the oxidation of inorganic compounds for the generation of metabolic energy; their energy metabolism can thus be classified as photolithotrophic or chemolithotrophic (Table 1). Since several of the organisms that carry out one of these two types of energy metabolism can also grow at the expense of organic energy sources (facultative autotrophs) or grow better when small amounts of organic carbon compounds are present in otherwise inorganic growth media (mixotrophs), the variety of nutritional types among prokaryotes can only be adequately described when the energy source, the electron donor, and the carbon source(s) are considered together. This may lead to a statement such as, “under the current conditions this organism grows chemolithoautotrophically”; this statement recognizes that the same organism under a different set of environmental conditions may grow chemoorganotrophically.

Heterotrophic bacteria obtain their carbon from organic nutrients. These have two major functions: they must supply the carbon skeletons to meet the biosynthetic requirements of the cell, and in addition they must supply the necessary energy for growth and maintenance. Here, the carbon source in most cases is also the energy source. Many bacteria grow with a single carbon compound added to the medium; others depend on additional organic compounds that must be present in small concentrations because the cells are unable to synthesize the entire complement of cell constituents from the single carbon source. These are called growth factors. Bacteria differ considerably with respect to the number and kind of carbon compounds that can be used. Some bacteria like the pseudomonads are versatile and are known to utilize over 100 different carbon compounds as the sole source of carbon and energy. These compounds belong to various groups of organic chemicals and include carbohydrates, sugar acids, poly-alcohols, fatty acids, primary alcohols, amino acids, and aromatic compounds. All compounds of natural origin are decomposable and can serve as carbon and energy source for certain bacteria or other microorganisms.

In contrast to the versatile bacteria, several groups exist that are limited in their ability to decompose organic compounds. In this category are the obligate methylotrophs that only use methane, methanol, dimethyl ethers, and a few other compounds; and the specialists that are restricted in their metabolism to only one type of organic compound (e.g., *Bacillus fastidiosus* grows only on uric acid).

Many chemoorganotrophs require carbon dioxide (mostly in small amounts) for specific purposes, for instance, for the synthesis of fatty acids and for “heterotrophic” fixation of carbon dioxide. Since carbon dioxide normally is produced during catabolism of organic compounds, it does not normally become a limiting nutritional factor. Some bacteria, such as *Neisseria* and *Brucella*, however, require higher concentrations of carbon dioxide (up to 10%) in the atmosphere for good growth in organic media, a need that must be considered in isolating and cultivating such organisms.

Nitrogen, Phosphorus, Sulfur, Potassium, and Magnesium Requirements.

Nitrogen comprises 8–14% of the dry weight of bacteria. In cellular material, nitrogen is incorporated in the reduced form. A wide range of inorganic and organic nitrogen compounds can be utilized to satisfy the requirement for this element. Most phototrophs take up nitrate as the nitrogen source, while the majority of the nonphototrophic bacteria also can assimilate nitrate. For biosynthesis, the anion must be reduced. However, some bacteria depend on reduced nitrogen in the form of ammonium salts. Other bacteria do not grow in the presence of inorganic nitrogen compounds, but depend on nitrogen present in amino acids or polypeptides. Such compounds may, in addition, also serve as a carbon and energy source. A number of aerobic and anaerobic bacteria are capable of fixing dinitrogen and thus can grow in media devoid of a combined nitrogen source.
Inorganic phosphate is the usual source of this element for microbial nutrition. It also is extensively used as a pH buffer and is often added in excess of the growth requirement to fulfill this role. Organic sources, e.g., glycerophosphate, are an alternative supply. Much of this element is bound up in RNA with the result that the demand increases with the specific growth rate. It is also an important structural component (as teichoic acids) in the cell wall of Gram-positive bacteria.

Most bacteria can utilize sulfate to satisfy the sulfur requirement. Since much sulfur is present in proteins in the form of sulfur-containing amino acids, the sulfate taken up from the environment must be reduced (a process called assimilatory sulfate reduction). Some organisms, however, require sulfur in a reduced form either as sulfide or an organic compound with an SH-group such as cysteine.

Potassium is the principal inorganic cation in the cell. Although much of it is bound up in the ribosomes of bacteria, it is a cofactor of some enzymes, is required for carbohydrate metabolism, and is involved in many transport processes and in osmoregulation. It is usual to add an inorganic potassium salt, e.g., potassium sulfate or potassium phosphate, to satisfy this requirement.

The magnesium requirement of bacteria is principally the result of a specific requirement of the ribosomes. Magnesium also functions as an enzyme cofactor and is present in cell walls and membranes. It is usually supplied as magnesium sulfate.

**Growth Factor Requirements.**

Not all bacteria can synthesize an entire complement of cell components from the compounds discussed so far. Some require an external supply of preformed organic molecules which form the building blocks of cell components. Such compounds are called growth factors. They generally stimulate the growth of organisms since they decrease the need for de novo biosynthesis in the cell, but certain bacteria have an absolute requirement for one or more of them since they cannot be synthesized by the organism. With respect to the chemical nature of the growth factors, three main classes can be differentiated: 1) amino acids (for the synthesis of proteins); 2) purines and pyrimidines (building blocks of nucleic acids and coenzymes); and 3) vitamins (required as coenzymes or prosthetic groups of certain enzymes). Vitamins are required only in small amounts (on the order of ppm). The biological functions of a number of vitamins are listed in Table 3. The lactic acid bacteria are well known for their complex vitamin requirements and for this reason they are often used in vitamin assay procedures. Certain bacteria depend on the presence of additional growth factors that belong chemically to rather unusual categories. Among these are porphyrin, the requirement for which is usually met by the addition of red blood cells; and short-branched or straight-chain fatty acids.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic acid</td>
<td>Precursoe of tetrahydrofolic acid; involved in one-carbon transfer</td>
</tr>
<tr>
<td>Biotin</td>
<td>Carbon dioxide fixation and release</td>
</tr>
<tr>
<td>Coenzyme M</td>
<td>Involved in methane formation</td>
</tr>
<tr>
<td>Cyanocobalamin (B₁₂)</td>
<td>Molecular-rearrangement reactions</td>
</tr>
<tr>
<td>Folic acid</td>
<td>One-carbon metabolism</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>Transfer of acyl groups</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>Precursor of CoA; metabolism of fatty acids</td>
</tr>
<tr>
<td>Pyridoxine (B₆)</td>
<td>Deamination/transamination</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>Precursor of NAD and NADP; involved in redox reactions</td>
</tr>
<tr>
<td>Riboflavin (B₂)</td>
<td>Precursor of FMN and FAD; involved in redox reactions</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>Precursor of menaquinone</td>
</tr>
</tbody>
</table>

Table 3. Functions of some vitamins and related compounds in bacteria.
**Principles of Bacterial Growth**

Viable bacteria have the ability to assimilate and metabolize substrates and to increase their biomass and/or cell number; in other words, they have the ability to grow. The rate at which growth takes place can be measured from the increase in bacterial density or cell number. This distinction has to be made since conditions may prevail in which growth is not completely balanced, and the increase in cell density is not strictly paralleled by an increase in cell numbers. The specific growth rate $\mu$ (h$^{-1}$) and the doubling time $t[d]$ (h) are thus based on changes in biomass whereas the equivalent specific division rate $v$ (h$^{-1}$) and the generation time $g$ (h) are based on changes in cell numbers. In most cases, a growing culture may be considered to be a system multiplying autocatalytically, in which case calculations based on bacterial density $x$ (biomass·1$^{-1}$) are most appropriate. Changes in $x$ are thus proportional to the initial value of $x$ at any time interval. In other words, the kinetics are first order, and the specific growth rate is described by:

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt}$$

and hence $x = x_0 \cdot e^{\mu t}$.

The biomass doubling time then is given by $t[d] = \ln 2/\mu$.

For a more thorough treatment of the description of microbial growth, the reader is referred to general textbooks on microbiology (Mandelstam and McQuillen, 1973; Pirt, 1975; Schlegel, 1986; reference is not an exact match Stanier et al., 1987; Brock and Madigan, 1991).

**Closed Systems**

**Batch Cultures.**

The occurrence of exponential growth is most clearly demonstrated in batch cultivation of bacteria. In a typical batch culture, bacteria grow suspended in a medium containing sufficient carbon and energy sources and other required nutrients to allow growth at their maximum rate. After an initial lag-phase while the cells adapt to the new culture conditions (through synthesis of new enzymes, etc.), the bacteria grow exponentially. Such a batch culture is termed a “closed system” if fresh supply and removal of essential medium components does not occur. Growth proceeds until one of the growth requirements becomes depleted. At that point the rate of growth drops and eventually becomes zero when the stationary growth phase is entered. Further incubation of such a culture will usually result in a more or less rapid decline in cell density due to lysis of the cells. Clearly, in such a closed culture system, cells grow in a continuously changing environment, due to their own metabolic activities; only during a relatively short period of time does balanced growth at $\mu_{\text{max}}$ occur. Nevertheless, thanks to its relative simplicity and ease of operation, batchwise cultivation is widely used as a routine procedure for propagation of bacteria both in research and in industry. To provide better command over the physiology of the bacteria grown in batch culture, various degrees of control can be obtained by regulating important parameters such as pH, temperature, and oxygen tension.

**Fed-Batch Cultures.**

A further elaboration of the batch cultivation technique was developed especially for certain industrial fermentations (Yoshida et al., 1973; Pirt, 1974, 1975). In such cultures, a continuous supply of nutrients is fed to a batch culture but no outflow of the culture occurs, which means that the system remains a closed one (according to the above terminology). Usually the initial nutrient concentration in this type of batch culture is allowed to drop to very low (sometimes...
growth-limiting) concentrations and only then is a fresh continuous supply provided at a low rate which keeps the cells growing under nutrient limitation. Due to the important characteristic of a fed-batch culture in which no medium is allowed to flow out, the culture volume and biomass increase continuously, thus making the nutrient supply ever more limiting per unit of biomass. The overall result of this is that growth of the cells can be extended over much longer periods of time than in a common batch culture but under conditions of nutrient limitation and at an ever decreasing rate. For some industrial fermentations, these very conditions have proved vital for optimal production of certain metabolites, e.g., citric acid, penicillin, some enzymes, and alcohols (Yoshida et al., 1973; Esener et al., 1981; Cleland and Enfors, 1983; Gond et al., 1986; Dawson et al., 1988; Kole et al., 1988). Especially for industrial fermentations, the possibility of turning a fed-batch culture into a “repeated fed-batch culture” by withdrawing part of the culture volume at regular intervals allows the productive growth phase in principle to be extended indefinitely. The major difference with truly continuous culture systems remains that the volume is not kept constant, thus introducing permanent transients in growth rate, possibly essential for certain metabolic activities.

In order to overcome the problem of a permanently changing volumes and concentrations of metabolic end-products, the recycling fermenter was introduced. In this type of fermenter, either complete or partial recycling of the biomass is accomplished but the culture liquid is continuously replenished with fresh medium and removed at the same rate. This type of fermenter has proven an especially valuable tool in studying growth rate dependence of microbial metabolism at very long generation times (i.e., very low specific growth rates) (Bulthuis et al., 1989; van Verseveld et al., 1984; Chesbro, 1988).

Open Systems

In contrast to batch-type cultures, a continuous culture is a typical “open” system. It is characterized by a well-mixed culture contained in a vessel to which fresh nutrient medium is added at a constant rate while the volume is kept constant through an overflow device.

Chemostats.

In most cases, this culture system is designed to provide continuous exponential growth at a constant sub-maximum rate which is dictated by the rate at which the limiting nutrient is fed to the culture. This type of a continuous culture is termed a chemostat. These characteristics have made the use of the chemostat one of the most widely used methods for studying microbial metabolism under carefully controlled environmental conditions both in pure and mixed cultures (Tempest, 1970a, 1970b; Veldkamp, 1977; Tempest and Neyssel, 1978; Bull and Brown, 1979; Matin, 1981; Kuenen and Harder, 1982; Gottschal, 1986, 1990; Gottschal and Dijkhuizen, 1988). Theory and practice of chemostat operation have been treated extensively many times in the literature (Monod, 1950; Novick and Szilard, 1950; Herbert et al., 1956; Evans, et al., 1970; Tempest, 1970b; Pirt, 1975; Bazin, 1981; Calcott, 1981; Gottschal, 1990). The key factors which determine the growth of bacteria in a chemostat are the dilution rate \( D \) (h\(^{-1}\)), defined as the rate of nutrient supply \( F \) divided by the volume \( V \) of the culture vessel, and the nature and concentration of the nutrient \( S_r \) in the reservoir medium that will first become limiting for growth. The combined effect of growth and dilution will eventually result in a steady state in which no further change in cell density \( x \) occurs:

\[
\frac{dx}{dt} = (\mu - D)x = 0
\]

At this point, the specific growth rate exactly balances the dilution rate, demonstrating that choosing a given rate of culture dilution fixes the specific growth rate at any value below \( \mu_{max} \). Assuming that the specific growth rate is adequately described by Monod-type kinetics (Monod, 1942) and thus

\[
\mu = \mu_{max} \cdot \frac{S}{(K_s + S)}
\]

with \( K[S] \) representing the half-saturation constant for growth, the actual substrate concentration \( S \) in the culture is fixed at a low, rate-limiting value. Steady-state substrate concentration and cell density are thus described as follows:
in which the cell yield coefficient \( Y \), the amount of cells produced per substrate consumed, is assumed constant over the range of dilution rates employed.

Chemostats offer a tremendously powerful tool for studying the physiology and ecology of bacteria, but for investigating the response of bacteria to conditions that are not constant, well mixed, or homogeneous in time and space, more elaborate culture systems are required. Very little modification of the general chemostat equipment is required to study microbial growth under controlled alternating conditions. Timed switching between different light intensities, degrees or types of nutrient limitation, rates of growth, pH values, temperatures, or combinations of these, is not difficult to implement using standard equipment. Although these types of studies will most certainly provide interesting information on the adaptability of bacteria to changing growth conditions, relatively little work of this kind has been published (see Gottschal, 1990, for some recent examples and further references).

**Coupled Chemostats.**

Bacteria growing in steady state in a chemostat have a physiological state which is independent of previous growth conditions. Although chemostats are very attractive for many laboratory studies of pure and mixed cultures, it has been pointed out that for optimal production of certain microbial metabolites, a continuous culture system is needed in which the cells pass through various stages of growth (Herbert, 1964; Ricica and Dobersky, 1981). This may be accomplished by coupling, in series, two or more chemostats (with the outflow of one chemostat connected to the inlet of the next one) in which the bacteria can be grown under different conditions, e.g., with respect to growth rate, aeration, temperature, and substrate. In ecologically oriented model studies, it is especially the spatial separation of different microbial populations in coupled chemostats that may prove useful for studying the metabolism of complex communities. For studies on the sequential mineralization of (recalcitrant) organic matter in anaerobic ecosystems, this seems a particularly useful approach (Thompson et al., 1983; Parkes and Senior, 1988; and several references therein). For aerobic cultures, the technique of a two-stage cascade of chemostats has also been used frequently in the study of predator-prey relationships, in which case bacteria grown in the first stage are transferred to the second one to serve as food for protozoan predators (Jost et al., 1973; Swift et al., 1982; Sambanis and Fredrickson, 1987; Glaser, 1988).

As opposed to such unidirectional multistage chemostats, recent years have seen the development of even more complex bidirectionally linked compound chemostats (Lovitt and Wimpenny, 1981; Keith and Herbert, 1985; Herbert, 1988; Wimpenny, 1988). The fundamental difference between the two types of multistage chemostats is the mutual exchange of bacteria and/or nutrients in the latter multistage systems as opposed to the one-way sequential passage of nutrients and bacteria in the former one. In a “gradostat” (Lovitt and Wimpenny, 1981), a series of chemostats (linked by tubing) is fed from both sides with media of different, sometimes complementary, composition. Following their inoculation bacteria will be exposed to differing physicochemical conditions in the different chemostat vessels. In “bidirectional compound diffusion-linked chemostats,” the various chemostat vessels are not linked by tubing but through membranes, permitting diffusion of solutes but not bacteria (Keith and Herbert, 1985). Both systems thus represent certain aspects of the heterogeneity so characteristic for most natural ecosystems and may be most applicable to studying the growth of bacteria influenced by the activity of neighboring organisms.

**Auxostats.**

An essential property of chemostat cultures is that their rate of growth is fixed by the external control of the rate at which fresh medium is fed to the culture. This rate can be varied over a considerable range of values but at the very high end of it (near the \( \mu_{\text{max}} \) of the bacteria) steady state cannot be obtained, and, at the critical dilution rate, washout occurs (Pirt, 1975). Furthermore, unbalanced growth and in some cases washout may also occur when inhibitory metabolites accumulate or potentially toxic substrates are used. A good alternative for obtaining controlled growth at an appreciable and constant cell density in such cases is by switching over to some type of internal control of the rate of medium supply. Such a control must be based on a growth-dependent
parameter. In the first published description of a continuous culture based on this principle, the equipment was termed a turbidostat because the feedback control was based on the measurement of the turbidity of the culture (Myers and Clark, 1944; Bryson and Szybalski, 1952); But reliable construction of such turbidostats has always been a major problem, mainly due to the unreliability of continuous and accurate turbidity measurement either directly inside the fermenter or in external culture loops (due to wall growth, inhomogeneity, etc.). To overcome such problems, newer designs often rely on sensors measuring various parameters directly dependent on the culture density, such as CO₂, O₂, pH, redox potential, fermentation products, and sulfide (Watson, 1969; Martin and Hempfling, 1976; Ottnann et al., 1978; Kjaergaard and Jørgensen, 1979; Schauer et al., 1982; Kistner et al., 1983; Cyponka, 1986; de la Broise and Durand, 1989; Minkevich et al., 1989). These more recent designs have proved reliable and their use is most rewarding in studying microbes in the presence of inhibitory concentrations of substrates and products and possibly also in selecting mutant strains exhibiting the highest growth rates under such conditions.

Growth on Mixed Substrates.

In spite of the fact that bacteria in natural environments grow in the presence of low concentrations of a large diversity of compounds, most laboratory studies have focused on growth on single nutrients. When bacteria are grown in batch culture with more than one growth substrate, sequential utilization of these substrates is often observed. In some cases this results in a typical diauxic growth pattern (Monod, 1942; Schlegel, 1986) in which the substrate that is used first represses the synthesis of enzymes required for subsequent substrates. Only after (almost) complete utilization of the first substrate is growth on the second one induced. However, this type of clear-cut diauxic pattern with a lag period between consumption of the first and the second substrate is by no means very common. More often a gradual transition between the use of two (or more) substrates is observed, and in some cases no enzyme repression is evident at all (Harder and Dijkhuizen, 1976, 1982; Weide, 1983; Gottschal, 1986). Moreover, it is questionable whether distinct preferences for one substrate would be functionally valuable in nutrient-poor natural environments with many different substrates available at the same time. Under nutrient limitation one would rather expect organisms to develop physiological strategies enabling them to make use of several nutrients simultaneously. Growth in chemostats under limitation of mixtures of different substrates has provided ample evidence that a multitude of substrates, serving similar physiological functions, can be growth limiting at the same time (Mateles et al., 1967; Harder and Dijkhuizen, 1976, 1982; Bull and Brown, 1979; Egli et al., 1983; Gottschal, 1986; Gottschal and Dijkhuizen, 1988). The question whether in practice truly multiple substrate-limited growth should also be possible with substrates that do not serve the same metabolic purpose (e.g., N, P, or C sources) has not been satisfactorily solved, but it seems quite likely that if one assumes a certain variability in the cell content of various nutrients, there should exist a range of nutrient ratios resulting in this type of multiple nutrient limitation (Bader, 1982; Thingstad, 1987).

Interactions of Prokaryotes with Their Environment

Abiotic Interactions

The growth of microorganisms is strongly influenced by a multitude of physicochemical properties of their immediate environment. Selection pressure ensures that the physiology of most microbial species will be adapted to the prevailing natural environmental conditions. Due to the large variety of these conditions it is not surprising that microorganisms have developed a wide range of physiological adaptations. Growth of microorganisms has been demonstrated at temperatures from approximately –10 to +110°C, at hydrostatic pressures of at least 110 MPa, at salt concentrations of more than 5 M, at water activity (\(a_{[\text{w}]}\)) values as low as 0.61, at pH values from 0.5 to 12, and at oxidation-reduction potentials from approximately –300 to +600 mV. The following sections describes the most important consequences of these environmental parameters for the survival, growth, and cultivation of microorganisms.
Temperature.

Temperature is one of the major physical factors that influence metabolic activity, growth, and survival of living organisms. Of the enormous range of temperatures from 0 to about $3 \times 10^9$ K known to occur in the universe, only a minute fraction is compatible with life as we know it (Henis, 1987). At both ends of this temperature range, only microorganisms have been shown to be capable of growth and survival. Although there are reports of organisms growing at a temperature of $-10^\circ$C for bacteria (Ingraham, 1969), $-20^\circ$C for some fungi (Hawker et al., 1960), and $-24^\circ$C for a yeast (Michener and Elliott, 1964), most data available so far concern bacteria with a minimum temperature for growth of $-5$ to $-10^\circ$C (Morita, 1975; Baross and Morita, 1978; Mazur, 1980).

The upper temperature limit at which microbial growth has been demonstrated has recently been established at a value of 105–110°C. The record is probably held by the anaerobic archaebacterium *Pyrodictium occultum* (Stetter, 1986). Although growth at 120°C (Deming and Baross, 1986) and even at 250°C (Baross and Deming, 1983) has been described, no unambiguous description of growth at these temperatures by an isolated species has appeared so far. In fact, the absolute upper limit is suggested to be somewhere between 110 and 150°C, due to constraints on the thermostability of several essential cell components (White, 1984; Stetter, 1986; Jaenicke, 1988). Although the total temperature range covered by microorganisms measures about 125 degrees Celsius, single organisms have never been shown to span more than 10 to maximally 60°C (Wiegel and Ljungdahl, 1986; Brock, 1987; Wiegel, 1990). Based on their so-called cardinal temperatures (i.e., minimum, optimum, and maximum temperatures for growth), organisms are currently divided into five major groups: psychrophiles, mesophiles, thermophiles, extreme thermophiles, and hyperthermophiles. This distinction is made purely for practical reasons, and the distinctive temperatures should not be taken as exact values. Considerable overlap in temperature ranges exists, which is caused in particular by the fact that it is very difficult to determine the minimum growth temperature, as doubling times can become extremely long at the low end of the temperature range.

Changes in temperature within the permissive range of all these different groups of microorganisms affect their metabolism, broadly speaking, in a similar way. This is to be expected as the rate of all chemical reactions is a function of temperature according to the following relationship (the Arrhenius equation):

$$v = Ae^{-H/RT}$$

Taking the logarithm of this equation yields the following familiar relationship:

$$10\log v = -\Delta H^*/2.303RT + C$$

If the logarithm of the reaction velocity is plotted versus the reciprocal of T, a straight line is obtained with a negative slope ($-\Delta H^*/2.303R$; in which $\Delta H^*$ represents the activation energy and R the gas constant). Plots of the logarithm of the specific growth rate versus $T^{-1}$ yield similar straight lines over a large part of the growth temperature range. Several examples of such plots can be found in the literature (Ingraham, 1962; Harder and Veldkamp, 1971; Herendeen et al., 1979; Ratkowski et al., 1983). Such curves deviate gradually from linearity at the low temperature end but usually more strongly at the high temperature end, caused by the abrupt fall of the growth rate near the maximum growth temperature as a result of thermal inactivation of enzymes and disruption of membrane structure. The factors that control the actual values of the minimum and maximum temperatures for growth have been discussed extensively (Innis and Ingraham, 1978; Amelunxen and Murdock, 1978; Russel, 1984; Herbert, 1986; Sundaram, 1986; Jaenicke, 1988; Russel and Fukunaga, 1990; Wiegel, 1990). From these studies, it has become evident that a multitude of different factors affecting enzyme activity and functional integrity both of enzymes and structural cell components are responsible for the temperature limits of an organism. Some of the most important ones currently recognized include:

1. The fluidity of the membrane lipids, affected particularly by the ratio of unsaturated and saturated fatty acids, their length and (a)polarity
2. The solute transport capabilities of the membrane
3. The forces governing the formation of proper tertiary and quaternary structure of proteins
4. The temperature sensitivity of regulatory mechanisms, which, in case of malfunctioning may cause fatal imbalances in cellular metabolism.

In some cases, growth is possible at temperatures outside the normal temperature range, provided certain additional medium components are added,
indicating that some enzymes (responsible for synthesis of these growth requirements) are no longer operative (Ingraham, 1962; Ron and Shani, 1971). These observations indicate that considerable differences may exist with respect to thermostability of the many enzymes involved in microbial metabolism. This is further supported by observed metabolic changes in response to a shift in temperature within the normal span of growth temperatures. Some metabolic alterations include different fermentation products (Jung et al., 1974), changes in yield and maintenance coefficients (Brooke et al., 1989), altered specific extracellular xylanase activity (Suh et al., 1988), changes in the affinity for $\text{H}_2$ and acetate consumption by Methanosarcina barkeri (Westermann et al., 1989), reflecting a metabolic flexibility which hitherto has remained virtually unexplored (Kogut, 1980; Wiegel, 1990).

Proton Concentration.

The pH value (the negative logarithm of the proton concentration) of natural environments varies from below 1 to over 12 (Grant and Tindall, 1986; Brock, 1978; Brock and Madigan, 1991). Many microorganisms have been isolated from these environments, and over this entire range of pH values microorganisms are able to grow (Brock, 1978; Langworthy, 1978; Horikoshi and Akiba, 1982; Krulwich and Guffanti, 1989); yet, the majority of natural environments possess pH values between 5 and 9 and the pH optima for the growth of most organisms (neutrophiles) fall well within this range. For practical use only, those species with optima below pH 5 are usually called acidophiles whereas alkalinophiles represent species with optima above 8. The most intriguing observation about these “extremophiles” is the fact that they possess many enzymes and structural components that cannot function properly at the very high or low pH values found in their external environment. From this it has been inferred that the internal pH is kept at much less extreme values and is probably relatively constant. Given the fact that some microbial species are able to grow over ranges of sometimes 4 to 5 pH units (a 100,000 fold change in proton concentration!), it is not surprising that many studies during the last decade have focused on the question whether, to what extent, and by what mechanisms an internal homeostasis with respect to pH can be established (Krulwich and Guffanti, 1983, 1989; Padan, 1984; Booth, 1985; Kashket, 1985; Padan and Schuldiner, 1986; Krulwich et al., 1988; Matin, 1990). The general picture that has emerged from these studies shows that the internal pH of acidophiles is regulated to a value of 6.0–7.0, that neutrophiles keep pH slightly more alkaline inside than outside, and that alkalinophiles maintain an internal value of 1 to 2 units lower than the external value. Major causes of external pH change in the cultivation of microorganisms are the growth-linked production of organic acids (fermentative organisms in particular), the utilization of anions from salts used as growth substrates, and the production of ammonia from nitrogen-containing compounds. Eventually this metabolic activity can result in total arrest of growth and metabolism if no precautions are taken to maintain the culture pH within the permissive range for growth. Interestingly, several studies on the activity of microorganisms close to or just beyond the pH values tolerated for normal growth have revealed metabolic activities not present at normal pH values (Gottschal and Morris, 1981; Graham and Lund, 1983; Gottwald and Gottschalk, 1985; Montville et al., 1985; Ferchichi et al., 1986; Huang et al., 1986; Bowden and Hamilton, 1987; Forsberg, 1987; Hüsemann and Papoutsakis, 1988; Hommes et al., 1989). Especially, fermentative organisms have thus been manipulated to produce nonacidic end products.

Osmolarity.

Life is strictly dependent on the availability of water, but microorganisms differ considerably in their requirements with respect to the actual water content of their immediate surroundings. A generally used measure of the water availability is water activity, $a[w]$, defined as the ratio of the vapor pressure of a given solution relative to the vapor pressure of pure water at the same temperature. Thus, water activity can have any value from 0 to 1. By far, most organisms known will only grow well at values between 0.9 and 1.0, values which are also most commonly found in aquatic and soil environments. Great variability exists in water activities of various materials and habitats and the types of organisms capable of growth under these conditions. Freshwater media used for routine cultivation of microorganisms usually have $a_w$ values not lower than 0.99, whereas seawater (3.5% NaCl) has a value of approximately 0.98. Salt lakes in which Halobacterium and Halococcus can be found have values of around 0.75 and NaCl concentrations of up to saturation. The lowest $a_w$ value reported at which growth of microorganisms still occurs is 0.61 for the xerophytic fungus Xeromyces bisporus, which can grow on dry foods. More detailed information on growth of microorganisms at low water potentials can be found in several text books and some specialized literature (Brown, 1976; Griffin.
A direct consequence of changes in water activity is variation in osmolarity. Bacteria appear to maintain their intracellular osmolarity at a value slightly above that of their surroundings, which is possible due to their rigid cell walls that can withstand considerable osmotic pressure. If internal osmolarity falls below that of the environment, dehydration and, as a consequence, damage to the cell membranes would be the result. The mechanisms through which microbial cells regulate their internal osmolarity in response to external change have become better understood only relatively recently. Although the precise characteristics of osmoregulation differ considerably for various organisms, the general strategy is based on selective accumulation of solutes that do not strongly interfere with cellular metabolism and have thus been termed compatible solutes (Brown and Simpson, 1972). A considerable list of such compatible solutes have been identified up to now; the most notable ones are: potassium ions, proline, glutamate, glutamine, alanine, α-aminobutyrate, glycine betaine, trehalose, sucrose, and choline. Some of these, especially proline, glycine betaine, and choline, are also termed osmoprotectants, as in some organisms they may alleviate osmotic stress effects through direct interactions with intracellular macromolecules.

Although this osmoregulatory potential allows growth of many organisms over a considerable range of osmotic conditions, this type of adaptation clearly has its limits, and microorganisms can be divided according to their osmotic tolerance. This classification has been used most often for NaCl tolerance. Nonhalophiles thus are organisms capable of growth at NaCl concentrations of less than 0.2 M, whereas moderate halophiles (and marine species) usually grow from 0.2–3.5 M NaCl, and extreme halophiles grow from 1–5.5 M NaCl. More detailed information on the mechanisms of osmoregulation and the properties of halophilic organisms is to be found elsewhere (Kushner, 1978; Griffin, 1982; Yancey et al., 1982; Epstein, 1986; Imhoff, 1986; Larsen, 1986; Higgins et al., 1987; Ingraham, 1987; Kogut and Russel, 1987; Walderhaug et al., 1987; Csonka, 1989).

### Hydrostatic Pressure.

In the deep sea, hydrostatic pressures prevail, due to the weight of the water column, to a maximum of approximately 1150 atm (= 115 Mpa) at the deepest places. Since the mean depth of the world's oceans is on the order of 3500–4000 m, and roughly 70% of the earth's surface is covered by oceans, a vast environment exists with a mean pressure of about 375 atm and a temperature of ≤5 degrees Celsius. As early as 1872 the Challenger expedition definitively revealed the occurrence of living material from depths of at least 8000 m. Only 12 years later, the bacteria found in samples from the deep sea were shown to be more pressure tolerant than terrestrial species (Marquis and Matsumura, 1978; and references therein). It took a long time before these findings were rigorously confirmed, and barophiles were defined as organisms well adapted to growth at high pressure (ZoBell and Johnson, 1949). Since then many bacteria have been isolated from the deep sea and classified as barotolerant (increased growth rates after decompression) or true barophiles (optimum growth rate at elevated pressures). As a rule these organisms are also psychrophiles (the reverse is definitely not true!) and until a few years ago were generally considered copiotrophs, i.e., growing optimally at relatively high nutrient concentrations (Morita, 1976, 1986; Jannasch and Taylor, 1984; Jannasch and Wirsen, 1984). However, most recent results seem to indicate that barophiles also include oligotrophic species capable of growth and metabolic activity at very low nutrient concentrations (<10 µg·L⁻¹) (Deming and Colwell, 1985; Deming, 1986). This indicates that barophiles are not confined to nutrient-rich niches, such as fecal pellets and inside higher organisms, but are also found free in the water column and in the sediment. In spite of this abundant occurrence of organisms adapted to grow optimally at elevated pressures, it must be remembered that nonbarophiles also abound at great depths. Some of these organisms are remarkably well adapted to survive under conditions of high pressure. High pressures affect many different aspects of a cell's metabolism and integrity, for example, membrane structure, transcription and translation, and enzyme activity are all sensitive to this type of stress (Marquis, 1976; Marquis and Matsumura, 1978; MacDonald, 1984; DeLong and Yayamos, 1986; Morita, 1986; Wirsen et al., 1987; Jaenicke, 1988). The exact mechanisms involved in the adaptive response to high pressure are largely unknown although it seems likely that membrane fluidity (which is negatively influenced by high pressures) is adjusted through changes in the ratio of unsaturated over saturated fatty acids in membrane phospholipids (MacDonald, 1984; DeLong and Yayamos, 1986; Wirsen et al., 1987) as occurs in response to temperature changes.
**Interactions Among Microorganisms**

In natural environments, prokaryotes are not only strongly influenced by the various physicochemical factors mentioned above but they are also subject to a plethora of interactions with other (micro) organisms. In the past, several schemes of classification have appeared in an attempt to aid description and understanding of the complexity of (microbial) community structure (Bungay and Bungay, 1968; Odum, 1971; Fredrickson, 1977; Bull and Slater, 1982; McInerney, 1986; Atlas and Bartha, 1987). However, in some of these classifications, attempts are made to describe too many of the numerous types of interactions and their various combinations, which makes the use of such schemes rather impractical. Fortunately, there appears to be agreement about a restricted number of terms that have proved quite useful in characterizing the essential traits of the known interactions. This terminology has been presented in various ways in previous publications and is summarized in Table 4. It is important to remember that in spite of the apparently clear distinctions between the different types of interactions it is more likely the rule than the exception that in natural communities all sorts of combinations of these relationships will occur. Even in laboratory studies on mixed populations, it will often be very difficult to decide about the precise nature of the interaction(s) between the organisms under study. In the sections below a brief outline is given of the most important interactions currently known to occur between microorganisms. No attempt has been made to present a comprehensive review but in most cases reference will be made to the pertinent and more detailed literature.

Table 4. The most common terminology used to describe interspecies interactions.

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Nature of the interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralism</td>
<td>Neither population affects the other</td>
</tr>
<tr>
<td>Competition</td>
<td>Populations inhibit each other when resources are in short (limiting) supply</td>
</tr>
<tr>
<td>Amensalism</td>
<td>Population 1 is influenced negatively by 2, but 2 is not affected by 1</td>
</tr>
<tr>
<td>Parasitism</td>
<td>Population 1 consumes population 2, but usually not in a destructive manner</td>
</tr>
<tr>
<td>Predation</td>
<td>Population 1 consumes population 2 in a destructive manner</td>
</tr>
<tr>
<td>Commensalism</td>
<td>Population 1 benefits from population 2 without affecting it in a negative sense</td>
</tr>
<tr>
<td>Protocooperation</td>
<td>Population 1 and 2 interact in a way that is favorable to both but is not obligatory</td>
</tr>
<tr>
<td>Mutualism</td>
<td>Like protocooperation, but interaction is obligatory</td>
</tr>
</tbody>
</table>

The ability to capture resources for growth from their immediate surroundings undoubtedly represents one of the most important properties of (micro) organisms to survive in nature. Since for most microorganisms energy and carbon sources and other inorganic nutrients will often be present in limiting quantities, competition for growth-limiting resources is a major interaction between microbes. It is a negative type of interaction in the sense that it adversely affects the competing species involved as it results in reduction of the rate of growth. It may eventually lead to the exclusion of the slower-growing species, which has led previous investigators in this field to formulate the “principle of competitive exclusion” (Gause, 1934; Hardin, 1960). Since the introduction of culture methods (see above) that allow continued cultivation under conditions of nutrient limitation, mixed culture studies have long been dominated by studies on the competitiveness of many different microorganisms under a great variety of environmental conditions. For extensive discussions and numerous examples of such studies, the reader is referred to the specialized literature (Powell, 1958; Veldkamp and Jannasch, 1972; Fredrickson, 1977; Tilman, 1977; Veldkamp, 1977; Fredrickson and Stephanopolous, 1981; Bull and Slater, 1982; Kuenen and Gottschal, 1982; Kuenen and Harder, 1982; Veldkamp et al., 1984; Gottschal, 1985; Gottschal and Dijkhuizen, 1988). These studies are mostly concerned with simple and pure competition (that is, no other interactions are involved) for a single growth-limiting nutrient and have clearly supported the competitive exclusion principle. The outcome of the competition between two or more species is merely dependent on the shape of the μ versus s relationships of the competing organisms. Organisms exhibiting the higher specific growth rate at a given substrate concentration always outcompeted the slower-growing species. Interestingly, in some cases, the outcome of the competition depended on the dilution rate chosen: the μ versus s relationships crossed. Apparently certain species are much better adapted to growth at very low substrate
concentrations (with relatively low $\mu_{\text{max}}$ values) whereas others are more specialized in growth at much higher rates in the presence of higher substrate concentrations. Examples have been reported for aerobic and anaerobic heterotrophs, for chemolithotrophic species, and also for phototrophic organisms (Harder and Veldkamp, 1971; Jannasch and Mateles, 1974; Fredrickson, 1977; Harder et al., 1977; Mur et al., 1977; Veldkamp, 1977; Matin and Veldkamp, 1978; Kristjansson et al., 1982; Lovley et al., 1982; Laanbroek et al., 1983, 1984; King, 1984; Kuenen and Robertson, 1984; Robinson and Tiedje, 1984; Veldkamp et al., 1984; Legan et al., 1987; Legan and Owens, 1988). Yet, although these examples of pure competition amply illustrate the principle of competitive exclusion, many examples of stable mixed cultures limited by only one primary substrate have been reported. In such cases, competition may have been not so pure. Indeed, reasons for stable coexistence include the occurrence of other interactions (especially commensalism and mutualism), formation of self-inhibitory products, the presence of predators, (selective) adhesion, fluctuations in physical parameters (pH, temperature, light, aerobic/anaerobic conditions, etc.), or an alternating supply of differing growth-limiting substrates (Bungay and Bungay, 1968; Megee et al., 1972; Jost et al., 1973; Meers, 1973; Van Gemerden, 1974; Meyer et al., 1975; Ellwood and Hunter, 1976; Lee et al., 1976; Fredrickson, 1977; de Freitas and Fredrickson, 1978; Harrison, 1978; Mayfield and Innis, 1978; Slater and Bull, 1978; Gottschal et al., 1979; Gottschal and Morris, 1981; Miura et al., 1980; Driessen, 1981; Slater, 1981; White and Kidney, 1981; Wood, 1981; Bull and Slater, 1982; Linton and Drozd, 1982; Kuenen and Robertson, 1984, 1988; Kuenen et al., 1985). A very thoroughly studied example of a commensalistic relationship based on cross-feeding involves the mixed continuous culture of Propionibacterium shermanii growing at the expense of lactate produced by Lactobacillus plantarum from glucose, the growth-limiting nutrient in the chemostat (Lee et al., 1976). Lactate was also the mediator in a commensal relationship between Streptococcus mutans and Veillonella alcalescens. Both strains are commonly found in dental plaque and were shown to coexist in mixed continuous cultures supplied with glucose as the limiting nutrient (Milk and van der Hoeven, 1975). In this mixed culture, the lactate produced by Streptococcus mutans was metabolized to acetate, propionate, and ethanol which, due to lower dissociation constants, might reduce the demineralization of enamel of the teeth surface. Several years later it was shown for Streptococcus cremoris that the consumption of lactate by a second organism (Pseudomonas stutzeri in this case) under anaerobic conditions in the presence of nitrate in a lactose-limited mixed chemostat culture resulted in a marked stimulation of the growth yield of Streptococcus cremoris (Otto et al., 1980). The explanation offered for this stimulatory interaction was that by maintaining very low external lactate concentrations, Streptococcus cremoris is able to gain more energy from the efflux of lactate than at higher external concentrations (Michels et al., 1979; ten Brink, 1984). This type of stimulatory interaction can also be encountered in mixed cultures in which stimulatory products are formed or inhibitory compounds are being removed. A well-known example of the latter situation was demonstrated in a mixed culture of methane-consuming pseudomonads with three other species present in which a Hyphomicrobium species grew at the expense of small inhibitory amounts of methanol formed by methane oxidation (Wilkinson et al., 1974). A good example of reciprocal stimulation of two species in mixed culture was demonstrated during attempts to produce yogurt by continuous cultivation. In this case, Lactobacillus bulgaricus produced amino acids which stimulated the growth of Streptococcus thermophilus, which in turn produced small quantities of formic acid which stimulated Lactobacillus bulgaricus (Driessen, 1981).

Chemolithotrophic and phototrophic (micro)organisms growing with CO$_2$ as a carbon source are known to excrete substantial quantities (values of up to 50% of the carbon fixed have been reported for phytoplankton) of various organic compounds (Clark and Schmidt, 1966; Fogg, 1971; Cohen et al., 1979; Lee, 1981; Jones, 1982; Coveney, 1982; Wolter, 1982; Azam et al., 1983; Soendergaard et al., 1985). The nature of these excretion products is very diverse indeed: vitamins, lipids, amino acids, peptides, enzymes, toxins, carbohydrates, organic phosphates, etc. This represents an excellent basis for commensalistic and mutualistic relationships (Jones, 1982; Bateson and Ward, 1988), in some cases accompanied by inhibitory and/or competitive interactions (Rhee, 1972; Gottschal et al., 1979; Jones, 1982; Kuenen and Gottschal, 1982; Currie and Kalff, 1984). Whereas the flow of carbon and other nutrients produced by autotrophic species obviously provides heterotrophic bacteria with growth substrates, the benefit of these interactions for the chemo- or photolithotrophic species often is less evident. Exchange of vitamins is most often cited as an important positive influence of bacteria on the growth of oxygenic phototrophic organisms (Jones, 1982). Stimulation of N$_2$ fixation in heterocysts of cyanobacteria due to oxygen consumption by bacteria has also been demonstrated (Bunt, 1961; Paerl, 1978). Enhanced growth of cyanobacteria in the presence of bacteria after addition of organic substrates has been interpreted as an effect of increased CO$_2$ concentration (Lange, 1971). Bateson and Ward (1988) suggested a mutualistic interaction by demonstrating the excretion of glycolate by...
**Synecococcus lividus** and subsequent uptake by filamentous heterotrophs, essential in maintaining the structure of a cyanobacterial mat in a hot spring. A mutualistic relationship was also suggested in a mesophilic estuarine mat ecosystem in which the cyanobacterium *Microcoleus chitonoplastes* excreted organic matter that stimulated growth of *Thiocapsa roseopersicina*, which in turn prevented accumulation of excess amounts of hydrogen sulfide (de Wit and van Gemen, 1988). Excretion of organic matter may play a significant role in the ecology of chemo- and photolithotrophic organisms but it represents a fundamental characteristic of the decomposition of all organic material by anaerobic microorganisms. In contrast to aerobic decomposition processes, mineralization in anaerobic ecosystems always proceeds stepwise through the tight cooperation of mixed populations each responsible for distinct phases in the total mineralization. The general outline of this anaerobic process has been reviewed repeatedly in the past (Hungate, 1960; Bryant, 1976; Hobson, 1981; McInerney and Bryant, 1981; Laanbroek and Veldkamp, 1982; Wolin, 1982; Gujer and Zehnder, 1983; Zeikus, 1983; Nedwell, 1984; Ljungdahl and Erikkson, 1985; Dolfing, 1988; McInerney, 1986; Oremland, 1988; Parkes and Senior, 1988). In this multistep process, polymeric organic matter (cellulose, proteins, lipids, etc.) is first hydrolyzed to oligomeric and monomeric compounds which subsequently are fermented predominantly to fatty acids, alcohols, CO₂, H₂, sulfide, and ammonia. In the presence of oxidized sulfur compounds, further mineralization may proceed directly through the activity of sulfate-reducing bacteria, which, as a group, have been shown capable of metabolizing an enormous range of (long-chain) fatty acids, amino acids, alcohols, and aromatic compounds, and hydrogen (Hansen, 1988; Widdel, 1988). In the absence of sulfate as a terminal electron sink, CO₂ will serve as the major electron acceptor resulting in the formation of methane. But due to the very limited range of suitable substrates for methanogenic bacteria (mainly H₂, acetate, formate, methanol, and several methylamines; Oremland, 1988), these “acetogenic” bacteria are involved in the formation of suitable methane precursors. For sugars, most amino acids, and other readily fermentable substrates, this results in the formation of hydrogen, CO₂, formate, alcohols, acetate, and other short-chain fatty acids. However the fermentative degradation of some organic compounds to hydrogen, carbon dioxide, and acetate is an endergonic process under standard conditions. These fermentations will only proceed if conditions are changed in such a way that these fermentation reactions become exergonic. It has been quite well established that this is accomplished in anaerobic systems by a decrease of the partial pressure of hydrogen (a reaction product in these fermentations) to very low values (typically below 10⁻⁴ atm) (Zehnder and Stumm, 1988). Such low pressures are obtained by the occurrence of exergonic hydrogen-consuming reactions performed by sulfate-reducing bacteria, methanogens, or other hydrogen-oxidizing bacteria. This so-called “interspecies hydrogen transfer” thus represents a very important syntrophic relationship between hydrogen-producing and hydrogen-consuming microorganisms. Several cocultures of obligate proton-reducing (or obligate hydrogen-producing) bacteria and methanogenic or sulfate-reducing bacteria have now been described. For example, *Syntrophomonas wolfei* is capable of metabolizing even-numbered fatty acids such as butyrate, caproate, and caprylate to H₂ and acetate; odd-numbered fatty acids such as valerate and heptanoate are metabolized to propionate, acetate, and hydrogen (Dwyer et al., 1988; McInerney et al., 1979, 1981). *Syntrophobacter wolinii* was shown to degrade propionate to acetate, CO₂, and H₂ in coculture with a H₂-consuming *Desulfovibrio* species (Boone and Bryant, 1980). Although acetate can be cleaved to CH₄ and CO₂ by several methanogenic species, a thermophilic acetate-oxidizing organism was recently shown to convert acetate to CO₂ and H₂ only in coculture with *Methanobacterium thermoautotrophicum* (Zinder and Koch, 1984). Benzoate and several other aromatic compounds were also shown to be degraded by cocultures of a proton-reducing species and methanogens, sulfate-reducing bacteria, or both (Shelton and Tiedje, 1984; Mountfort and Bryant, 1985; Dolfing and Tiedje, 1986; Mountfort and Asher, 1986; Stevens et al., 1988; Gentner et al., 1989; Szewzyk and Schink, 1989). In those fermentations for which proton reduction and subsequent hydrogen transfer is not a strict requirement (the overall conversion is exergonic), hydrogen consumption very often appears to shift the fermentation pattern toward the production of more oxidized products plus hydrogen at the expense of reduced fermentation products. This was shown first for *Ruminococcus albus*, which no longer produced ethanol but fermented glucose to acetate, H₂, and CO₂ only if cocultured with *Wolinella succinogenes*, which used hydrogen as electron donor in the reduction of fumarate to succinate (Ianotti et al., 1973). Theoretically, this results in an energy gain for *Ruminococcus albus* as more acetyl-CoA is converted in an ATP-yielding route to acetate. Similar shifts in fermentation products were shown for *Clostridium thermocellum* if grown in the presence of *Methanobacterium thermoautotrophicum* (Weimer and Zeikus, 1977) and for an anaerobic fungus from the rumen in coculture with *Methanobrevibacter* species and *Methanosarcina barkeri* (Mountfort et al., 1982; Joblin et al., 1989; Marvin-Sikkema et al., 1990). Further examples can be found in Wolin, (1982), McInerney (1986), Dolfing (1988), and references therein. Although hydrogen represents the
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best-documented vehicle through which fermentative species can transfer reducing equivalents generated in the glycolysis to other species, formate may serve the same purpose, especially in species lacking hydrogenases (Thauer et al., 1975; Thielean Zeikus, 1988; Thiele et al., 1988).

Other intriguing types of interactions are those which occur between organisms with strongly opposing growth requirements such as aerobic and anaerobic species. In heterogeneous ecosystems, like soils, sediments, suspended particles, and stratified water bodies, anaerobic and aerobic microorganisms have been shown to occur and to be active virtually next to each other; very high (micro) biological activities are often associated with such sites (Jones, 1982; Revsbech and Jørgensen, 1986; Murray et al., 1989; Sweerts and de Beer, 1989) These aerobic and anaerobic populations are tightly linked to each other by diffusion of metabolic products. It is also feasible that certain compounds requiring both oxygen-dependent and strictly anoxic steps for complete degradation could be metabolized optimally under such conditions. In an elegant study on the degradation of the insecticide DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl] ethane), this principle was demonstrated in vitro using beads as a model particulate system (Guenzi and Beard, 1968; Beunink and Rehm, 1988). Calcium alginate-immobilized mixed cultures of Alcaligenes species and Enterobacter cloacae performed reductive dechlorination of DDT inside the beads, whereas on the outside of the beads, the partly dechlorinated products were metabolized oxidatively. Since highly chlorinated compounds generally are dehalogenated more readily under anaerobic conditions, this same principle may hold for many other halogenated xenobiotics as well.

A fascinating network of interspecies interactions can be found not only between aerobic and anaerobic organotrophic bacteria but also among oxygenic and anoxygenic phototrophs in laminated microbial ecosystems, often referred to as microbial mats. These intricate microbial communities have been found on seashores, estuarine areas, salt marshes, and along the effluents from geothermal springs. The formation of microbial mats usually starts with the colonization of sandy sediments by N₂-fixing oxygenic cyanobacteria (Stal et al., 1985; Ward et al., 1987). This eventually results in organic enrichment of the sediment due to excretion by and lysis of these primary colonizers. The subsequent mineralization of this organic matter by aerobic heterotrophs soon results in depletion of oxygen at the most productive sites. The resulting anoxic conditions allow the development of fermentative bacteria and eventually the building up of active sulfate-reducing and/or methane-producing populations. The sulfide produced supports the development of anoxygenic phototrophs, and at sites where sulfide comes in contact with oxygen it results in the establishment of sulfur-oxidizing chemolithotrophic bacteria. In those cases in which methane is produced (in habitats with limiting sulfate availability) methane-oxidizing bacteria develop at the oxygen interface. Although the basic structure and species composition of several microbial mat ecosystems are known, the multitude of these microbial species and the complexity of their interaction is such that the details of these extremely complicated communities are still poorly understood. For a good survey of the current knowledge in this field the reader is referred to several recent overviews (Cohen, 1984; Herbert et al., 1986; Cohen and Rosenberg, 1989).

A special type of negative interaction is exerted by species of the genus Bdellovibrio (Burnham and Conti, 1984). Bdellovibrios are bacteria having the unique ability to attack Gram-negative bacteria, by attaching to their prey, penetrating the cell wall, and subsequently multiplying within the periplasmic space. In the course of this process, the host cell envelope is converted into a so-called “bdelloplast,” a globular envelope with a drastically altered morphology and composition. Within this bdelloplast, the Bdellovibrio multiplies at the expense of cellular components of the host cell. Approximately 2–3 hours after infection, up to 20–30 daughter cells are released, which are immediately capable of swarming out and attacking new prey cells (Varon and Shilo, 1980; Rittenberg, 1982; Shilo, 1984). Bdellovibrio species and similar organisms (Varon and Shilo, 1980; Burnham and Conti, 1984) appear to be widespread, having been isolated from many different aquatic and terrestrial ecosystems. Although the growth and survival of bdellovibrios is strictly dependent on the availability of appropriate prey cells, the mechanism differs fundamentally from that adopted by viruses, as the latter depend on the metabolic machinery of the host cell. In spite of their ubiquitous occurrence in nature, the importance of bdellovibrio-(like) cells for natural communities is still obscure.

A similar situation exists with respect to the significance of bacterial lysis caused by viruses, especially since recent evidence suggests that viral abundance in marine habitats is far greater than previously believed (Bergh et al., 1989). If these observations should also indicate an abundance of bacteriophages in natural environments, it could mean a significant involvement of phages in the transduction of genetic information between bacteria in natural communities. This type of interaction between prokaryotes may be quite important for microbial communities to meet the challenge of developing pathways for the degradation of unusual and/or xenobiotic compounds and to increase drug resistance (Slater and Bull, 1982; Reanney et al., 1982, 1983; Slater, 1984).
Bacteria, faced with compounds which they cannot or can only partly catabolize, could attain the ability to fully metabolize such compounds through mutational changes in their genetic material. However, it is perhaps more probable that microbial communities acquire such novel capabilities by rearranging the pool of enzymes through an exchange of genetic information within the entire mixed population (Reaney et al., 1983). Numerous synergistic communities capable of catabolizing many different xenobiotic compounds have been readily isolated (Reaney et al., 1983; and references therein). In some cases individual strains were obtained from such (defined) communities that had acquired complementary pathways for the degradation of the xenobiotic on their own (Reineke and Knackmuss, 1988; Reaney et al., 1983). However, this is by no means a general rule, as exemplified by the inability to demonstrate the occurrence of such new strains in communities which nevertheless have successfully degraded xenobiotics for very long periods of time (Senior et al., 1976; Daughton and Hsieh, 1977).

The reasons for these different results remain obscure, illustrating our current lack of knowledge of the conditions required for this highly complex process of horizontal gene transfer within laboratory mixed cultures, let alone in natural ecosystems. Recent surveys of our knowledge about the survival and genetic transfer of natural and engineered bacteria in natural environments further emphasize the need for developing means of studying the influence of engineered organisms on the ecological structure and functioning of natural communities (Stotzky and Babich, 1986; Trevors et al., 1987).

General Techniques for the Cultivation, Enumeration, and Isolation of Bacteria

The study of prokaryotes usually requires their culture in the laboratory. In order to allow development of the desired organisms, sterile culture media have been developed that not only contain the water, nutrients, and gases required, but also provide the appropriate physicochemical environment, while contamination is prevented. The isolation of a clone or a population of bacteria, all derived from a single cell, is usually called “the isolation of a bacterium.” In order to isolate and culture a particular bacterium, its nutritional requirements and physicochemical environmental needs have to be met. Although much has been learned from studies with pure cultures of bacteria, these remain artificial in the sense that most bacteria in nature live together with other organisms in multispecies communities. In various habitats, microbes have been shown to interact in various ways ranging from straightforward antagonistic behavior up to mutual interdependencies, forming associations or consortia of a more or less obligatory nature.

While many bacteria have been isolated and described from their natural habitat, our understanding of how well these pure-culture organisms represent the diversity of microorganisms within these environments is unknown. Some experts believe that 80% of all bacteria in nature still have not been grown in pure culture (Wayne et al., 1987). Among the bacteria that have never been isolated are various morphologically conspicuous large bacteria living in sewage sludge, sediments, and the animal gastrointestinal tract, as well as many bacteria that live as endosymbions or parasites in protozoa. It is now recognized that certain bacteria from low-nutrient environments (probably living as oligotrophs in soil and in the aquatic environment) are viable, but nevertheless resist attempts at isolation when common media are used. This “viable but nonculturable” phenomenon is of considerable importance to public hygiene since it also poses a problem for the isolation of species such as Campylobacter and Vibrio.

Handling of Glassware and Equipment

In each microbiological laboratory—in particular when work is done with pathogens—there should be a service unit run by professionals who are responsible for decontamination, washing, and sterilization of glassware and equipment, as well as for the provision of sterile material and media. Because of the potential danger involved in working with infectious microorganisms, irrespective of their known pathogenic nature, the requirements for sterile working conditions and safe handling of contaminated glassware and equipment should be rigid. A continuous and cyclic flow of glassware and media should be secured: i.e., contaminated glassware will go into a dirty area, used materials are first autoclaved, and then unloaded in the wash-up room. Thereafter, the cleaned glassware is brought into the media room where culture media and solutions are prepared, from where they go to the autoclave room. Sterile media
are distributed for use or stored in a special room at 2–4°C.

Sterile rooms and sterile cabinets supplied with gas, electricity, sterile air, and ultraviolet (UV) irradiation are useful, because they considerably reduce the possibility of air contamination. For the sterilization of laboratory inoculating chambers and other work areas, UV irradiation (wavelength 260 nm) is sometimes applied. Laminar-flow clean air stations are cabinets designed to provide a work area which is protected from the environment, preventing airborne contamination (for safety cabinets, see below). Filtered air is passed—in a vertical or horizontal unidirectional (laminar) flow—through the cabinet. The air is made sterile by filtering through high-efficiency air filters that can remove particles down to 0.3 µm. Cabinets in which the flow of filtered air is directed toward the front are useful for preventing contamination when handling sterile media, such as during aseptically dispensing sterile fluids and culture media. In other types of cabinets the flow is directed vertically downward and is then usually recirculated. Laminar-flow cabinets are not to be confused with safety cabinets and should not be used for handling bacteria or tissue cultures, although a class II microbiology safety cabinet will also protect against contamination.

General information on large equipment in the microbiology laboratory can be found in Gerhardt et al. (1981). For a handbook on microscopy, see Locquie and Langeron (1983), while Pettipher (1983) has details on the use of the fluorescence microscope for epifluorescent filter counting. Advanced light microscopy techniques are dealt with in the book by Pluta (1988). Techniques for photography of colonies is treated by Shapiro (1985). Information on the staining of bacteria is to be found in G. Clark (1983) and Clark and Kasten (1983). Information on smaller equipment can be found in Gerhardt et al. (1981), Collins et al. (1989), O'Leary (1989), and in various volumes of the series Methods in Microbiology edited by Norris and Ribbons.

Culture Media

Bacteriological culture media usually are made as liquid broths or as semisolid or solid media. Liquid media that are prepared by including a solidifying agent like gelatin or agar were originally designed for isolation of organisms in pure culture but now they are also used routinely for general culture work.

Liquid and Solid Media.

Liquid media (broths) are used in studies of growth and metabolism in which it is necessary to have homogenous media conditions, to follow optical density, and to allow easy sampling for analysis of substrates and metabolic products. Tubes and flasks with liquid cultures can be incubated either static or shaken. With aerobes the gas phase should not be too small and because of the poor solubility of oxygen, aeration or vigorous shaking is necessary to increase the air-water contact.

Solid media are often used in the enumeration and isolation of bacteria from a mixed population by diluting the original bacterial suspension and spreading a small inoculum over the surface of the solidified medium. Using solid media, colony morphology and other properties such as swarming over the agar surface can be easily observed. Extracellular enzymes diffusing away from the colony can be detected as a result of their action on insoluble substrates (cellulose, starch, lipid emulsions) present in the agar medium. Because degraded amylase fractions of starch do not produce a blue color when stained with iodine, starch plates can be flooded with iodine to render clearing zones more evident. To improve visibility of clearing zones in opaque media, methods have been devised (Smith, 1977) involving the use of dyed substrates (cellulose-azure, lipids stained with Sudan black). Starch can be complexed with the blue dye Cibacron blue F3 G-A to yield a water-insoluble substrate for incorporation in a plating medium (Klein et al., 1969). Other applications of solid media are: the detection of microbial excretion products, such as fermentation acids, amino acids (LiMuti and Paulson, 1989), and the testing of the effects of antibiotic substances on colony growth.

Although agar is most commonly used as the solidifying agent, silica gel and Gelrite are used for particular purposes. Agars constitute a family of algal polysaccharides which range from the neutral agarose to highly charged sulfated galactans (Winterburn, 1974). These polysaccharides occur in nature as hydrated visco-elastic gels. Each gel melts when warmed at a temperature characteristic of the particular polysaccharide, but resets on cooling. The process is reversible and reproducible, but the interesting aspect is that the melting and setting temperatures are not the same and may differ by as much as 50°C. Agar
from various red algae (*Gelidium* species) is the most commonly used solidifying agent. It has a large water-binding capacity, melts at 80–95°C, and solidifies at 35–40°C; it is broken down by only a few microorganisms, and it can be used for the cultivation of thermophiles on the one hand, but solidifies at such a low temperature that temperature-sensitive constituents can be generally added without problems. Various batches of normal agar may contain variable amounts of impurities such as Ca (*Bromke and Hammel, 1987*), Mg, and other minerals. For defined culture media, various purified agars are available. *Akagi et al. (1977)* described a method for direct plating of oligotrophs from the marine environment using a glass filter substitute for agar.

Agar-containing media should not be adjusted to pH values below 6.0 before sterilization, because the agar may be hydrolyzed. If low pH values are required, the adjustment should be done by aseptic addition of acid after heat treatment.

Gelatin is an animal protein that can be broken down by many bacterial species and binds less water than agar and melts at 30°C. It is mostly used to identify proteolytic activity of bacteria that shows as liquefaction of the gelatin medium.

Gelrite (*Kelco Div., Merck & Co. Inc., San Diego, CA*) was proposed by *Harris (1985)* as an agar substitute since at high concentrations, agar may be toxic to methanogens.

Media can be classified into various groups: isolation/culture media, media for identification/differentiation, and assay media. Isolation media can be nonselective or selective. Selective media favor the growth of particular bacteria by the inclusion of a particular substrate (e.g., inulin, benzoate) as a carbon and/or energy source, by the presence or absence of specific nutrients (nitrogen, vitamins, etc.) or by the presence of compounds with differential toxicity (crystal violet, antibiotics). Media for the identification of organisms are designed to differentiate between morphologically similar organisms (hemolytic reaction on blood agar, urease, production of acetoin). Assay media are used for quantifying various organic substances (vitamins, amino acids, growth factors) in bioassays, in which the growth response of a certain organism (e.g., requiring the factor to be assayed) is directly proportional to the concentration of the factor under investigation.

**Preparation of Media**

In microbiology laboratories, glass-washing machines are often used for the cleaning of glassware, which should be rinsed thoroughly afterwards to remove traces of detergents, because some bacteria are very sensitive to such compounds. Electrically heated drying cabinets with wire-rack shelves are used in large laboratories. New glassware needs special treatment for the removal of free alkali. Bidistilled water should be used in media for certain sensitive and exacting organisms, e.g., bidistilled water had to be used in our laboratory for the successful isolation and cultivation of certain cyanobacteria and marine oligotrophs.

Heat-labile supplements (serum, peptide mixtures, vitamins, growth factors) are added to the basal medium preferably as filter-sterilized solutions after sterilization of the basal medium to avoid deterioration of such compounds. This is also the recommended practice for compounds that might react with other medium ingredients during autoclaving. Glucose and other sugars, when autoclaved with salts such as phosphate, may form inhibitory sugar phosphates. Sugars and free amino groups may result in the formation of toxic Maillard reaction products. Reducing agents such as cysteine will be oxidized by other medium ingredients during autoclaving and have to be added separately. The formation of mineral precipitates sometimes has to be avoided by separate sterilization of solutions of the calcium, magnesium, and/or iron salts, and the additions of these to the cooled medium. For certain media the order in which the salts are dissolved is crucial. Addition of chelating agents may help to prevent the precipitation in some cases.

It may be necessary to check and adjust the pH of the medium after autoclaving. Changes in pH can be minimized by the inclusion of buffers or by the addition of insoluble carbonate to culture media. Dispensing of media is usually carried out after cooling to below 50°C to avoid formation of condensation. Media are dispensed aseptically into sterile tubes, flasks, or petri dishes and immediately recapped to reduce the chance of contamination. Petri dish covers are left slightly open for 1–2 hours if a dry surface is required. The use of robotics to dispense culture media is described by *Brewer et al. (1989)*. Anaerobic dispensing is described by *Holdeman et al. (1977)* and *Roche et al. (1973)*.

A large variety of complex medium ingredients (yeast extract, beef extract, meat extract) and even the readymade complete media can be obtained in dehydrated form from a number of commercial suppliers. Descriptions of numerous bacteriological media for various purposes can be found in series like

Sterilization of Media and Equipment

Since most experiments in microbiology are performed with pure cultures or defined mixed cultures in which the presence of undesired microorganisms should be avoided, sterilization has become an important part of microbiology practice. Sterilization—the complete inactivation or removal of microorganisms—can be achieved by procedures causing the death of all microorganisms by applying heat or irradiation (physical methods) or by treatment with toxic compounds and gases (chemical methods). Gases and liquids can also be sterilized by filtration, i.e., the removal of microorganisms by passing the liquid or the gas through filters with extremely small pores. No single sterilization procedure is suitable for all materials and therefore an appropriate method has to be selected. When only a fraction of the microbial population is removed from an object or from a culture, this is called disinfection, a procedure often used to indicate the removal of pathogens.

Death is defined as the irreversible loss of viability—the loss of the ability to reproduce as a result of irreversible damage to enzymes (denaturation) and other macromolecules of the microbial cell. All members of a population of a given microbial species are assumed to be comparably sensitive to a physical or chemical treatment, which means that the death of an individual cell becomes a matter of probability. Therefore, when a pure culture is exposed to a lethal agent, the kinetics of death are exponential, i.e., when the logarithm of the number of survivors (usually determined by counting colony-forming units on a suitable medium) is plotted against time a straight line is obtained whose downward slope is called the death rate. The actual number of survivors is determined by the initial size of the population and the death rate. There are differences among microbial species in their resistance to heat, radiation, and other treatments. Since, in practice, mixed microbial populations have to be destroyed in the sterilization process and since all microorganisms have to be killed, sterilization procedures should be designed to kill the most resistant forms of life, i.e., they should destroy bacterial endospores (preferably of a thermophilic species of the genus *Bacillus* or *Clostridium*). Purified suspensions of bacterial endospores are therefore used as indicators for the effectiveness of the sterilization process (Brown, 1988; Brown and Gaze, 1988). Routine sterilization procedures are designed to provide a wide margin so that the chance of having even a single survivor is less than one in a million.

Further information on methods for sterilization and decontamination can be found in Russell et al. (1982), Block (1983), and Gardner and Peel (1986).

Sterilization with Heat.

Direct heat, dry heat, and moist heat (autoclaving) are the three most common methods of sterilization. When exposed to elevated temperatures, microorganisms die at a certain rate, which is a function of the medium composition and the temperature. McKee (1988) published a simple mathematical model of the thermal death of microorganisms. The higher the temperature is raised above the microorganisms' maximum growth temperature, the faster the organism dies. Four parameters are used to compare the relative effectiveness of heat treatment procedures. The thermal death time (TDT) is the time required to kill all the organisms in a liquid suspension at a given temperature. The thermal death point (TDP) is the lowest temperature at which all organisms in a suspension are killed within a 10-minute exposure time. The D value is the decimal reduction time, i.e., the time it takes for a 10-fold reduction in the microbial population at a particular temperature. The Z value is the number of degrees that the temperature must be raised to reduce the D value 10-fold, thus: $Z = (T_2 - T_1)/(\log D_1 - \log D_2)$. 

Direct heat or incineration exposes the objects to be sterilized to an open flame, and the adhering microorganisms are quickly burned. Small equipment, inoculating needles, forceps, open ends of culture tubes, and Pasteur pipettes are routinely flame-sterilized. In hospitals, incineration is used for the destruction of contaminated wound dressings and disposables. Carcasses of diseased animals are also disposed of in this way.

Dry heat is used to sterilize empty glassware and other heat-resistant objects (laboratory instruments, surgical tools, glass syringes, needles, mineral oils, and dry powders) by baking for 2 h at 170°C in a hot-air oven. The objects should be protected from subsequent contamination, e.g., by wrapping them in Kraft paper or aluminum foil.

Heat conduction in dry air is less rapid than in moist air. Also, dry bacterial cells and spores intrinsically have a higher heat resistance than wet cells and wet spores. This means that longer exposure times and higher temperatures are required in dry sterilization than with moist objects. Obviously, dry sterilization cannot be used to sterilize liquids since these would boil at a temperature of around 100°C, while viruses or bacterial spores would still survive. Neither is the method suited for heat-labile or heat-sensitive materials such as cotton wool, plastics, or rubber.

Moist heat is the most effective and most commonly used method for sterilizing heat-labile liquids and culture media, but equally often for sterilizing equipment and instruments, glassware, hospital linen, surgical utensils, infected materials, and spent cultures. The type of container in which the microbiological waste is placed, the physical characteristics of the load, and of the autoclave bag influence heat transfer and thus sterilization time (Rutala et al., 1982). Autoclaving is the term used to denote sterilizing treatment with moist heat under pressure. In principle, an autoclave is a large pressure cooker in the form of a small chamber. After the materials are placed in the chamber, the autoclave door is tightly closed, and the air is driven out through an exhaust by allowing pressurized steam to flow into the chamber. When the chamber is completely filled with steam, the exhaust is closed while steam is still forced into the chamber until the pressure reaches 15 pounds per square inch (1 psi or 1.06 k⋅cm²) above atmospheric pressure. At this pressure—but only when the autoclave chamber is entirely filled with saturated steam—a temperature of 121°C is reached, which is high enough to kill even microbial endospores. At high altitude, a somewhat higher pressure will be required. The pressure is only needed to achieve the high temperature and is not itself involved in the killing action. It is therefore the temperature that should be monitored when checking the effectiveness and reliability of the sterilization procedure. Most commonly, materials are kept in the autoclave for 15–20 min at 121°C, not counting the time involved in heating up and cooling down. After sterilization, the steam is allowed to escape slowly to prevent boiling of liquids as a result of the sudden drop in pressure. At atmospheric pressure, the autoclave door can be opened and objects removed.

While test tubes with 10 ml of medium are safely sterilized at 121°C for 15 min, large volumes of medium take longer to heat up. It is best therefore for autoclaves to have short heat-up times to avoid unwanted changes in culture media as a result of overheating.

A mild moist heat treatment given to sensitive materials, most often to control spoilage of food products and to extend their shelf life, is called pasteurization. One treatment is the low-temperature-long-time (LTLT) procedure in which, e.g., milk in tanks is heated for 30 min at 62.9°C, but in the more-often-used high-temperature-short-time (HTST) or flash procedure, heating is for 15 sec at 71.6°C. This is followed by rapid cooling in order to minimize undesirable changes in taste and nutrient content. Pasteurization reduces the microbial population by between 97 and 99% and is intended to eliminate pathogens. This method was used first by Pasteur to control the spoilage of wine.

Sterilization Without Heat.

Filtration is the method of choice for the sterilization of heat-sensitive liquids and gases. Microorganisms are not destroyed but are removed by passing liquids or gases through filters that have pores small enough to retain the microbes. Many types of filters made of porcelain, diatomaceous earths, asbestos, and glass were used in the past, but membrane filters are used most often today. Membrane filters consist of a rigid uniform mesh of polymeric materials, such as mixed cellulose esters, polycarbonate, polytetrafluoroethylene bonded to polyethylene, or polypropylene, with pore sizes precisely determined during manufacture. Membrane (screen) filters with different pore-size diameters are available from commercial suppliers. One standard type of filter has an average pore-size diameter of 0.45 ± 0.02 µm, but for safe sterilization an 0.22-µm or 0.10-µm filter is necessary. It should be borne in mind that passage time is inversely correlated to pore-size diameter, and that the permeability of a filter medium can be affected by the chemical or electrostatic properties of
the filtrate. Moreover, it should be realized that viruses are not necessarily retained on the filters and may be present in the filtrate. Some of the advantages of the use of membrane filters are: 1) microbes are quantitatively retained on the filter surface and can be analyzed for various components or enumerated by placing the filters with bacteria on solid nutrient media for cultivation, a method used in quantitative water microbiology: 2) filtration allows the concentration of bacteria from dilute suspensions: and 3) there is a minimal loss of the suspending fluid.

In some cases, as in the sterilization of seawater, it is necessary to combine filtration with pasteurization, since certain organisms (e.g., *Flexibacter*) may even pass membrane filters (Little et al., 1987). Both methods increase the concentration of dissolved organic carbon and nitrogen as well as dissolved inorganic nitrogen. Pasteurization and sterilization in glass vessels also increases dissolved silica.

Pretreatment methods and the application of prefilters will prevent the clogging of membrane filters by colloidal or proteinaceous materials. Membrane filters can be used for final filtration or prefiltration, while depth filters generally are used in the clarification of liquids. Depth filters consist of a matrix of randomly oriented fibers wound or otherwise bonded together into a tortuous maze of flow channels. Depth filters differ considerably from membrane filters, e.g., organisms trapped in the matrix eventually grow through and contaminate the filtrate. Also, depth filters will absorb and retain large volumes of liquid. Both hydrophilic and hydrophobic filters are available. A large variety of filter holders, filter units, and other specialized ultrafiltration equipment is on the market.

For the filtration of gases, high-efficiency particulate air filters (HEPA) are used instead of membrane filters when large volumes of air have to be filtered, e.g., to supply clean rooms or laminar-flow cabinets.

Chemical sterilization is the method of choice for sterilizing solid objects that cannot be sterilized without damage by physical methods, e.g., disposable plastic ware, plastic tubing, surgical supplies (sponges), optical equipment, artificial medical implants, and samples of plant material (Shockey and Dehority, 1989). Microorganisms are killed by exposing them to toxic chemicals, e.g., propylene oxide and \( \beta \)-propiolactone, but the most widely used is ethylene oxide (EtO), an alkylating agent that reacts with hydroxyl-, sulfhydryl-, and other reactive groups in proteins and nucleic acids. EtO is a liquid that volatilizes above 10.8°C, and the sterilization involves the exposure of materials for at least 4 to EtO gas in a closed chamber, after which they must be thoroughly flushed for 8–12 with an inert gas or air. The flushing is essential since residual EtO may interfere with the experiments. EtO is explosive, flammable, causes skin burns, and is highly toxic. In practice, nonflammable mixtures of EtO and freon or carbon dioxide with the same microbicidal activity as EtO alone are in use. The high cost of the sterilizing chambers restricts the use of the EtO method to hospitals and large laboratories.

Ionizing radiation may be used in large laboratories for the sterilization of heat-sensitive solid objects such as powered pharmaceuticals, disposable plastics, clothing, hospital bedding, and mattresses. Because radiation has the power to penetrate solids, it can also be used to retard or eliminate spoilage of foods (Murray, 1989). Gamma radiation emitted by a radioactive cobalt source is most often employed. A high dose of 2.5 Mrad is sufficient to kill living cells, spores, and viruses, but chemical changes in the media are possible. The gamma rays interact with water molecules to produce ions (OH\(^{-}\)) and free radicals (·OH) that can destroy almost any type of molecule in the cell.

Sterilized culture media should be stored in a cold room at 4°C or in a refrigerator and routinely examined for contamination. The whole batch must be discarded in case of contamination. Agar slants and plates will dry out and need to be replaced from time to time.

### Aseptic Techniques

When working with a pure culture or with a defined mixed culture, it is essential to prevent chance contamination, and thus aseptic techniques are necessary. All growth media and their containers and all instruments (inoculating and sampling devices) that touch the inside of the culture or its container must be sterile. Entry of unwanted microorganisms (contaminants) has to be prevented. Containers should be covered with sterile lids or stoppers, while drafts or the creation of aerosols, e.g., by blowing out pipettes vigorously or plunging into cultures with hot loops, must be avoided, and the work area must be kept as clean as possible. The work bench can be wiped with a disinfectant to reduce the number of possible contaminants.
Sampling Procedures

General information on sampling of food and clinical material can be found in Collins et al. (1989), and general information on sampling for microbiological monitoring of the environment is found in Board and Lovelock (1973).

Equipment of various different designs is available for the sampling of air to be used in studies of environmental microbiology. Various types of slit samplers (Casella, Reynier) exist in which agar plates receive particles from the air passed through. Such samplers are used to monitor air contamination in hospitals or in the food industry. In field studies, it is easier to use the Fisons model, which draws the air through a membrane filter (Kitchell et al., 1973). Also convenient for field studies are portable, battery-operated, hand-held samplers (SAS, surface air system; RCS, Reuter Centrifugal Sampler), which contain agar-coated strips that receive particles from centrifuged air (Casewell et al., 1986). Alternative methods exist in which air is passed through broth (e.g., in an impinger) and then through a membrane filter. The filter is placed on agar or is transferred to a pad wetted with a suitable medium.

Water samples are best collected in sterilized glass bottles. Bottles should contain fresh thiosulfate solution (0.1 ml of 3%, w/v) when chlorinated water (swimming pools) is sampled. Fast transport to the laboratory and processing within 6 h is required to obtain meaningful results. Methods for the sampling of freshwater, marine, and estuarine habitats are described by Herbert (1988) and include descriptions of surface film samplers, water column samplers, bottom samplers, and procedures for the removal of sediment cores. Phelps et al. (1989) compared four types of sampling tools for the aseptic recovery of sediments from the terrestrial deep subsurface sediment. Correct sampling procedures for food are discussed in a publication of the International Commission of Microbiological Specifications for Foods (ICMSF, 1986), Mossel (1982), and Speck (1984).

Containers and sample jars are available in many sizes and forms but large screw-capped glass jars are suitable for most purposes, although leak-proof, strong and self-sealing plastic bags are preferred for pathogenic materials. Anaerobic samples should be protected from contact with air.

Nonselective Isolation and Enumeration

Quantification of the total number of microorganisms in a sample can be carried out by performing microscopic counts, using counting chambers with a ruled area, or a microscope slide with a grid. Modern image analysis systems using video cameras and epifluorescence microscopy provide accurate cell counts and size measurements of aquatic bacteria (David and Paul, 1989).

For the estimation of the viable count in a sample, the material is serially diluted and a known amount of each dilution is placed on a general (all-purpose) culture medium. Each colony that develops is assumed to have grown from a single cell, a viable “colony-forming” unit. As there is no single medium that will allow growth of all bacteria in a sample, and since a fraction of the population will be dead or nonculturable, this method gives lower numbers than the total count.

For quantification of the number of viable cells (cells capable of growing to colonies in or on a culture medium), a small amount (0.1 ml with not more than a few hundred cells) of a dilute suspension is brought on the surface of an agar plate and spread evenly with a sterile Drigalski spatula. Care should be taken to ensure that the liquid from the inoculum is readily absorbed by the agar after inoculation to prevent unreliable results caused by multiplication of bacteria in the film of liquid with subsequent lateral spreading. Motile and swarming species of bacteria cannot easily be enumerated in a plate count, unless the plates are thoroughly dried, and chemicals are added to stop motility. The plate count is used for performing viable counts of strictly aerobic bacteria, to determine the proportions of cell types in mixed populations of aerobes or to check culture purity. Some bacteria cannot be cultivated or isolated on solid agar media and require sloppy agar or a liquid medium.

In the pour-plate method, a known volume of a serial dilution of a bacterial suspension is thoroughly mixed with molten nutrient agar (agar tempered at 40°C) and then poured into a sterile petri dish where the mixture is allowed to solidify. Upon incubation, cells will grow into colonies that mostly develop below the surface. Such colonies often show a lenticular shape. Gasforming colonies may crack the agar. The pour-plate technique is easy to perform and is suited for the isolation of organisms requiring oxygen at pressures lower than atmospheric (anaerobes, facultative aerobes, microaerobic bacteria). The
method cannot be used for quantifying strict aerobes. Moreover, it will not be easy to count or isolate subsurface colonies or to assess colony morphology as a criterion for culture purity. Also, some organisms may become less viable or may be killed by even a short time of exposure to the rather high temperature of the molten agar.

Instead of using plates, one may use tubes with molten agar that are allowed to solidify after inoculation from serial dilutions. These dilution-shake cultures have the same advantages and disadvantages as the pour-plate procedure. They are especially used for the enumeration of anaerobic bacteria in tubes that can be closed with rubbers stoppers and contain an oxygen-free gas phase above the prerelaxed medium.

Plate counts, roll-tube counts, and drop count methods are described and commented on by Collins et al. (1989). The use of spiral plating machines equipped with a laser colony counter is increasingly popular for handling large numbers of specimens. The merit of the total viable count procedure in microbial ecology relative to other methods of quantification is discussed by Karl (1986). For a rapid and easy estimation of the total viable count, the most probable number (MPN) estimate is the best choice. By inoculating tubes with cell suspensions so dilute that there are some tubes that receive just one single cell or no cell at all, quantification of the total count is possible. The method is based on the assumption that bacteria are normally distributed in liquid media. Applications of this technique are diverse and range from the routine measurement of coliforms in water to the counting of oligotrophic bacteria that use naturally occurring organics in fresh water (Kimio et al., 1988) or the enumeration of sulfate-reducing bacteria (Fedorak et al., 1987). Haas (1989) proposed a procedure for the correction of biases encountered with maximum-likelihood methods, which are generally used to estimate microbial densities from dilution count experiments. Various publications provide programs for the computation of MPN by computer (e.g., MacDonell et al., 1984; Nagel et al., 1989). Rapid enumeration of viable bacteria by image analysis is described by Singh et al. (1989). A computer-enhanced dark-field microscopy method for the quantitative analysis of bacterial growth and behavior on surfaces is given by Lawrence et al. (1989).

Cultivation of Anaerobic Bacteria

The Hungate technique for the cultivation of anaerobes was discussed earlier. Later modifications of the Hungate technique included the use of butyl rubber stoppers (Hungate et al., 1966), the use of a combined screw-cap and rubber-stopper closure (Attebery and Finegold, 1969), the use of serum bottles closed with crimp-closure metal seals holding slotted butyl rubber stoppers (Miller and Wolin, 1974), the syringe technique (Macy et al., 1972), and the use of pressurized tubes and vessels for the culture of methanogens (Balch and Wolfe, 1976). Caldwell et al. (1973) designed an inexpensive apparatus facilitating the storage of oxygen-sensitive compounds. Many useful details on the cultivation of strict anaerobes and modifications for routine use can be found in Holdeman et al. (1977).

Procedures and techniques for the isolation, enumeration, and cultivation of anaerobic rumen bacteria on various defined and nondefined media were most often variants of the Hungate technique and have been reviewed by Stewart and Bryant (1988). The authors provide details and references to the sampling of rumen contents and the preparation of nonselective media for the enumeration or isolation of functional groups of rumen bacteria. Nonselective, nondefined, rumen-fluid media usually contain clarified rumen fluid, a bicarbonate-CO$_2$ buffer system, minerals, demineralized water, low concentrations of yeast extract, a source of organic nitrogen, a mixture of carbohydrates, cysteine/Na$_2$S as reducing agents, and a redox indicator (e.g., resazurin).

A sulfur-free reductant is titanium (III) citrate. Due to complex formation between titanium and citrate, Ti(OH)$_3$-precipitate formation is minimized (Zehnder and Wuhrman, 1976). The solution can be prepared by adding 5 ml of a 15% (w/v) titanium (III) chloride solution to 50 ml of a 0.2 M sodium citrate solution, followed by neutralization with saturated sodium carbonate. Moench and Zeikus (1983) reported an easy method for preparing this reductant with the use of nitriloacetic acid instead of citrate as the complexing agent.

It is now also possible to grow fastidious anaerobic bacteria, including methanogens, on common petri dishes in anaerobic chambers, thus allowing the application of replica-plating techniques to ecological and genetic studies of bacterial populations in anaerobic habitats. Leedle and Hespell (1980) introduced the use of anaerobic chambers in rumen bacteriology and employed replica-plating techniques for delineating carbohydrate-utilizing subgroups in
rumen bacterial populations. Balch et al. (1979) described this technique for use in the cultivation of methanogens in cylinders pressurized with H₂/CO₂ mixtures. Long equilibration times are required to ensure removal of O₂ adhering to dishes and media, and sufficient CO₂ should be in the gas phase to maintain the pH of the medium if a bicarbonate buffer system is used. Another advantage of the anaerobic glove-box is that miniaturized identification systems can be used without much difficulty.

Methods for Preparation of Pure Cultures

Pure cultures are required for much of the laboratory work on microorganisms, and they are essential for unambiguous studies of the (ultra) structure, physiology, molecular biology, genetics, and autecology of microorganisms. A pure culture of a microorganism represents a population (clone, strain) derived from an individual cell and when free from other (contaminating) microorganisms is called an axenic culture. The term “bacterium” is most often used to indicate a population of cells of a certain clone or strain. Not all individuals in such a population need to be genetically identical as mutation and selection of mutants can take place, but this heterogeneity is accommodated in the pure culture concept. Since the occurrence of a pure culture in nature is rare (but see below on pathogens) and usually only complex microbial communities are found in all kinds of habitats, the preparation of a pure culture is based on: 1) physical separation of a cell from its community members, 2) inoculation into a sterilized medium; and 3) incubation under conditions that allow axenic growth. The various techniques for preparing pure cultures are discussed below. Aseptic techniques as outlined earlier must be maintained throughout the work.

Plates.

A practical method for obtaining a pure culture is by dipping a sterile loop into a bacterial suspension and streaking a drop of inoculum on the surface of a solidified medium (streak plate), until bacterial cells are separated from each other. This can be achieved by continuous streaking or by fractional streaking with intermittent sterilization of the loop. The latter procedure improves the chance that cells in thick inocula will be separated and saves space on the agar plate. Physically separated cells will grow out into distinct populations visible on the solidified medium as small heaps or spots called colonies, varying in shape, color, and size. All colonies of a pure culture should be identical in appearance, color, and texture but in crowded parts of plates, the size of the colonies will be smaller because of nutrient depletion. Size difference as a criterion can only be applied to plates with well-separated (> 5-mm separation) colonies.

Serial Dilutions.

Before inoculation in a sterilized medium, most laboratory cultures and environmental samples have to be diluted, as these normally contain very high numbers of bacteria per milliliter. In case of a heterogeneous sample, a known portion of the original material is first homogenized in the dilution fluid (diluent) by shaking the mixture in a container or by agitation in some sort of mechanical device, e.g., by short-term blending in a mixer. A nontoxic detergent may be added for detachment of microbes adhering to solids. Procedures that cause some kind of injury to the cells may be detrimental to viability and should obviously be avoided. The diluent must be appropriately chosen in order to prevent loss of viability, and at the same time the conditions should be such that no growth is possible before plating. The diluent (water, saline, buffer, liquid broth) is designed to mimic the conditions of the habitat but at the same time should lack energy sources.

After preparation of the homogenous suspension, one part is placed in nine parts of diluent in the first tube of the series to obtain a 1:10 dilution. This tube is shaken thoroughly and using a fresh pipette or syringe, one part of its contents are placed in the next tube with nine parts of sterile diluent to obtain a 1:100 dilution. The denominator of the final dilution is called the dilution factor.

The pipettes used in most microbiological laboratories are either serological pipettes or measuring pipettes. Serological pipettes (marked with two concentrically etched rings near the top) are graduated all the way to the tip and are calibrated to be blown out for delivery of the exact amount shown on the scale. Measuring pipettes are not calibrated to the tip and one has to look at the position of the fluid meniscus for exact delivery.
**Single Cell Isolation.**

Microscopically controlled isolation of cells can be performed by placing microdroplets of a diluted bacterial suspension on the lower surface of a sterile cover glass and searching for particular droplets containing only one cell. Such droplets are then transferred to a nutrient medium and incubated. For precisely controlled movements a micromanipulator can be used (Skerman, 1968). Various groups of microorganisms such as cyanobacteria, myxobacteria, budding bacteria, and manganese-oxidizing bacteria (Sly and Arunpairojana, 1987) have been isolated in this way.

**Culture Purity.**

When a colony is picked for pure culturing, it is common practice to perform a number of successive streakings or platings before a culture is called a pure culture. Certain bacterial species from the original inoculum that did not multiply in the isolation medium, but are still present and viable can contaminate the selected colony and may cause problems upon subculture. The danger of contamination of this kind is especially great with species that produce sticky extracellular capsules and slime. Sometimes one can detect a mixed culture by the appearance of the colony itself (segmented colonies). To certify culture purity one has to repeat subculturing until all subsequent plates show only one type of colony, and until cells from this colony all show the same morphology, staining reactions, and physiological criteria.

**Special Aspects of the Isolation and Detection of Medically Important Bacteria**

**Safety in the Clinical Laboratory.**

The nature of pathogens as causal agents of disease requires that handling material harboring such bacteria be subject to special precautions. More than 4000 laboratory-associated infections have been reported in this century while many more probably were unreported (Collins, 1988; Collins et al., 1989). Most of these infections were caused by organisms whose potential pathogenicity was unknown at the time or when techniques were used that are known to be hazardous. On the basis of our increasing knowledge of the potential hazards of microorganisms, of the routes by which microorganisms may enter the body, and of methods for good laboratory practice and containment measures, it should be possible to avoid unnecessary risks.

In principle, the techniques used are similar in working with pathogens and nonpathogens, but certain aspects of the isolation of pathogens require special attention. These are safety and the need for rapid direct isolation or rapid isolation following selective enrichment and antibiotic sensitivity measurement. Quick isolation and identification can hasten disease treatment. Much information can be found in general books on medical bacteriology (Joklik et al., 1984; Linton, 1982; Stokes and Ridgway, 1986; Lenette, 1985); in monographs (e.g., Bartlett et al., 1988; Harrison and Taylor, 1980; Newell, 1982; WHO, 1981); in series on various genera of pathogens like the Public Health Laboratory Service Monographs (HMSO, London) or publications of the World Health Organization (WHO, Geneva), or in the chapters on medically important bacteria in this Handbook.

Recent research innovations and techniques have changed the laboratory environment. Safety management, hazard assessment, containment equipment, and techniques are discussed by Miller (1986). Although microorganisms can enter the human body in various ways, it seems that most laboratory-associated infections are caused by inhalation of infected airborne particles or aerosols (Collins, 1988; Pike, 1979).

Three main categories of laboratory aerosols are distinguished: 1) droplet nuclei, generated by mechanical forces on liquids—the droplets remain in the air and are inhaled; 2) dried materials, in particular lyophilized infective bacteria; and 3) dust particles contaminated with pathogens (Darlow, 1972). Aerosols of the first type can be created, e.g., by the opening of petri dishes and culture tubes, during transfer of cultures with loops and syringes, by various actions involved in laboratory work such as the aeration and shaking of liquid cultures, and by pipetting, homogenization, centrifugation, and ultrasonic disintegration of cells (Blumberg, 1975). Other routes of infection are by ingestion during pipetting or via contaminated food, pencils, and cigarettes, by injection such as accidental stabbing with Pasteur pipettes and hypodermic needles, and by splashing of infected liquids in the eyes. Ampoules should
preferably be wrapped in a disinfectant-soaked swabs before breaking them to minimize the risk of releasing an aerosol of the dried material.

To ensure safety, the three following rules are essential:

1. All clinical material should be regarded as potentially infectious. Tissue specimens, infected clinical materials, body fluid samples must be autoclaved and incinerated after use. Contaminated paper and cotton wool must be burnt.

2. Contaminated reusable materials must be adequately decontaminated or sterilized before washing. Immediately after use, glassware and contaminated utensils must be placed in a disinfectant solution and subsequently autoclaved before washing. Disinfectants with laboratory application are discussed in Gardner and Peel (1986). Clear phenolics and hypochlorites are most commonly used, but alcohols and mixtures of alcohols and formaldehyde and iodospheres are more effective against spores. Laboratory testing of disinfectants is discussed by Russell et al. (1982) and Collins et al. (1989).

3. Pipettes must be plugged with cotton wool and used only with a pipetting aid. All manipulations that could cause splashing and spattering are avoided. All workers must protect themselves by wearing gloves and a face mask.

### Use of Safety Cabinets.

With respect to their ability to cause disease, microorganisms are classified into four classes—hazard groups with increasing risks to workers in the microbiological laboratory and to the community. Lists of such groups have been published by the Public Health Service (USPHS, 1981) and the UK Advisory Committee on Dangerous Pathogens (ACDP, 1984). In addition, a third system for classifying microorganisms was published by the World Health Organization (WHO, 1983). Most bacteria belong to WHO risk groups I and II and carry no or minimal risk to individual laboratory workers and the community; thus they do not require special safety equipment. Strains belonging to the genus *Brucella*, the plague bacillus *Yersinia pestis*, *Francisella tularensis*, the three species of tubercle bacilli *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium africanum*; Johnne's bacillus *Mycobacterium paratuberculosis*; and the leprosy bacillus *Mycobacterium leprae* are risk/hazard group III pathogens. All manipulations with such strains that might cause aerosols have to be carried out in microbiological safety cabinets in biosafety/containment laboratories.

Safety cabinets are of three kinds: class I, II, and III. The first two classes are used in diagnostic and containment laboratories for work on risk/hazard group III organisms, and class III cabinets are used for work with risk/hazard group IV viruses. In class I cabinets, the air is sucked into the cabinet and passed out through a filter. The operator and the environment are protected, but the cultures are not. In class III cabinets, sterile filtered air enters the gas-tight chamber and the air is filtered before discharge. During operation a negative pressure is maintained inside the cabinet. Work is done via arm-length rubber gloves fitted into the front panel and access is via a separate two-door sterilizing disinfecting chamber. Detailed descriptions of the use of safety cabinets is given by Collins et al. (1989) and Clark (1988). Safety cabinets must comply with national standards (BSI, 1979; reference is not an exact match SAA, 1980; NSF, 1984). Liberman and Gordon (1989) integrate principles of biosafety with those of industrial hygiene, environmental health and safety, and occupational health. Biosafety in the large-scale fermentation laboratory is discussed by Maigetter et al. (1990).

### Sampling and Handling of Clinical Specimens.

Sampling, transport, and microbiological examination of body fluids and excretions, such as cerebrospinal fluid, eye and ear discharges, feces and rectal swabs, nasal and throat swabs, pus, serous fluids, sputum, urethral discharges, urine, vaginal discharges, wounds, and burns, are described in detail by Collins et al. (1989). Surgical specimens can be expected to contain anaerobes and hence require anaerobic transport. Blood samples are collected with great care to avoid extravasous contamination. Timing of sampling, descriptions of biphasic Castenada blood culture bottles, vacuum blood collection systems, and rapid systems for detection of growth in blood cultures are also discussed by Collins et al. (1989).

Since all clinical material should be regarded as potentially infectious and since in most cases the sampling for microbiological examination is not carried out by laboratory staff, specimen containers should be designed in order to avoid problems. Preferably such containers are robust screw-capped bottles that can be opened easily and are autoclavable and leak-proof. For certain body fluids and for tissue samples, it may be necessary to homogenize or grind up the sample before analysis.
Transport media are used when samples cannot be delivered to the laboratory immediately. Sometimes it is advisory to refrigerate and store samples at 4°C to further reduce chances of bacterial overgrowth. Boric acid is added to urine samples when neither direct delivery nor refrigeration is possible (Lum and Meers, 1989).

Selective Isolation of Pathogens.

For the isolation, detection, and recognition of pathogens from a mixed culture, direct isolation on a selective medium usually is the most rapid method. A selective medium contains at least one ingredient that inhibits the growth of unwanted microorganisms without preventing the growth of the desired species (repression selection). The selective agents most commonly used are antibiotics, dyes, or other inhibitory chemicals, but in a very strict sense all media are at least slightly selective.

A wide choice of media for the selective isolation of medically important bacteria and/or various growth-promoting or inhibitory supplements and additives for the more exacting organisms are described in manuals of commercial manufacturers. Collins et al. (1989) also provide a list of laboratory-prepared media for the identification, transport, and maintenance of pathogens and other bacteria.

Differentiating media are designed to distinguish one type of microorganism from another in a mixed culture. A differentiating medium contains a special ingredient that changes during growth of a certain type of bacterium. Differentiating media can be selective or nonselective, and the latter differ from all-purpose media only because they permit the recognition of a special type of microorganism on the plate.

When the desired organism is present in very small numbers, the enrichment-culture technique is used to enable this particular type of microorganism to grow faster than all others in the sample. No inhibitory ingredient is added to the medium, but the enrichment medium is designed to favor the growth of the desired organism. Examples of such enrichments are given elsewhere in this Handbook.

Selective Enrichments

In a selective enrichment culture, a (natural) sample, containing many different microorganisms, is kept under conditions that favor a particular physiological type of organism or a group of organisms, thus allowing them to increase in number relative to other physiologically different types of microorganisms. Invariably the mechanism involved is that the chosen growth conditions allow the favored type to grow faster than other types of microorganisms. The “art” of enriching for certain bacteria has been exploited for many years. The technique was essentially developed by microbiologists like Beijerinck and Winogradsky early this century. The basic techniques and later refinements have been reviewed extensively several times (Schlegel and Jannasch, 1967; Skerman, 1967; Aaronson, 1970; Norris and Ribbons, 1970a, 1970b; Veldkamp, 1977; Brown et al., 1978; Gerhardt et al., 1981; Parkes, 1982; Poindexter and Leadbetter, 1986; Austin, 1988).

For many laboratory studies, especially those aiming to understand the role of microorganisms in their natural environment, selective enrichment represents the initial and very often a most critical step. The choice of the method and the precise selective conditions determine to a high degree the properties of the organism that will be obtained for further study. The type of enrichment is also of great importance with respect to the question whether such an organism may be expected to play a role of any significance in the ecosystem it came from. This relates not only to actual cell numbers but also to the isolate’s metabolic activity and ability to grow in its natural environment. It should be borne in mind that in most cases even the most sophisticated enrichment culture systems will not truly mimic the actual growth conditions in a natural habitat, neither is it usually meant to do so! Only a very limited number of elements are chosen to exert a selective pressure, and in most cases it will be virtually impossible to decide to what extent these factors control growth, metabolic activity, and survival in the ecosystem from which the sample was taken. Against this background three main investigative objectives can be formulated:

1. To isolate a microorganism or a microbial community with particular metabolic properties that is known to exist. Thus selective enrichment in this case is used simply as a powerful tool in obtaining the right organisms to work with. By continued cultivation of such cultures under the same selection pressure for much longer periods of time than strictly needed to obtain the desired species, enrichment may allow further selection of even better adapted species due to
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In deciding on the precise setup of the enrichment culture system, one has to consider carefully the objective of the particular enrichment. A major distinction in this respect is between batch-type (closed) and continuous-flow-type (open) culture systems. By far the most common technique involves batch cultivation, and most of the bacteria known to date have been isolated using this method. Its major characteristics are simplicity, versatility, growth in the presence of relatively high substrate concentrations, and in most cases the occurrence of a succession of dominantly active species since the culture conditions usually change continuously. This also implies that the precise selective conditions leading to the enrichment of a certain species remains largely unknown. Yet, it is evident that in most batch-type enrichments in which all substrates are in excess, selection is based on the maximum specific growth rate of the microorganisms. Therefore it is not surprising that bacteria isolated according to such enrichment procedures generally possess high specific growth rates and a low substrate specificity—the characteristics of opportunistic, ymogenous organisms (Winogradsky, 1949) sometimes referred to as r-strategists (MacArthur and Wilson, 1967; Parkes, 1982; Andrews, 1984). Although this approach may be particularly useful in obtaining organisms capable of performing certain (desirable) biochemical conversions at high rates (objectives 1 and 2; see above), it has been argued for many years and in some cases substantiated that these microorganisms are not representative of those really responsible for the microbiological activities in the environment, in most cases characterized by conditions of nutrient limitation (Jannasch, 1967a, 1967b; Veldkamp and Jannasch, 1972; Veldkamp, 1976, 1977; Kuenen and Harder, 1982; Parkes, 1982; Gottschal, 1986; Poindexter and Leadbetter, 1986; Gottschal and Dijkhuizen, 1988). Notwithstanding the general validity of the latter consideration, the possible importance of nonlimiting growth conditions in many ecosystems, albeit for relatively short periods of time, should not be overlooked. Transient phenomena like burial of organic matter (leaves, fecal pellets, etc.), algal blooms, tidal flooding, sudden changes in physicochemical conditions (oxygenation, temperature changes, availability of light, etc.), feeding in gastrointestinal tracts may all provide for conditions most suitable for a rapid response of opportunistic microbial species. Batch enrichments and further batch-wise cultivation do represent a quite reasonable means for studying the potential of microbial populations most active under such conditions. Consequently it must be emphasized that both the closed system approach of batch-wise enrichment and cultivation and enrichments under conditions of various types of continuous nutrient limitation are required to understand more completely the properties of microbial ecosystems. The most widely used technique for growth and enrichment in such an open system is the use of a chemostat with one or more growth-limiting nutrients in the inflowing medium. Using this approach, organisms specialized in growing at very low substrate concentrations and usually exhibiting relatively low $\mu_{\text{max}}$ values and fairly high substrate specificities can be obtained and studied. Extensive information on the isolation of such “autochtonous” bacteria, sometimes referred to as K-strategists (Andrews, 1984), has accumulated over the last three decades (Jannasch, 1967a, 1967b; Schlegel and Jannasch, 1967; Veldkamp, 1976; Harder et al., 1977; Brown et al., 1978; Kuenen and Harder, 1982; Parkes, 1982; Slater and Hardman, 1982; Gottschal, 1985; Poindexter and Leadbetter, 1986; Gottschal and Dijkhuizen, 1988; Gottschal, 1990). In addition to the important fact that this technique selects for organisms that are optimally adapted to growth under severe nutrient limitation, many other physical and chemical parameters and hence the overall selection pressure can be maintained constant over prolonged periods of time. Alternatively, these conditions may be changed in a controlled way, for example, resulting in a defined pattern of continued environmental changes such as light/dark cycles, and pH, temperature, aerobic/anaerobic, and osmotic transitions. This clearly adds enormously to the precision of the selective pressure that may be applied. A further particularly interesting aspect of chemostat enrichments is the possibility of selecting organisms under conditions of multiple substrate limitation. Chemostat enrichment under such circumstances has demonstrated that metabolically versatile bacteria could be selected that are specialized in using several different substrates at the same time if present at growth-limiting concentrations (Laanbroek et al., 1979; Dykhuizen and Davies, 1980; Gottschal and Kuenen, 1980; Beudeker et al., 1982; Legan and Owens, 1988). Since such bacteria usually display lower maximum growth rates relative to more specialized species, they will in most cases be missed by employing batch-type enrichment techniques even if mixtures of several substrates were used. Finally, chemostat enrichments provide excellent possibilities for selective enrichment of entire microbial communities. Mixed cultures of bacteria representing simple microbial food chains or more complex food webs usually appear in batch cultures as successional changes in populations; however, in continuous flow systems, they are all able to grow.
simultaneously for extended periods of time. Fascinating examples of enrichments of such complex microbial communities are to be found, especially in the literature concerning microbial degradation of synthetic recalcitrant compounds such as herbicides and various halogenated aromatics (Dorn et al., 1974; Senior et al., 1976; Daughton and Hsieh, 1977; Hartmann et al., 1979; Reineke and Knackmuss, 1984; Schmidt et al., 1985; Schmidt, 1987; Taeger et al., 1988; Oltmanns et al., 1988).

In the short introduction above to some of the major aspects of enrichment of microorganisms, no attempt has been made to be complete with respect to the many possibilities one has to select for a given organism or physiological type. For that kind of more specific information the reader is referred to other chapters in this Handbook.

**Selective Isolation of Mutants**

In the selective isolation of mutants, the method used for screening is of paramount importance. Direct selection is possible with mutants resistant to antibiotics, antimetabolites, bacteriophages, or bacteriocins. To select these resistance mutants from a sensitive wild-type population, large numbers of cells are plated in the presence of the agent which may then give rise to resistant colonies. Antibiotic-resistant mutants can be separated from wild-type susceptible strains by growing both types of bacteria on a medium containing the antibiotic; only the mutant will grow. In some cases mutants can easily be distinguished on the basis of changes in phenotype such as cell or colony morphology, pigmentation, and motility. A commonly used technique to identify mutants is replica plating. Wild type and mutant bacteria are grown in a rich complex medium on which each cell develops into an individual colony. The colonies of both types are then transferred either to several media, each of which lacks a certain type of nutrient (auxotrophic mutants, i.e., mutants defective in the synthesis of this nutrient do not grow) or to the same medium incubated under different environmental conditions. The transfer from the original (master) plate to all subsequent plates is performed by carefully placing a pad of sterile velvet on the surface of the master plate to pick up cells from each colony. The inoculated velvet pad can be used to inoculate several replica plates by stamping on the sterile agar surface.

Prokaryotes can acquire new metabolic activities as a result of mutations affecting the rate of enzyme synthesis or the structure of enzyme proteins. Although the selection of mutants in itself is not part of the classical use of the enrichment culture, the selective isolation of mutants is a powerful application of the chemostat technique receiving more and more attention. Examples of regulatory mutations are the constitutive β-galactosidase mutants of *Escherichia coli* originally described by Novick and Szilard (1950), the xylitol-using mutants of *Aerobacter aerogenes* found by Hartley and co-workers, and the mutant of *Pseudomonas putida* capable of using the herbicide 2,2-dichloropropionic acid as a substrate for growth found by Senior and colleagues (for references, see Harder et al., 1977; Slater and Hardman, 1982).

Loss of certain biosynthetic functions also may lead to a selective advantage of a mutant over the parent strain. In the classical experiments of Zamenhof and Eichhorn (1967), a histidine-requiring mutant (his⁻) of *Bacillus subtilis* had a strong selective advantage over the nonrequiring spontaneous revertant (his⁺) when grown together in a chemostat in the presence of histidine.

The carryover of genetic information in mixed cultures with strains of *Pseudomonas* species either capable of using 3-chlorobenzoate but not 4-chlorobenzoate, or containing the TOL plasmid coding for a benzoate-1,2-dioxygenase with broad substrate specificity, has been the principle behind the experiments of Knackmuss and colleagues when selecting for exconjugants capable of growing on 3- and 4-chlorobenzoate.

Instability of chimeric plasmids encoding commercially important foreign proteins may result in the loss of the inserted genes during culture, a problem in many biotechnological applications. Fleming et al. (1988) were able to improve the plasmid-stability characteristics of a strain of *Bacillus subtilis* carrying a chimeric plasmid (derived from pUB110) encoding chloramphenicol and kanamycin resistance and high-temperature α-amylase by growing the organism in the chemostat in the presence of rising levels of chloramphenicol. Interestingly, the improved strains with higher plasmid stability isolated at the end of the chemostat run had almost three times higher maximum growth rates as compared to the original strain, which was outcompeted both in batch and in
Conservation of Bacterial Cultures

The enormous growth of biotechnology and various branches of microbiology has resulted in an increased interest in techniques for the maintenance and long-term preservation of cultures.

The maintenance of bacterial cultures usually requires propagating working cultures by subculture and keeping strains of bacteria alive and growing in the course of investigations or in order to provide inocula of “standard strains” for industrial processes, certain assays, and tests, and routine investigations. For long-term maintenance, cultures are usually stored in a lyophilized or deep-frozen form to prolong their viability and to reduce changes due to the occurrence of mutations. A practical book with information on various techniques and procedures from subculture to storage in frozen nitrogen and freeze-drying is Kirsop and Snell (1984). The book is an extension of the courses given by the UK Federation for Culture Collections and as such reflects the techniques used in the United Kingdom.

Maintenance of Working Cultures

Usually, maintenance of working cultures requires the periodic transfer of strains to fresh minimal media, streaked on cotton-plugged agar slants (aerobes) or stabbed in agar deeps in screw-capped tubes (facultative anaerobes, microaerobes, anaerobes). The cultures can then be incubated and then are stored in the refrigerator in the dark at 5–8°C to reduce metabolic activity, while maintaining viability. Frequency of transfer should be kept to a minimum, but it has to be separately determined for each organism: some require transfer after just a few days, while some sporeformers can survive for years. Drying of the agar must be prevented. To avoid selection, it is advisable to subculture from the whole plate or slant and not just from one colony. For different physiological groups of bacteria, specific maintenance requirements exist. Working cultures of rumen bacteria and other fermentative anaerobes can be maintained in slants or sloppy agars (0.7–1.2%, w/v agar) prepared from nonselective media. The cultures are incubated at 39°C until growth is just apparent and then stored at 4°C. Such cultures remain viable for a few weeks at least, although the viable count is decreased.

Lists of maintenance media can be found in culture collection manuals, such as of the Deutsche Sammlung für Mikroorganismen. Some serious problems that may be encountered in maintenance of strains are infection, mislabelling of tubes, and plates, mutation and genetic variation, and selection.

Preservation of Stock Cultures

The above-mentioned problems made it necessary to find ways for the long-term storage of bacterial strains in national and international culture collections. Furthermore, long-term preservation of cultures is of importance to taxonomy, because it allows confirmation and extension of research findings. It is also essential to many kinds of industrial applications, and it is mandated by patent laws and regulations.

A method of long-term preservation that can be followed with sporeforming bacteria is based on the use of sterile soil or sand. A sample of soil or sand is sterilized in a screw-capped bottle by autoclaving for several hours on at least two successive days. Then, 1 ml of a suspension of the organism is added, and the contents are dried in a vacuum desiccator with the cap of the tube loosely closed. When the contents are dry, the cap is closed tightly and the bottle is stored in the refrigerator. Even easier is the use of sterile filter paper discs or strips soaked with the bacterial suspension. The discs are stored as described for sand above. One may keep several discs in one screw-cap container and remove one disk at a time, taking care not to contaminate the remaining discs.
Lyophilization.

Cultures maintained on agar slants at 4°C or kept on oil still show slow growth and cell turnover with the potential of genetic and metabolic alterations. Starter cultures for microbiological research or for processes in industry have to be maintained in a state that allows reproducible rapid growth upon inoculation. Most strains therefore are stored frozen or as freeze-dried (lyophilized) cultures.

The organisms are placed in an ampoule with suspending medium and frozen at –60 to –80°C, most often in a metal chamber with solid carbon dioxide and ethanol. The water is then removed from the frozen state directly by sublimation in a vacuum. Freeze-dryers consist of a manifold connected to a vacuum pump capable of reducing the pressure to less than 0.01 mm Hg. The ampoule is sealed while under vacuum and stored in a cool place. The preserved culture weighs little, takes little space, and can be easily stored, handled, and mailed. The suspending medium is critical for the rate of survival during the freeze-drying process and for the rate of death of dried bacteria during storage. Among the most commonly used suspending media are serum plus 30% glucose nutrient broths and sterile skimmed milk fortified with 5% sucrose, sterilized in 3 ml amounts in bijou bottles.

A procedure for the freeze-drying of anaerobes is given by Phillips et al. (1975). A freeze-drying method suitable for the preservation of nitrogen-fixing and other fragile bacteria was described by Malik (1988a). No loss of diazotrophy, plasmids, or other desirable qualities was observed. References for the freeze-drying of several bacterial groups can be found in Malik (1988a, 1988b). A simple method for long-term preservation of luminous bacteria (often used in microbial toxicity tests) has been described by Janda and Opekarová (1989).

Survival of the population during freezing and thawing depends on a number of factors (Calcott, 1986), but growth characteristics and pretreatment and posttreatment manipulations are as important as the way cells are frozen and thawed. Each type of cell appears to exhibit its own set of optimal conditions for maximum survival. Irreversible injury will lead to death or loss of viability. The last point is a subjective determination that depends as much on the choice of the medium composition and incubation conditions (Postgate and Calcott, 1985). Reversible cell damage due to freezing and thawing includes altered plating ability reflecting both structural and metabolic injury. Damage may occur to the cell’s basic structure including the membrane, cell wall, cytoskeleton, and DNA secondary structure. Damage to the metabolic machinery may include impairment of active transport, protein synthesis, and the induction of the synthesis of inducible operons. Mutagenic effects of freeze-thaw stress are shown to be related to single-stranded breaks in DNA, an effect that might be similar to that of ionizing radiation. The damage can be repaired in nutrient media. In Escherichia coli, both the uvrA-, polA-dependent (excision repair) and the error-prone rec-, polA-dependent (recombinational repair) DNA repair pathways are required for repair of freeze-thaw-induced DNA damage.

Calcott et al. (1983; cited in Calcott, 1986) examined the incidence of plasmid loss from the survivors of freeze-thaw, namely the loss of plasmid RP4 from Escherichia coli and the loss of plasmid pPL1 from Pseudomonas aeruginosa, two natural non-engineered plasmids known to be extremely stable under a variety of growth conditions. Freezing and thawing did cure the strains at a low rate of loss in a dose-dependent manner. Frozen storage of the population itself, although detrimental to survival, did not increase the incidence of plasmid loss. When populations were protected by either glycerol or sucrose viability of the preparation remained high with minimal loss of plasmids from the culture. A percentage of the survivors of freeze-thaw stress may show “metabolic injury,” i.e., the loss of the ability to form colonies on a minimal salts-glucose (“poor”) medium. This was studied in Escherichia coli and was shown not to be due to a transient requirement for a particular nutrient that was absent, but rather due to a perturbation of the cell control systems. Cyclic GMP and ppGpp singly or in combination partly restored the efficiency of plating on a minimal medium of frozen-thawed cells.

Effects of specific harmful agents and potential damage sites in the microbial cell are reviewed by Hurst and Nasim (1984), while Andrew and Russell (1984) report on problems associated with enumerating and recovering microbes exposed to various forms of stress.

Freezing and Ultrafreezing

For the preservation of cultures of rumen bacteria for up to one year at –20°C, Teather (1982) recommended freezing after the addition of 20% (v/v) glycerol as a cryoprotectant to cultures that have been incubated at 39°C for 12–24 h. Long-term storage under liquid nitrogen (–196°C) in the presence of...

Culture Collections and Shipment of Cultures

Culture collections and their role in biotechnology and microbiology are described by Lapage et al. (1970), Malik (1987), and Malik and Claus (1987). Iizuka (1987) provides an overview of new microbial resources and world culture collections. Certain collections are very specific and deal only with bacteria from a certain habitat, e.g., the Australian Collection of Antarctic Microorganisms (ACAM), or a certain taxonomic group of bacteria, e.g., the Oregon Graduate Center Collection of Methanogenic Archaeabacteria (OGC/CMA), Oregon Graduate Center, 19600 N.W. Von Neumann Drive, Beaverton, OR, USA.

Regulations and guidelines for packaging and requirements for labeling and shipping of cultures and biological materials are described in a reference manual of the American Type Culture Collection (ATCC). These guidelines and regulations also describe restrictions on quantities and a list of countries that prohibit movement of perishable biological substances through the postal service (Alexander and Daggett, 1988). Many diagnostic specimens (human or animal excreta, secreta, blood, tissues, and tissue fluids shipped for diagnosis believed not to contain infectious substances) are exempt from the regulations defined in the International Mail Manual (IMM 137.2). For International Postal Shipments, noninfectious biological substances that are freeze-dried (nonperishable) have no special packaging and shipment requirements, except that a high-quality box must be used.

Drying or freeze-drying undoubtedly is the safest and most practical method of preparing specimens for shipment. In air transport, a package may be subjected to a wide range of temperatures from −40 to +55°C. Packages sealed at atmospheric pressure (100 kilopascals) may become subject to reduced pressures as low as two-thirds of atmospheric pressure (60 kilopascals). Samples sent as liquids, e.g., in test tubes, should be heat-sealed in 5 ml of polyethylene tubing before shipment, a good packaging practice for all infectious substances.

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