BACTERIOLOGY

FOR 2^{d} YEAR STUDENTS

MICROBIOLOGY/CHEM & MICROBIOLOGY

FACULTY OF SCIENCE

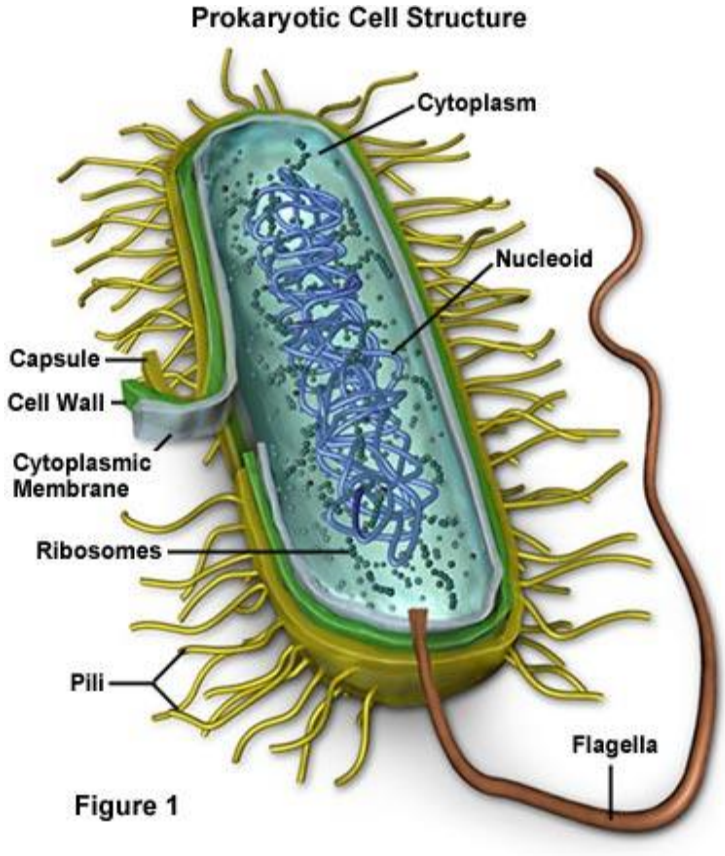

Figure 1

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أهداف المقرر ونواتج التعلم المستهدفة

أهداف المقرر: فهم ودراسة أساسيات النمو للبكتريا والكشف عن النشاط الحيوى وطرق الفحص للعينات البكتيرية

التعرف على طرق العزل والتعريف المبدئى وطرق التعامل مع البكتريا فى بعض التطبيقات وطرق حل المشكلات المرتبطة بها فى مجالات الحياة المختلفة.

نواتج التعلم المستهدفة

1- المعلومات والمفاهيم:

- **يصف الطالب تركيب الخلية البكتيرية ويتعرف على وضع الكائنات الأولية بالنسبة لباقى الكائنات ويسمى وظائف المحتويات الخلوية والأغلفة**
- **يحدد الطالب مراحل النمو المختلفة للبكتريا (منحنى النمو) والظروف التى تتحكم فيه وخاصة التعقيم وأنواع مثبطات النمو المختلفة والمضادات الحيوية وطرق قياس النمو**
- **يذكر الطالب دور الأنواع البكتيرية المختلفة فى المحافظة على العناصر فى الطبيعة ويتعرف على صفات بعض المجموعات الهامة ودور البكتريا فى التطبيقات المختلفة والأمراض وتفادى أضرارها**

2- المهارات الذهنية:

- **يميز الطالب بين الأنواع الهامة للبكتريا ودورها فى البيئات المختلفة**
- **يربط الطالب بين نظم نقل الجزيئات من والى الخلية وميكانيكية عملها وحاجتها للطاقة**
- **يوضح العناصر الرئيسية للتحكم فى النمو والأيض الخلوى وطرق قياسه باستخدام تقنيات مختلفة**
- **يحلل بعض التطبيقات الهامة ودور البكتريا فيها وكيفية توفير الظروف المثلى**
- **يقارن بين البكتريا الضارة (المسببة للأمراض الشائعة وفساد الأغذية- مع طرق حفظها) وبين الأنواع الأخرى النافعة**
-

3- المهارات المهنية:

- **يستخدم الميكروسكوب الضوئى ووسائل وأدوات التعقيم المختلفة**
- **يتناول الأشكال الرئيسية للبكتريا تحت الميكروسكوب وبعض التركيبات الخلوية الداخلية**
- **يستخدم طرق تقدير نمو البكتريا وبعض التفاعلات الحيوية كدليل على النمو وللتفرقة بين الأنواع**
- **يتناول تأثير مضادات النمو الفيزيائية والكيميائية على البكتريا وخاصة المضادات الحيوية**
- **يستخدم الأدوات المعملية المناسبة لقياس تأثير بعض المواد الأخرى على الخلية**
- **يستخدم طرق عزل البكتريا من البيئات المختلفة ويجرى خطوات التعريف والتحكم فى النمو بالتحفيز أو التثبيط**

4- المهارات العامة:

- **المناقشة واستحضار المعلومات الأساسية خلال المحاضرات والدروس العملية**
- **التعامل المناسب والتواصل والتفاعل بالعمل الجماعى والمشاركة.**

OVERVIEW OF BACTERIOLOGY

The Scope of Bacteriology

The Bacteria are a group of single-cell microorganisms with **procaryotic** cellular configuration. The genetic material (DNA) of procaryotic cells exists unbound in the cytoplasm of the cells. There is **no nuclear membrane**, which is the definitive characteristic of eucaryotic cells such as those that make up plants and animals. Until recently, bacteria were the only known type of procaryotic cell, and the discipline of biology related to their study is called **bacteriology**. In the 1980's, with the outbreak of molecular techniques applied to phylogeny of life, another group of procaryotes was defined and informally named "archaebacteria". This group of procaryotes has since been renamed **Archaea.** The current science of bacteriology includes the study of both domains of procaryotic cells. but the name "bacteriology" is not likely to change to reflect the inclusion of archaea in the discipline.

The Origin of Life

When life arose on Earth about 4 billion years ago, the first types of cells to evolve were procaryotic cells. For approximately 2 billion years, procaryotic-type cells were the only form of life on Earth. The oldest known sedimentary rocks, from Greenland, are about 3.8 billion years old. The oldest known fossils are procaryotic cells, 3.5 billion years in age, found in Western Australia and South Africa. The nature of these fossils, and the chemical composition of the rocks in which they are found, indicate that **lithotrophic** and **fermentative** modes of metabolism were the first to evolve in early procaryotes. **Photosynthesis** developed in bacteria a bit later, at least 3 billion years ago. **Anoxygenic photosynthesis** (bacterial photosynthesis, which is anaerobic and does not produce O_2) preceded **oxygenic photosynthesis** (plant-type photosynthesis, which yields O_2). However, oxygenic photosynthesis also arose in procaryotes, specifically in the cyanobacteria, which existed millions of years before the evolution of green algae plants. Larger, more complicated eucaryotic cells did not appear until much later, between 1.5 and 2 billion years ago.

Figure 1. Opalescent Pool in Yellowstone National Park, Wyoming USA. Conditions for life in this environment are similar to Earth over 2 billion years ago. In these types of hot springs, the orange, yellow and brown colors are due to pigmented photosynthetic bacteria which make up the microbial mats. The mats are literally teeming with bacteria. Some of these bacteria such as *Synechococcus* conduct oxygenic photosynthesis, while others such as *Chloroflexus* conduct anoxygenic photosynthesis. Other non-photosynthetic bacteria, as well as thermophilic and acidophilic Archaea, are also residents of the hot spring community.

The archaea and bacteria differ fundamentally in their structure from eucaryotic cells, which always contain a membrane-enclosed nucleus, multiple chromosomes, and various other membranous organelles such as mitochondria, chloroplasts, the golgi apparatus, vacuoles, etc. Unlike plants and animals, archaea and bacteria are unicellular organisms that do not develop or differentiate into multicellular forms. Some bacteria grow in filaments or masses of cells, but each cell in the colony is identical and capable of independent existence. The cells may be adjacent to one another because they did not separate after cell division or because they remained enclosed in a common sheath or slime secreted by the cells, but typically there is no continuity or communication between the cells.

Size and Distribution of Bacteria and Archaea

Most procaryotic cells are very small compared to eucaryotic cells. A typical bacterial cell is about 1 micrometer in diameter or width, while most eucaryotic cells are from 10 to 100 micrometers in diameter. Eucaryotic cells have a much greater volume of cytoplasm and a much lower surface: volume ratio than procaryotic cells. A typical procaryotic cell is about the size of a eucaryotic mitochondrion. Since procaryotes are too small to be seen except with the aid of a microscope, it is usually not appreciated that they are the most abundant form of life on the planet, both in terms of biomass and total numbers of species. For example, in the sea, procaryotes make up 90

percent of the total combined weight of all organisms. In a single gram of fertile agricultural soil there may be in excess of 10^9 bacterial cells, outnumbering all eucaryotic cells there by 10,000: 1. About 3,000 distinct species of bacteria and archaea are recognized, but this number is probably less than one percent of all the species in nature. These unknown procaryotes, far in excess of undiscovered or unstudied plants, are a tremendous reserve of genetic material and genetic information in nature that awaits exploitation.

Procaryotes are found in all of the habitats where eucaryotes live, but, as well, in many natural environments considered too extreme or inhospitable for eucaryotic cells. Thus, the outer limits of life on Earth (hottest, coldest, driest, etc.) are usually defined by the existence of procaryotes. Where eucaryotes and procaryotes live together, there may be mutualistic associations between the organisms that allow both to survive or flourish. The organelles of eucaryotes (mitochondria and chloroplasts) are thought to be remnants of Bacteria that invaded, or were captured by, primitive eucaryotes in the evolutionary past. Numerous types of eucaryotic cells that exist today are inhabited by endosymbiotic procaryotes.

From a metabolic standpoint, the procaryotes are extraordinarily diverse, and they exhibit several types of metabolism that are rarely or never seen in eucaryotes. For example, the biological processes of **nitrogen fixation** (conversion of atmospheric nitrogen gas to ammonia) and **methanogenesis** (production of methane) are metabolically-unique to procaryotes and have an enormous impact on the nitrogen and carbon cycles in nature. Unique mechanisms for energy production and photosynthesis are also seen among the Archaea and Bacteria.

The lives of plants and animals are dependent upon the activities of bacterial cells. Bacteria enter into various types of symbiotic relationships with plants and animals that usually benefit both organisms, although a few bacteria are agents of disease.

The metabolic activities of procaryotes in soil habitats have an enormous impact on soil fertility that can affect agricultural practices and crop yields. In the global environment, procaryotes are absolutely essential to drive the cycles of elements that

make up living systems, i.e., the carbon, oxygen, nitrogen and sulfur cycles. The origins of the plant cell chloroplast and plant-type (oxygenic) photosynthesis are found in procaryotes. Most of the earth's atmospheric oxygen may have been produced by free-living bacterial cells. The bacteria fix nitrogen and a substantial amount of CO_2, as well.

Bacteria or bacterial products (including their genes) can be used to increase crop yield or plant resistance to disease, or to cure or prevent plant disease. Bacterial products include antibiotics to fight infectious disease, as well as components for vaccines used to prevent infectious disease. Because of their simplicity and our relative understanding of their biological processes, the bacteria provide convenient laboratory models for study of the molecular biology, genetics, and physiology of all types of cells, including plant and animal cells.

TAXONOMY AND CLASSIFICATION OF PROCARYOTES

Haeckel (1866) was the first to create a natural Kingdom for the microorganisms, which had been discovered nearly two centuries before by van Leeuwenhoek. He placed all unicellular (microscopic) organisms in a new kingdom, **"Protista"**, separated from plants (**Plantae**) and animals (**Animalia**), which were multicellular (macroscopic) organisms. The development of the electron microscope in the 1950's revealed a fundamental dichotomy among Haeckel's **"Protista"**: some cells contained a membrane-enclosed nucleus, and some cells lacked this intracellular structure. The latter were temporarily shifted to a fourth kingdom, **Monera** (or **Moneres**), the procaryotes (also called **Procaryotae**). **Protista** remained as a kingdom of unicellular eucaryotic microorganisms. Whittaker refined the system into five kingdoms in 1967, by identifying the **Fungi** as a separate multicellular eucaryotic kingdom of organisms, distinguished by their absorptive mode of heterotrophic nutrition.

In the 1980's, Woese began phylogenetic analysis of all forms of cellular life based on comparative sequencing of the small subunit ribosomal RNA (ssrRNA) that is contained in all organisms. A new dichotomy was revealed, this time among the procaryotes: there existed two types of procaryotes, as fundamentally unrelated to

one another as they are to eucaryotes. Thus, Woese defined the **three cellular Domains of life** as **Eucarya**, **Bacteria** and **Archaea**. Whittaker's Plant, Animal and Fungi kingdoms (all of the multicellular eucaryotes) are at the end of a very small branch of the tree of life, and all other branches lead to microorganisms, either procaryotes (Bacteria and Archaea), or protists (unicellular algae and protozoa), thus establishing clearly that microbial life is the predominant form of life on the planet.

Although the definitive difference between Woese's **Archaea** and **Bacteria** is based on fundamental differences in the nucleotide base sequence in the 16S ribosomal RNA, there are many biochemical and phenotypic differences between the two groups of procaryotes. (Table 1). The phylogenetic tree indicates that **Archaea** are more closely related to **Eucarya** than are **Bacteria**. This relatedness seems most evident in the similarities between transcription and translation in the **Archaea** and the **Eucarya**. However, it is also evident that the **Bacteria** have evolved into chloroplasts and mitochondria, so that these eucaryotic organelles derive their lineage from this group of procaryotes. Perhaps the biological success of eucaryotic cells springs from the evolutionary merger of the two procaryotic life forms.

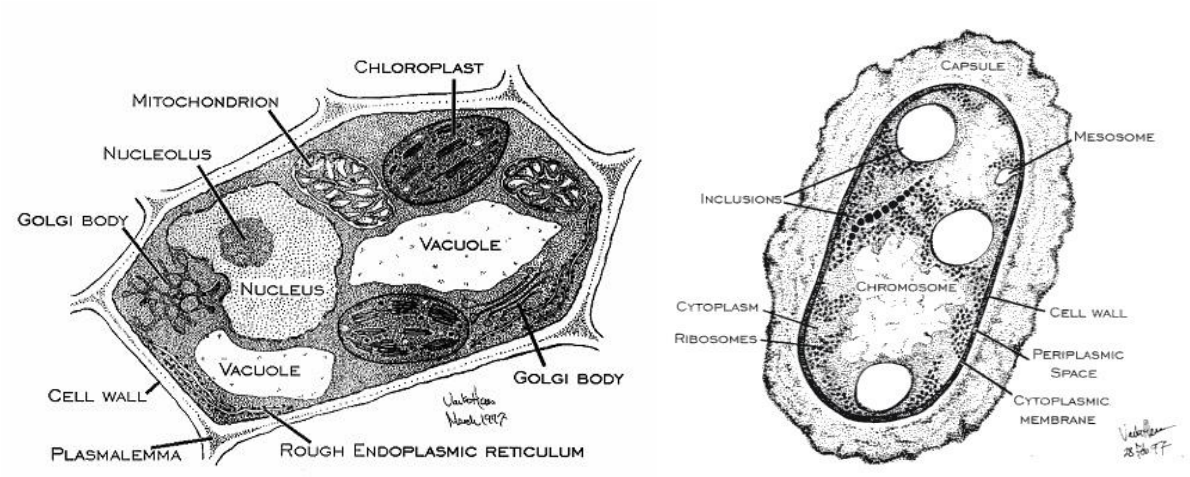


Figure 2 (Right) The structure of a typical procaryotic cell, in this case, a Gram-negative bacterium, compared with (Left) a typical eucaryotic cell (plant cell). The procaryote is about 1 micrometer in diameter and about the size of the eucaryotic chloroplast or mitochondrion.

Table 1. Phenotypic properties of Bacteria and Archaea compared with Eucarya.

Property	Biological Domain		
	Eucarya	Bacteria	Archaea
Cell configuration	eucaryotic	Prokaryotic	Prokaryotic
Nuclear membrane	present	Absent	Absent
Number of chromosomes	>1	1	1
Chromosome topology	linear	Circular	circular
Murein in cell wall	-	+	-
Cell membrane lipids	ester-linked glycerides; unbranched; polyunsaturated	ester-linked glycerides; unbranched; saturated or monounsaturated	ether-linked branched; saturated
Cell membrane sterols	present	Absent	Absent
Organelles (mitochondria and chloroplasts)	present	Absent	Absent
Ribosome size	80S (cytoplasmic)	70S	70S
Cytoplasmic streaming	+	-	-
Meiosis and mitosis	present	Absent	Absent
Transcription and translation coupled	-	+	+
Amino acid initiating protein synthesis	methionine	N-formyl methionine	methionine
Protein synthesis inhibited by streptomycin and chloramphenicol	-	+	-
Protein synthesis inhibited by diphtheria toxin	+	-	+

IDENTIFICATION OF BACTERIA

The criteria used for microscopic identification of procaryotes include cell shape and grouping, Gram-stain reaction, and motility. Bacterial cells almost invariably take one of three forms: rod (**bacillus**), sphere (**coccus**), or spiral (**spirilla** and **spirochetes**). Rods that are curved are called **vibrios**. Fixed bacterial cells stain either Gram-positive (purple) or Gram-negative (pink); motility is easily determined by observing living specimens. Bacilli may occur singly or form chains of cells; cocci may form chains (**streptococci**) or grape-like clusters (**staphylococci**); spiral shape cells are almost always motile; cocci are almost never motile. This

nomenclature ignores the **actinomycetes**, a prominent group of branched bacteria which occur in the soil. But they are easily recognized by their colonies and their microscopic appearance.

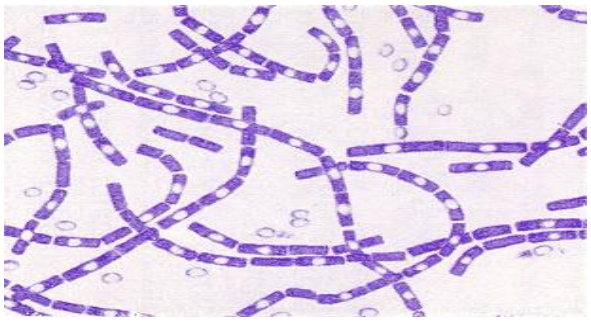


Figure 3. Gram stain of *Bacillus anthracis, the* cause of anthrax.

Such easily-made microscopic observations, combined with knowing the natural environment of the organism, are important aids to identify the group, if not the exact genus, of a bacterium - providing, of course, that one has an effective key. Such a key is **Bergey's Manual of Determinative Bacteriology**, the "field guide" to identification of the bacteria. Bergey's Manual describes affiliated groups of **Bacteria** and **Archaea** based on a few easily observed microscopic and physiologic characteristics. Further identification requires biochemical tests which will distinguish genera among families and species among genera. Strains within a single species are usually distinguished by genetic or immunological criteria.

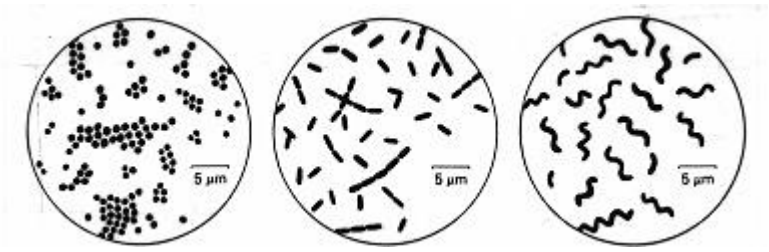


Figure 4. Size and fundamental shapes of procaryotes revealed by three genera of Bacteria (l to r): *Staphylococcus* (spheres), *Lactobacillus* (rods), and *Aquaspirillum* (spirals).

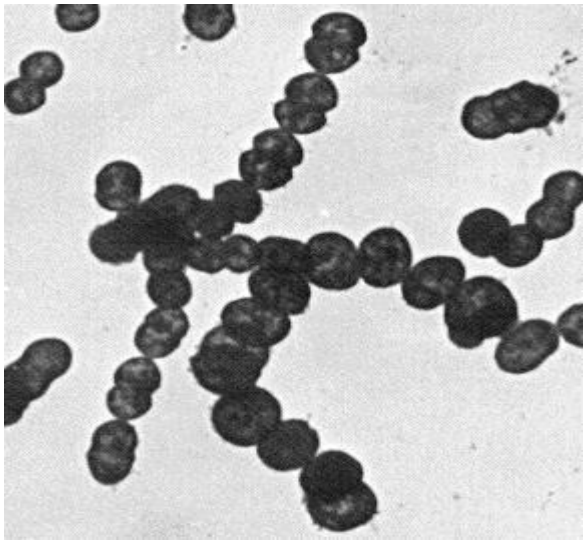


Figure 5. Chains of dividing streptococci. Electron micrograph of *Streptococcus pyogenes*.

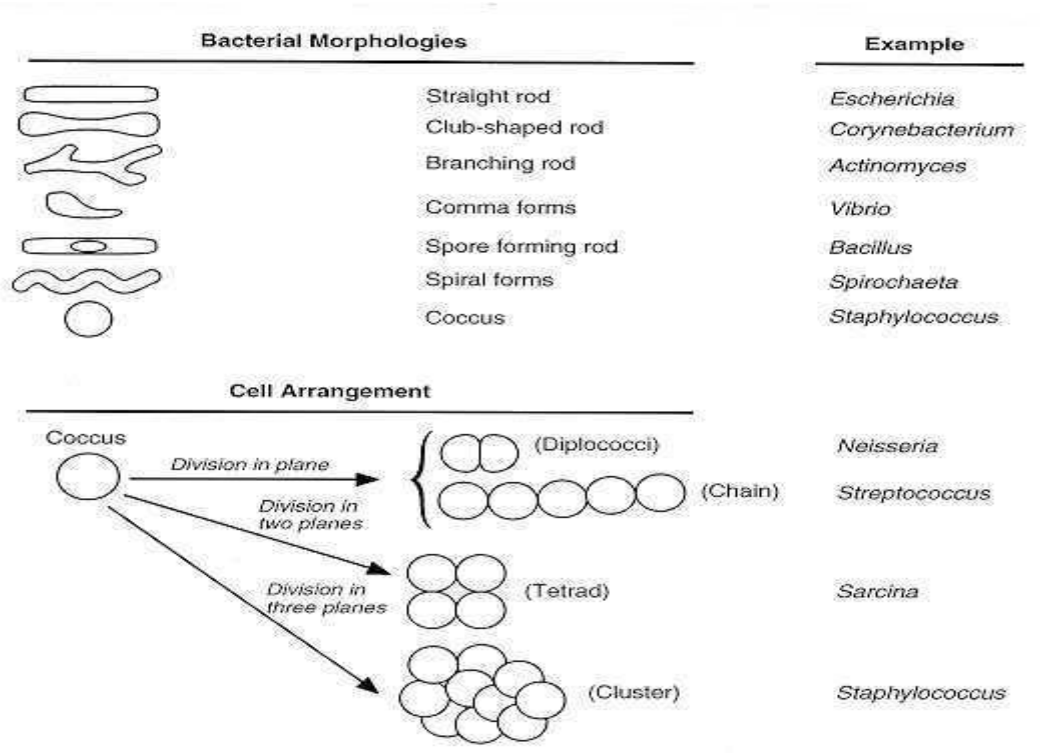


Figure 6. Different shapes and arrangements of bacterial cells, with examples.

STRUCTURE AND FUNCTION OF PROCARYOTIC CELLS

Procaryotes are unicellular organisms of relatively simple construction, especially if compared to eukaryotes. Whereas eukaryotic cells have a preponderance of organelles with separate cellular functions, procaryotes carry out all cellular functions

as individual units. A procaryotic cell has five essential structural components: a **genome (DNA)**, **ribosomes**, **cell membrane**, **cell wall**, and some sort of **surface layer** which may or may not be an inherent part of the wall. Other than enzymatic reactions, all the cellular reactions incidental to life can be traced back to the activities of these macromolecular structural components. Thus, functional aspects of procaryotic cells are related directly to the structure and organization of the macromolecules in their cell make-up, i.e., DNA, RNA, phospholipds, proteins and polysaccharides. Diversity within the primary structure of these molecules accounts for the diversity that exists among procaryotes.

At one time it was thought that bacteria were essentially "bags of enzymes" with no inherent cellular architecture. The development of the electron microscope, in the 1950s, revealed the distinct anatomical features of bacteria and confirmed the suspicion that they lacked a nuclear membrane. Structurally, a procaryotic cell (Figure 1) has three architectural regions: **appendages** (attachments to the cell surface) in the form of **flagella** and **pili (or fimbriae)**; a **cell envelope** consisting of a **capsule**, **cell wall** and **plasma membrane**; and a **cytoplasmic region** that contains the cell **genome** (**DNA**) and **ribosomes** and various sorts of **inclusions**. In this chapter, we will discuss the anatomical structures of procaryotic cells in relation to their adaptation, function and behavior in natural environments.

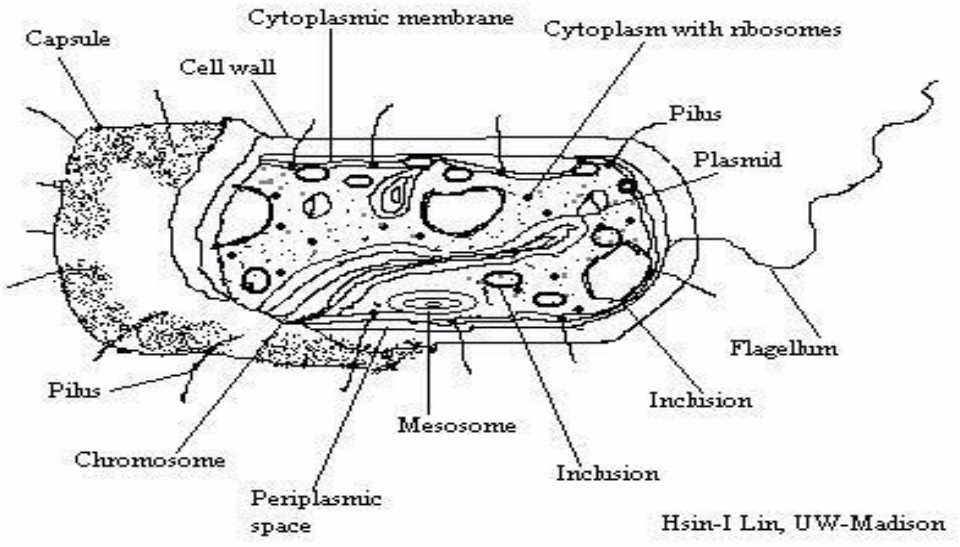


Figure 1. Schematic drawing of a typical bacterium.

Table 1. Summary: Characteristics of typical bacterial cell structures.

Structure	Function(s)	Predominant chemical composition
Flagella	Swimming movement	Protein
Pili		
Sex pilus	Mediates DNA transfer during conjugation	Protein
Common pili or fimbriae	Attachment to surfaces; protection against phagotrophic engulfment	Protein
Capsules (includes "slime layers" and glycocalyx)	Attachment to surfaces; protection against phagocytic engulfment, occasionally killing or digestion; reserve of nutrients or protection against desiccation	Usually polysaccharide; occasionally polypeptide
Cell wall		
Gram-positive bacteria	Prevents osmotic lysis of cell protoplast and confers rigidity and shape on cells	Peptidoglycan (murein) complexed with teichoic acids
Gram-negative bacteria	Peptidoglycan prevents osmotic lysis and confers rigidity and shape; outer membrane is permeability barrier; associated LPS and proteins have various functions	Peptidoglycan (murein) surrounded by phospholipid protein-lipopolysaccharide "outer membrane"
Plasma membrane	Permeability barrier; transport of solutes; energy generation; location of numerous enzyme systems	Phospholipid and protein
Ribosomes	Sites of translation (protein synthesis)	RNA and protein
Inclusions	Often reserves of nutrients; additional specialized functions	Highly variable; carbohydrate, lipid, protein or inorganic
Chromosome	Genetic material of cell	DNA
Plasmid	Extrachromosomal genetic material	DNA

Appendages

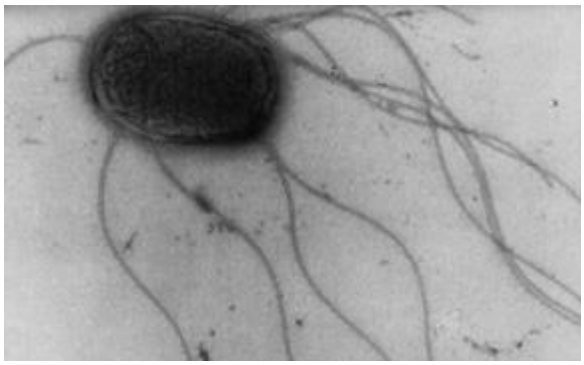


Figure 2. *Salmonella enteritidis* TEM about 10,000X. *Salmonella* is an enteric bacterium related to *E. coli.* The enterics are motile by means of peritriochous flagella.

Flagella

Flagella are filamentous protein structures attached to the cell surface that provide the swimming movement for most motile procaryotes. Procaryotic flagella are much thinner than eukaryotic flagella. The diameter of a procaryotic flagellum is about 20 nanometers, well-below the resolving power of the light microscope. The flagellar filament is rotated by a motor apparatus in the plasma membrane allowing the cell to swim in fluid environments. Bacterial flagella are powered by proton motive force (chemiosmotic potential) established on the bacterial membrane, rather than ATP hydrolysis which powers eukaryotic flagella. About half of the bacilli and all of the spiral and curved bacteria are motile by means of flagella. Very few cocci are motile, which reflects their adaptation to dry environments and their lack of hydrodynamic design.

The ultrastructure of the flagellum of *E. coli* is illustrated in (Figure 3) below. About 50 genes are required for flagellar synthesis and function. The flagellar apparatus consists of several **distinct proteins**: a system of **rings** embedded in the cell envelope (the **basal body**), a **hook-like structure** near the cell surface, and the **flagellar filament**. The innermost rings, the **M** and **S** rings, located in the plasma membrane, comprise the motor apparatus. The outermost rings, the **P** and **L** rings, located in the periplasm and the outer membrane respectively, function as bushings to support the rod where it is joined to the hook of the filament on the cell surface.

As the **M** ring turns, powered by an influx of protons, the rotary motion is transferred to the filament which turns to propel the bacterium.

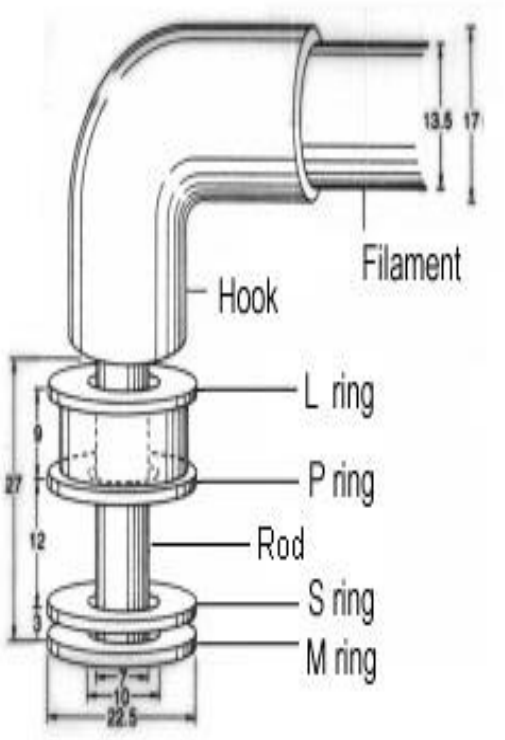


Figure 3. The ultrastructure of a bacterial flagellum. Measurements are in nanometers. The flagellum of *E. coli* consists of three parts, filament, hook and basal body, all composed of different proteins. The basal body and hook anchor the whip-like filament to the cell surface. The basal body consists of four ring-shaped proteins stacked like donuts around a central rod in the cell envelope. The inner rings, associated with the plasma membrane, are the flagellar powerhouse for activating the filament. The outer rings in the peptidoglycan and outer membrane are support rings or "bushings" for the rod. The filament rotates and contracts which propels and steers the cell during movement. Compare with Figure 21 below.

Flagella may be variously distributed over the surface of bacterial cells in distinguishing patterns, but basically flagella are either **polar** (one or more flagella arising from one or both poles of the cell) or **peritrichous** (lateral flagella distributed over the entire cell surface). Flagellar distribution is a genetically-distinct trait that is occasionally used to characterize or distinguish bacteria. For example, among Gram-negative rods, pseudomonads have polar flagella to distinguish them from enteric bacteria, which have peritrichous flagella.

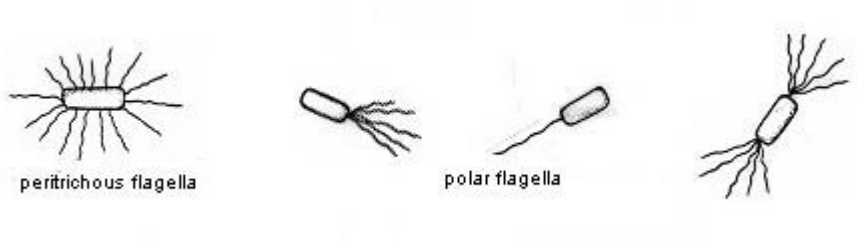


Figure 4. Different arrangements of bacterial flagella. Swimming motility, powered by flagella, occurs in half the bacilli and most of the spirilla. Flagellar arrangements, which can be determined by staining and microscopic observation, may be a clue to the identity of a bacterium. See Figure 5 below.

Flagella were proven to be organelles of bacterial motility by shearing them off (by mixing cells in a blender) and observing that the cells could no longer swim although they remained viable. As the flagella were regrown and reached a critical length, swimming movement was restored to the cells. The flagellar filament grows at its tip (by the deposition of new protein subunits) not at its base (like a hair).

Procaryotes are known to exhibit a variety of types of **tactic behavior**, i.e., the ability to move (swim) in response to environmental stimuli. For example, during **chemotaxis** a bacterium can sense the quality and quantity of certain chemicals in its environment and swim towards them (if they are useful nutrients) or away from them (if they are harmful substances). Other types of tactic response in procaryotes include **phototaxis**, **aerotaxis** and **magnetotaxis**. The occurrence of tactic behavior provides evidence for the ecological (survival) advantage of flagella in bacteria and other procaryotes.

Detecting Bacterial Motility

Since motility is a primary criterion for the diagnosis and identification of bacteria, several techniques have been developed to demonstrate bacterial motility, directly or indirectly.

1. **Flagellar stains** outline flagella and show their pattern of distribution. If a bacterium possesses flagella, it is presumed to be motile.

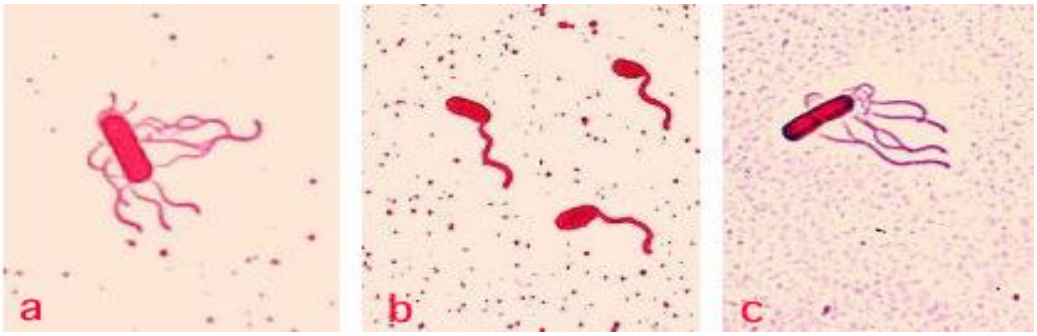


Figure 5. Flagellar stains of three bacteria a. *Bacillus cereus* b. *Vibrio cholerae* c. *Bacillus brevis*. Since the bacterial flagellum is below the resolving power of the light microscope, although bacteria can be seen swimming in a microscope field, the organelles of movenent cannot be detected. Staining techniques such as Leifson's method utilize dyes and other components that precipitate along the protein filament and hence increase its effective diameter. Flagellar distribution is occasionally used to differentiate between morphologically related bacteria. For example, among the Gram-negative motile rod-shaped bacteria, the enterics have peritrichous flagella while the pseudomonads have polar flagella.

2. **Motility test medium** demonstrates if cells can swim in a semisolid medium. A semisolid medium is inoculated with the bacteria in a straight-line stab with a needle. After incubation, if turbidity (cloudiness) due to bacterial growth can be observed away from the line of the stab, it is evidence that the bacteria were able to swim through the medium.

OFF THE WALL. Julius Adler exploited this observation during his studies of chemotaxis in *E. coli.* He prepared a gradient of glucose by allowing the sugar to diffuse into a semisolid medium from a central point in the medium. This established a concentration gradient of glucose along the radius of diffusion. When *E. coli* cells were seeded in the medium at the lowest concentration of glucose (along the edge of the circle), they swam up the gradient towards a higher concentration (the center of the circle), exhibiting their chemotactic response to swim towards a useful nutrient. Later, Adler developed a tracking microscope that could record and film the track that *E. coli* takes as it swims towards a chemotactic attractant or away from a chemotactic repellent. This led to an understanding of the mechanisms of bacterial chemotaxis, first at a structural level, then at a biomolecular level.

3. **Direct microscopic observation** of living bacteria in a wet mount. One must look for transient movement of swimming bacteria. Most uincellular bacteria, because of their small size, will shake back and forth in a wet mount observed at 400X or 1000X. This is Brownian movement, due to random collisions between water

molecules and bacterial cells. True motility is confirmed by observing the bacterium swim from one side of the microscope field to the other side.

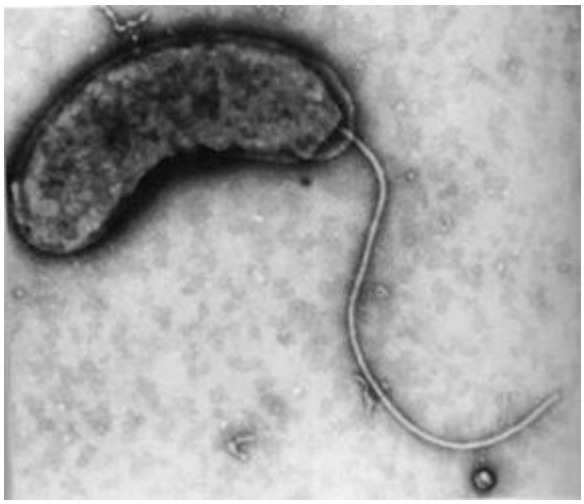


Figure 6. A *Desulfovibrio* species. TEM. About 15,000X. The bacterium is motile by means of a single polar flagellum. Of course, one can determine the presence of flagella by means of electron microscopy. Perhaps this is an alternative way to determine bacterial motility, if you happen to have an electron microscope.

Fimbriae

Fimbriae and **pili** are interchangeable terms used to designate short, hair-like structures on the surfaces of procaryotic cells. Like flagella, they are composed of protein. Fimbriae are shorter and stiffer than flagella, and slightly smaller in diameter. Generally, fimbriae have nothing to do with bacterial movement (there are exceptions, e.g. twitching movement on *Pseudomonas*). Fimbriae are very common in Gram-negative bacteria, but occur in some archaea and Gram-positive bacteria as well. Fimbriae are most often involved in adherence of bacteria to surfaces, substrates and other cells or tissues in nature. In *E. coli*, a specialized type of pilus, the **F or sex pilus**, mediates the transfer of DNA between mating bacteria during the process of **conjugation**, but the function of the smaller, more numerous common pili is quite different.

Common pili (almost always called **fimbriae**) are usually involved in specific adherence (attachment) of procaryotes to surfaces in nature. In medical situations, they are major determinants of bacterial virulence because they allow pathogens to attach to (colonize) tissues and/or to resist attack by phagocytic white blood cells.

For example, pathogenic *Neisseria gonorrhoeae* adheres specifically to the human cervical or urethral epithelium by means of its fimbriae; enterotoxigenic strains of *E. coli* adhere to the mucosal epithelium of the intestine by means of specific fimbriae; the M-protein and associated fimbriae of *Streptococcus pyogenes* are involved in adherence and to resistance to engulfment by phagocytes.

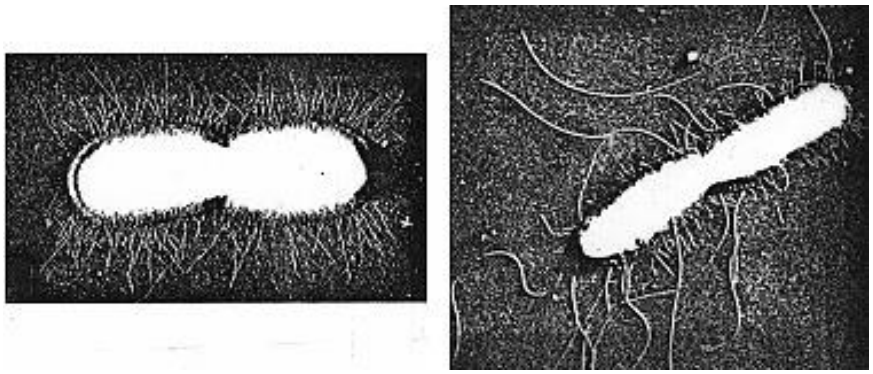


Figure 7. Fimbriae (common pili) and flagella on the surface of bacterial cells. Left: dividing *Shigella* enclosed in fimbriae. The structures are probably involved in the bacterium's ability to adhere to the intestinal surface. Right: dividing pair of *Salmonella* displaying both its peritrichous flagella and its fimbriae. The fimbriae are much shorter and slightly smaller in diameter than flagella. Both *Shigella* and *Salmonella* are enteric bacteria that cause different types of intestinal diarrheas. The bacteria can be differentiated by a motility test. *Salmonella* is motile; *Shigella* is nonmotile.

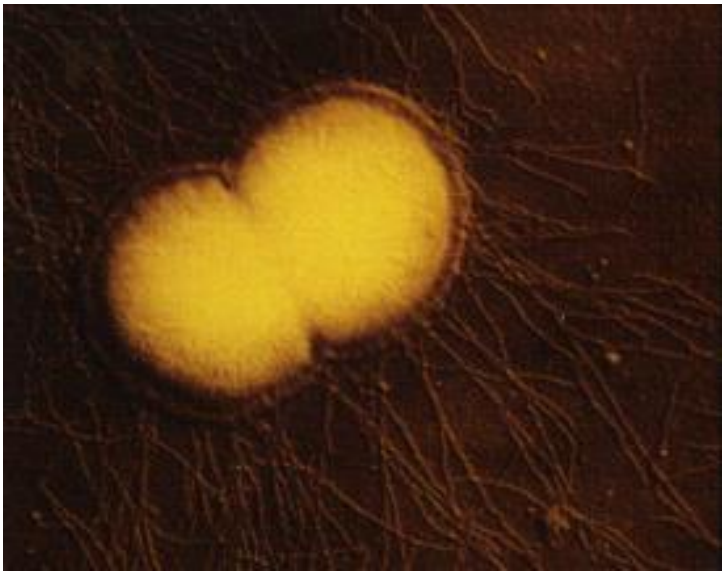


Figure 8. Fimbriae of *Neisseria gonorrhoeae* allow the bacterium to adhere to tissues. Electron micrograph.

Table 2. Some properties of pili and fimbriae.

Bacterial species where observed	Typical number on cell	Distribution on cell surface	Function
Escherichia coli (F or sex pilus)	1-4	uniform	mediates DNA transfer during conjugation
Escherichia coli (common pili or Type 1 fimbriae)	100-200	uniform	surface adherence to epithelial cells of the GI tract
Neisseria gonorrhoeae	100-200	uniform	surface adherence to epithelial cells of the urogenital tract
Streptococcus pyogenes (fimbriae plus the M-protein)	?	uniform	adherence, resistance to phagocytosis; antigenic variability
Pseudomonas aeruginosa	10-20	polar	surface adherence
Sulfolobus acidocaldarius (an archean)	?	?	attachment to sulfur particles

The Cell Envelope

The **cell envelope** is a descriptive term for the several layers of material that envelope or enclose the protoplasm of the cell. The cell protoplasm (**cytoplasm**) is surrounded by the **plasma membrane**, a **cell wall** and a **capsule**. The cell wall itself is a layered structure in Gram-negative bacteria. All cells have a membrane, which is the essential and definitive characteristic of a "cell". Almost all procaryotes have a cell wall to prevent damage to the underlying **protoplast**. Outside the cell wall, foremost as a surface structure, may be a polysaccharide **capsule**, or at least a **glycocalyx**.

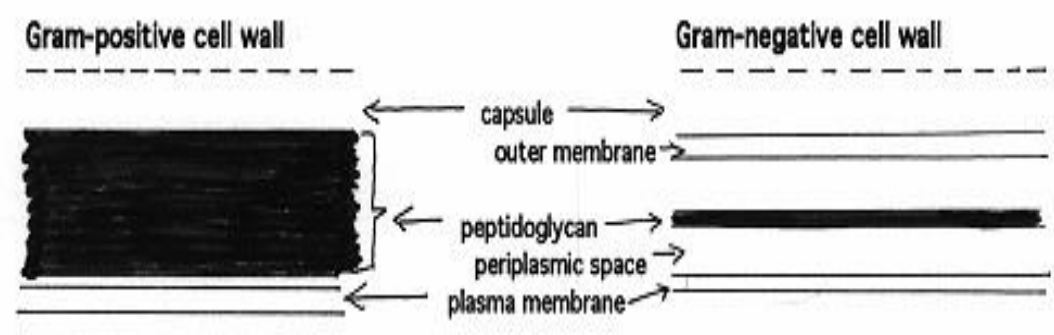


Figure 9. Profiles of the cell envelope the Gram-positive and Gram-negative bacteria. The Gram-positive wall is a uniformly thick layer external to the plasma membrane. It is composed mainly of peptidoglycan (murein). The Gram-negative wall appears thin and multilayered. It consists of a relatively thin peptidoglycan sheet between the plasma membrane and a phospholipid-lipopolysaccharide outer membrane. The space between the inner (plasma) and outer membranes (wherein the peptidoglycan resides) is called the periplasm.

Capsules

Most procaryotes contain some sort of a polysaccharide layer outside of the cell wall polymer. In a general sense, this layer is called a **capsule**. A **true capsule** is a discrete detectable layer of polysaccharides deposited outside the cell wall. A less discrete structure or matrix which embeds the cells is a called a **slime layer** or a **biofilm**. A type of capsule found in bacteria called a **glycocalyx** is a thin layer of tangled polysaccharide fibers which is almost always observed on the surface of cells growing in nature (as opposed to the laboratory).

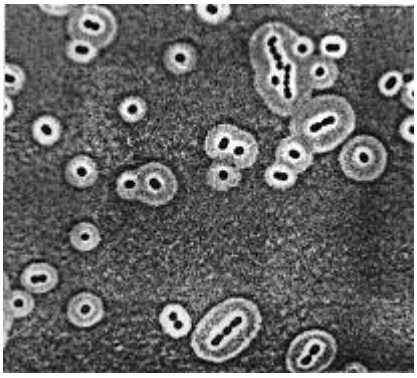


Figure 10. Bacterial capsules outlined by India ink viewed by light microscopy. This is a true capsule, a discrete layer of polysaccharide surrounding the cells. Sometimes bacterial cells are embedded more randomly in a polysaccharide matrix called a slime layer or biofilm. Polysaccharide films that may inevitably be present on the surfaces of bacterial cells, but which cannot be detected visually, are called glycocalyx.

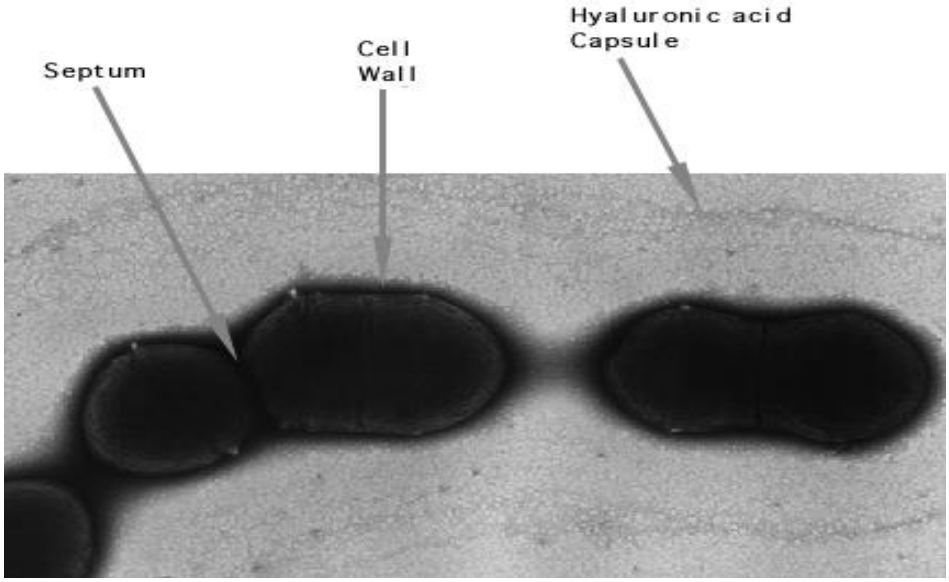


Figure 11. Negative stain of *Streptococcus pyogenes* viewed by transmission electron microscopy (28,000X). The halo around the chain of cells is the hyaluronic acid capsule that surrounds the exterior of the bacteria. The septa between dividing pairs of cells may also be seen. Electron micrograph of *Streptococcus pyogenes*.

Capsules are generally composed of polysaccharide; rarely they contain amino sugars or peptides (see Table 3).

Table 3. Chemical composition of some bacterial capsules.

Bacterium	Capsule composition	Structural subunits
Gram-positive Bacteria		
Bacillus anthracis	polypeptide (polyglutamic acid)	D-glutamic acid
Bacillus megaterium	polypeptide and polysaccharide	D-glutamic acid, amino sugars, sugars
Streptococcus mutans	polysaccharide	(dextran) glucose
Streptococcus pneumoniae	polysaccharides	sugars, amino sugars, uronic acids
Streptococcus pyogenes	polysaccharide (hyaluronic acid)	N-acetyl-glucosamine and glucuronic acid
Gram-negative Bacteria		
Acetobacter xylinum	polysaccharide	(cellulose) glucose
Escherichia coli	polysaccharide (colonic acid)	glucose, galactose, fucose glucuronic acid
Pseudomonas aeruginosa	polysaccharide	mannuronic acid
Azotobacter vinelandii	polysaccharide	glucuronic acid

Agrobacterium tumefaciens	polysaccharide	(glucan) glucose

Capsules have **several functions** and often have multiple functions in a particular organism. Like fimbriae, capsules, slime layers, and glycocalyx often **mediate adherence** of cells to surfaces. Capsules also **protect bacterial cells from engulfment** by predatory protozoa or white blood cells (phagocytes), or from attack by antimicrobial agents of plant or animal origin. Capsules in certain soil bacteria **protect cells from perennial effects of drying** or desiccation. Capsular materials (e.g. dextrans) may be overproduced when bacteria are fed sugars to become **reserves of carbohydrate** for subsequent metabolism.

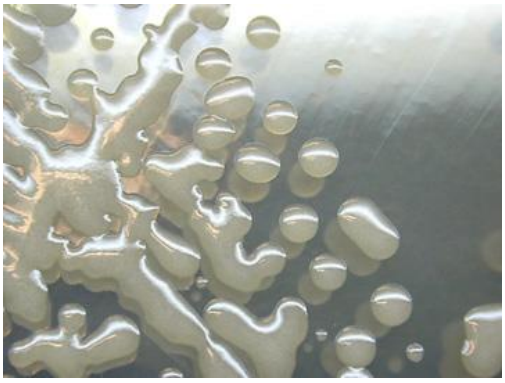


Figure 12. Colonies of *Bacillus anthracis*. The slimy or mucoid appearance of a bacterial colony is usually evidence of capsule production. In the case of *B. anthracis,* the capsule is composed of poly-D-glutamate. The capsule is an essential determinant of virulence to the bacterium. In the early stages of colonization and infection the capsule protects the bacteria from assaults by the immune and phagocytic systems.

Some bacteria produce slime materials to adhere and float themselves as colonial masses in their environments. Other bacteria produce slime materials to attach themselves to a surface or substrate. Bacteria may attach to surface, produce slime, divide and produce microcolonies within the slime layer, and construct a **biofilm**, which becomes an enriched and protected environment for themselves and other bacteria.

A classic example of biofilm construction in nature is the formation of **dental plaque** mediated by the oral bacterium, *Streptococcus mutans*. The bacteria adhere specifically to the pellicle of the tooth by means of a protein on the cell surface. The

bacteria grow and synthesize a dextran capsule which binds them to the enamel and forms a biofilm some 300-500 cells in thickness. The bacteria are able to cleave sucrose (provided by the animal diet) into glucose plus fructose. The fructose is fermented as an energy source for bacterial growth. The glucose is polymerized into an extracellular dextran polymer that cements the bacteria to tooth enamel and becomes the matrix of dental plaque. The dextran slime can be depolymerized to glucose for use as a carbon source, resulting in production of lactic acid within the biofilm (plaque) that decalcifies the enamel and leads to dental caries or bacterial infection of the tooth.

Another important characteristic of capsules may be their ability to block some step in the phagocytic process and thereby prevent bacterial cells from being engulfed or destroyed by phagocytes. For example, the primary determinant of virulence of the pathogen *Streptococcus pneumoniae* is its polysaccharide capsule, which prevents ingestion of pneumococci by alveolar macrophages. *Bacillus anthracis* survives phagocytic engulfment because the lysosomal enzymes of the phagocyte cannot initiate an attack on the poly-D-glutamate capsule of the bacterium. Bacteria such as *Pseudomonas aeruginosa*, that construct a biofilm made of extracellular slime when colonizing tissues, are also resistant to phagocytes, which cannot penetrate the biofilm.

Cell Wall

Most procaryotes have a rigid **cell wall**. The cell wall is an essential structure that protects the cell protoplast from mechanical damage and from osmotic rupture or **lysis**. Procaryotes usually live in relatively dilute environments such that the accumulation of solutes inside the procaryotic cell cytoplasm greatly exceeds the total solute concentration in the outside environment. Thus, the osmotic pressure against the inside of the plasma membrane may be the equivalent of 10-25 atm. Since the membrane is a delicate, plastic structure, it must be restrained by an outside wall made of porous, rigid material that has high tensile strength. Such a material is **murein**, the ubiquitous component of bacterial cell walls.

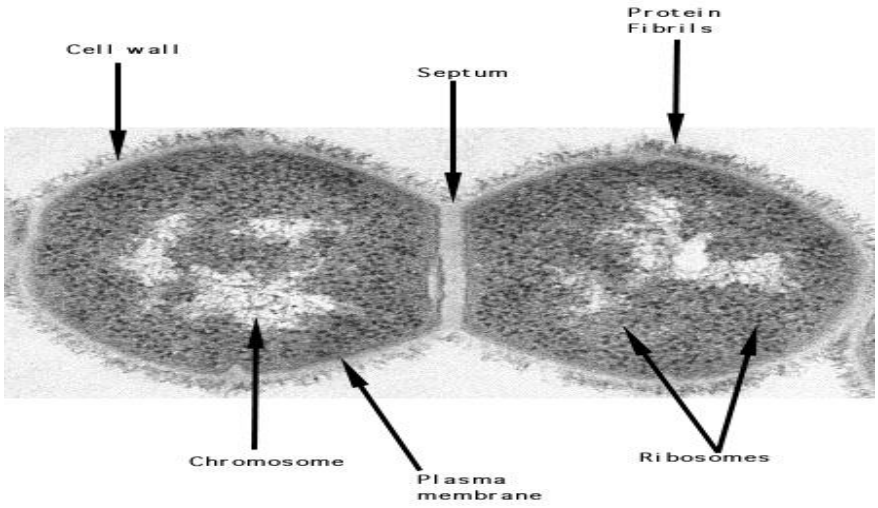


Figure 13. Electron micrograph of an ultra-thin section of a dividing pair of group A streptococci (20,000X). The cell surface fibrils, consisting primarily of M protein, are evident. The bacterial cell wall, to which the fibrils are attached, is also clearly seen as the light staining region between the fibrils and the dark staining cell interior. Cell division in progress is indicated by the new septum formed between the two cells and by the indentation of the cell wall near the cell equator. The streptococcal cell diameter is equal to approximately one micron. Electron micrograph of *Streptococcus pyogenes*.

The cell walls of bacteria deserve special attention for several reasons:

1. They are an essential structure for viability, as described above.
2. They are composed of unique components found nowhere else in nature.
3. They are one of the most important sites for attack by antibiotics.
4. They provide ligands for adherence and receptor sites for drugs or viruses.
5. They cause symptoms of disease in animals.
6. They provide for immunological distinction and immunological variation among strains of bacteria.

The cell walls of all **Bacteria** contain a unique type of **peptidoglycan** called **murein**. Peptidoglycan is a polymer of disaccharides (a glycan) cross-linked by short chains of amino acids (peptides), and many types of peptidoglycan exist. All **Bacterial** peptidoglycans contain **N-acetylmuramic acid**, which is the definitive component of **murein**. The cell walls of **Archaea** may be composed of protein, polysaccharides, or peptidoglycan-like molecules, but never do they contain murein. This feature distinguishes the **Bacteria** from the **Archaea**.

In the **Gram-positive Bacteria** (those that retain the purple crystal violet dye when subjected to the Gram-staining procedure) the cell wall is thick (15-80 nanometers), consisting of several layers of peptidoglycan. In the **Gram-negative Bacteria** (which do not retain the crystal violet) the cell wall is relatively thin (10 nanometers) and is composed of a single layer of peptidoglycan surrounded by a membranous structure called the **outer membrane**. The outer membrane of Gram-negative bacteria invariably contains a unique component, **lipopolysaccharide** (**LPS** or **endotoxin**), which is toxic to animals. In Gram-negative bacteria the outer membrane is usually thought of as part of the cell wall.

Peptidoglycan structure and arrangement in *E. coli* is representative of all *Enterobacteriaceae*, and many other Gram-negative bacteria, as well. The glycan backbone is made up of alternating molecules of N-acetylglucosamine (G) and N-acetylmuramic acid (M) connected by a beta 1,4-glycoside bond. The 3-carbon of N-acetylmuramic acid (M) is substituted with a lactyl ether group derived from pyruvate. The lactyl ether connects the glycan backbone to a peptide side chain that contains L-alanine, (L-ala), D-glutamate (D-glu), Diaminopimelic acid (DAP), and D-alanine (D-ala). MurNAc is unique to bacterial cell walls, as is D-glu, DAP and D-ala. The muramic acid subunit of *E. coli* is shown in Figure 15.

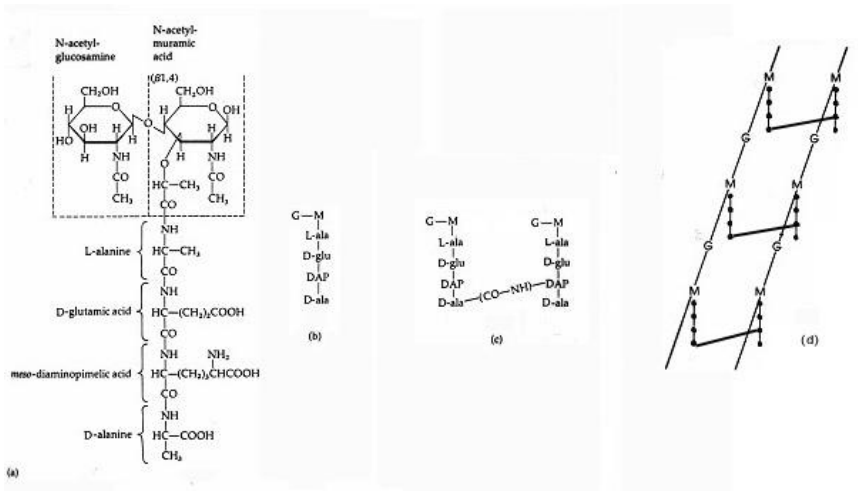


Figure 14. The structure of the muramic acid subunit of the peptidoglycan of *Escherichia coli*. This is the type of murein found in most Gram-negative bacteria. The glycan backbone is a repeat polymer of two amino sugars, N-acetylglucosamine (G) and N-acetylmuramic acid (M). Attached to the N-acetylmuramic acid is a tetrapeptide consisting of L-ala-D-glu-DAP-D-ala.b. Abbreviated structure of the muramic acid subunit. c. Nearby

tetrapeptide side chains may be linked to one another by an interpeptide bond between DAP on one chain and D-ala on the other. d. The polymeric form of the molecule.

Strands of murein are assembled in the periplasm from about 10 muramic acid subunits. Then the strands are connected to form a continuous glycan molecule that encompasses the cell. Wherever their proximity allows it, the tetrapeptide chains that project from the glycan backbone can be cross-linked by an **interpeptide bond** between a free amino group on DAP and a free carboxy group on a nearby D-ala. The assembly of peptidoglycan on the outside of the plasma membrane is mediated by a group of periplasmic enzymes which are transglycosylases, transpeptidases and carboxypeptidases. The mechanism of action of penicillin and related beta-lactam antibiotics is to **block transpeptidase and carboxypeptidase enzymes** during their assembly of the murein cell wall. Hence, the beta lactam antibiotic are said to "block cell wall synthesis" in the bacteria.

The glycan backbone of the peptidoglycan molecule can be cleaved by an enzyme called **lysozyme** that is present in animal serum, tissues and secretions, and in the phagocytic lysosome. The function of lysozyme is to lyse bacterial cells as a constitutive defense against bacterial pathogens. Some Gram-positive bacteria are very sensitive to lysozyme and the enzyme is quite active at low concentrations. Lachrymal secretions (tears) can be diluted 1:40,000 and retain the ability to lyse certain bacterial cells. Gram-negative bacteria are less vulnerable to attack by lysozyme because their peptidoglycan is shielded by the outer membrane. The exact site of lysozymal cleavage is the beta 1,4 bond between N-acetylmuramic acid (M) and N-acetylglucosamine (G) , such that the muramic acid subunit shown in Figure 13(a) is the result of the action of lysozyme on bacterial peptidoglycan.

In Gram-positive bacteria there are numerous different peptide arrangements among peptidoglycans. The best studied is the murein of *Staphylococcus aureus* shown in Figure 16 below. In place of DAP (in *E. coli*) is the diamino acid, L-lysine (L-lys), and in place of the interpeptide bond (in Gram-negatives) is an **interpeptide bridge** of amino acids that connects a free amino group on lysine to a free carboxy group on D-ala of a nearby tetrapeptide side chain. This arrangement apparently allows for more frequent cross-bonding between nearby tetrapeptide side chains. In *S. aureus*,

the interpeptide bridge is a peptide consisting of 5 glycine molecules (called a **pentaglycine bridge**). Assembly of the interpeptide bridge in Gram-positive murein is inhibited by the beta lactam antibiotics in the same manner as the interpeptide bond in Gram-negative murein. Gram-positive bacteria are more sensitive to penicillin than Gram-negative bacteria because the peptidoglycan is not protected by an outer membrane and it is a more abundant molecule. In Gram-positive bacteria, peptidoglycans may vary in the amino acid in place of DAP or L-lys in position 3 of the tetrapeptide, and in the exact composition of the interpeptide bridge. At least eight different types of peptidoglycan exist in Gram-positive bacteria.

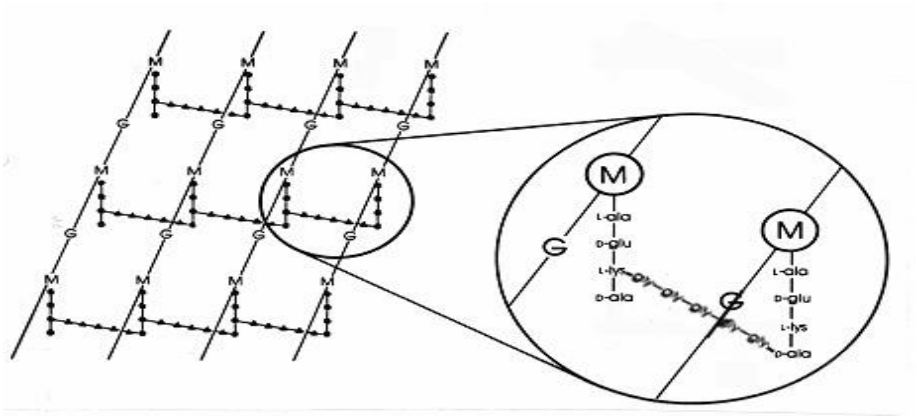


Figure 15. Schematic diagram of the peptidoglycan sheet of *Staphylococcus aureus*. G = N-acetyl-glucosamine; M = N-acetyl-muramic acid; L-ala = L-alanine; D-ala = D-alanine; D-glu = D-glutamic acid; L-lys = L-lysine. This is one type of murein found in Gram-positive bacteria. Compared to the *E. coli* peptidoglycan there is L-lys in place of DAP (diaminopimelic acid) in the tetrapeptide. The free amino group of L-lys is substituted with a glycine pentapeptide (gly-gly-gly-gly-gly-) which then becomes an interpeptide bridge forming a link with a carboxy group from D-ala in an adjacent tetrapeptide side chain. Gram-positive peptidoglycans differ from species to species, mainly in regards to the amino acids in the third position of the tetrapeptide side chain and in the amino acid composition of the interpeptide bridge.

Gram-negative bacteria may contain a single monomolecular layer of murein in their cell walls while Gram-positive bacteria are thought to have several layers or "wraps" of peptidoglycan. Closely associated with the layers of peptidoglycan in Gram-positive bacteria are a group of molecules called teichoic acids. **Teichoic acids** are linear polymers of polyglycerol or polyribitol substituted with phosphates and a few amino acids and sugars. The teichoic acid polymers are occasionally anchored to the plasma membrane (called **lipoteichoic acids**) apparently directed outward at right angles to the layers of peptidoglycan. The functions of teichoic acid are not known.

They are essential to viability of Gram-positive bacteria in the wild. One idea is that they provide a channel of regularly-oriented negative charges for threading positively charged substances through the complicated peptidoglycan network. Another theory is that teichoic acids are in some way involved in the regulation and assembly of muramic acid subunits on the outside of the plasma membrane. There are instances, particularly in the streptococci, wherein teichoic acids have been implicated in the adherence of the bacteria to tissue surfaces.

The Outer Membrane of Gram-negative Bacteria

Of special interest as a component of the Gram-negative cell wall is the **outer membrane**, a discrete bilayered structure on the outside of the peptidoglycan sheet (see Figure 16). For the bacterium, the outer membrane is first and foremost a permeability barrier, but primarily due to its lipopolysaccharide content, it possesses many interesting and important characteristics of Gram-negative bacteria. The outer membrane is a lipid bilayer intercalated with proteins, superficially resembling the plasma membrane. The inner face of the outer membrane is composed of phospholipids similar to the phosphoglycerides that compose the plasma membrane. The outer face of the outer membrane may contain some phospholipid, but mainly it is formed by a different type of amphiphilic molecule which is composed of lipopolysaccharide (LPS). Outer membrane proteins usually traverse the membrane and in one case, anchor the outer membrane to the underlying peptidoglycan sheet.

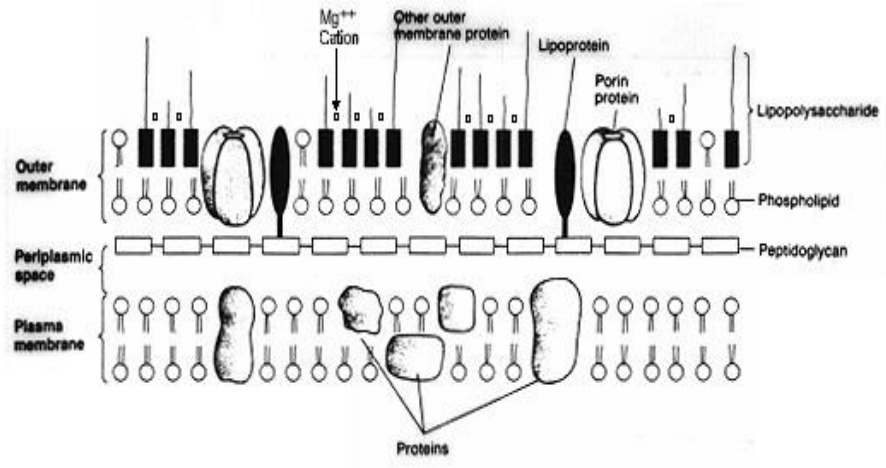


Figure 16. Schematic illustration of the outer membrane, cell wall and plasma membrane of a Gram-negative bacterium. Note the structure and arrangement of molecules that constitute the outer membrane.

The LPS molecule that constitutes the outer face of the outer membrane is composed of a hydrophobic region, called **Lipid A**, that is attached to a hydrophilic linear polysaccharide region, consisting of the **core polysaccharide** and **the O-specific polysaccharide**.

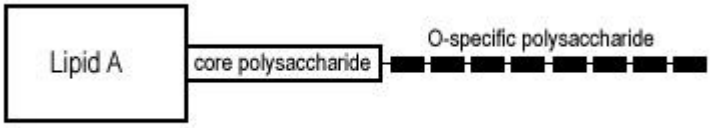


Figure 17. Structure of LPS

The Lipid A head of the molecule inserts into the interior of the membrane, and the polysaccharide tail of the molecule faces the aqueous environment. Where the tail of the molecule inserts into the head there is an accumulation of negative charges such that a magnesium cation is chelated between adjacent LPS molecules. This provides the lateral stability for the outer membrane, and explains why treatment of Gram-negative bacteria with a powerful chelating agent, such as EDTA, causes dispersion of LPS molecules.

Bacterial lipopolysaccharides are toxic to animals. When injected in small amounts LPS or **endotoxin** activates macrophages to produce pyrogens, activates the complement cascade causing inflammation, and activates blood factors resulting in intravascular coagulation and hemorrhage. Endotoxins may play a role in infection by

any Gram-negative bacterium. The toxic component of endotoxin (LPS) is Lipid A. The O-specific polysaccharide may provide ligands for bacterial attachment and confer some resistance to phagocytosis. Variation in the exact sugar content of the O polysaccharide (also referred to as the O antigen) accounts for multiple antigenic types (serotypes) among Gram-negative bacterial pathogens. Therefore. even though Lipid A is the toxic component in LPS, the polysaccharides nonetheless contribute to virulence of Gram-negative bacteria.

The proteins in the outer membrane of *Escherichia coli* are well characterized (see Table 4). About 400,00 copies of the **Braun lipoprotein** are covalently attached to the peptidoglycan sheet at one end and inserted into the hydrophobic interior of the membrane at the opposite end. A group of trimeric proteins called **porins** form pores of a fixed diameter through the lipid bilayer of the membrane. The **omp C** and **omp F** porins of *E. coli* are designed to allow passage of hydrophilic molecules up to mw of about 750 daltons. Larger molecules or harmful hydrophobic compounds (such as bile salts in the intestinal tract) are excluded from entry. Porins are designed in Gram-negative bacteria to allow passage of useful molecules (nutrients) through the barrier of the outer membrane, but to exclude passage harmful substances from the environment. The ubiquitous **omp A** protein in the outer membrane of *E. coli* has a porin like structure, and may function in uptake of specific ions, but it is also a receptor for the F pilus and an attachment site for bacterial viruses.

Table 4. Functions of the outer membrane components of *Escherichia coli*.

Component	Function
Lipopolysaccharide (LPS)	Permeability barrier
Mg++ bridges	Stabilizes LPS and is essential for its permeability characteristics
Braun lipoprotein	Anchors the outer membrane to peptidoglycan (murein) sheet
Omp C and Omp F porins	proteins that form pores or channels through outer membrane for passage of hydrophilic molecules
Omp A protein	provides receptor for some viruses and bacteriocins; stabilizes mating cells

	during conjugation

A correlation between Gram stain reaction and cell wall properties of bacteria is summarized in Table 5. The Gram stain procedure contains a "destaining" step wherein the cells are washed with an acetone-alcohol mixture. The lipid content of the Gram-negative wall probably affects the outcome of this step so that Gram-positive cells retain a primary stain while Gram-negative cells are destained.

Table 5. Correlation of Grams stain with other properties of Bacteria.

Property	Gram-positive	Gram-negative
Thickness of wall	thick (20-80 nm)	thin (10 nm)
Number of layers	1	2
Peptidoglycan (murein) content	>50%	10-20%
Teichoic acids in wall	present	absent
Lipid and lipoprotein content	0-3%	58%
Protein content	0	9%
Lipopolysaccharide content	0	13%
Sensitivity to Penicillin G	yes	no (1)
Sensitivity to lysozyme	yes	no (2)

(1) A few Gram-negative bacteria are sensitive to natural penicillins. Many Gram-negative bacteria are sensitive to some type of penicillin, especially semisynthetic penicillins. Gram-negative bacteria, including *E. coli*, can be made sensitive to natural penicillin by procedures that disrupt the permeability characteristics of the outer membrane.

(2) Gram-negative bacteria are sensitive to lysozyme if pretreated by some procedure that removes the outer membrane and exposes the peptidoglycan directly to the enzyme.

Cell Wall-less Forms

A few bacteria are able to live or exist without a cell wall. The mycoplasmas are a group of bacteria that lack a cell wall. Mycoplasmas have sterol-like molecules

incorporated into their membranes and they are usually inhabitants of osmotically-protected environments. *Mycoplasma pneumoniae* is the cause of primary atypical bacterial pneumonia, known in the vernacular as "walking pneumonia". For obvious reasons, penicillin is ineffective in treatment of this type of pneumonia. Sometimes, under the pressure of antibiotic therapy, pathogenic streptococci can revert to cell wall-less forms (called **spheroplasts**) and persist or survive in osmotically-protected tissues. When the antibiotic is withdrawn from therapy the organisms may regrow their cell walls and reinfect unprotected tissues.

The Plasma Membrane

The **plasma membrane**, also called the **cytoplasmic membrane**, is the most dynamic structure of a procaryotic cell. Its main function is a s a **selective permeability barrier** that regulates the passage of substances into and out of the cell. The plasma membrane is the definitive structure of a cell since it sequesters the molecules of life in a unit, separating it from the environment. The bacterial membrane allows passage of water and uncharged molecules up to mw of about 100 daltons, but does not allow passage of larger molecules or any charged substances except by means special membrane **transport processes** and **transport systems**.

Since procaryotes lack any intracellular organelles for processes such as respiration or photosynthesis or secretion, the plasma membrane subsumes these processes for the cell and consequently has a variety of functions in **energy generation**, and **biosynthesis**. For example, the **electron transport system** that couples **aerobic respiration** and **ATP synthesis** is found in the procaryotic membrane. The **photosynthetic chromophores** that harvest light energy for conversion into chemical energy are located in the membrane. Hence, the plasma membrane is the site of **oxidative phosphorylation** and **photophosphorylation** in procaryotes, analogous to the functions of mitochondria and chloroplasts in eukaryotic cells. Besides **transport proteins** that selectively mediate the passage of substances into and out of the cell, procaryotic membranes may contain **sensing proteins** that measure concentrations of molecules in the environment or **binding proteins** that translocate signals to genetic and metabolic machinery in the cytoplasm. Membranes

also contain **enzymes** involved in many metabolic processes such as cell wall synthesis, septum formation, membrane synthesis, DNA replication, CO_2 fixation and ammonia oxidation. The predominant functions of bacterial membranes are listed in the table below.

Table 6. Functions of the procaryotic plasma membrane.

1. Osmotic or permeability barrier

2. Location of transport systems for specific solutes (nutrients and ions)

3. Energy generating functions, involving respiratory and photosynthetic electron transport systems, establishment of proton motive force, and transmembranous, ATP-synthesizing ATPase

4. Synthesis of membrane lipids (including lipopolysaccharide in Gram-negative cells)

5. Synthesis of murein (cell wall peptidoglycan)

6. Assembly and secretion of extracytoplasmic proteins

7. Coordination of DNA replication and segregation with septum formation and cell division

8. Chemotaxis (both motility per se and sensing functions)

9. Location of specialized enzyme system

Bacterial membranes are composed of 40 percent phospholipid and 60 percent protein. The phospholipids are amphoteric molecules with a polar hydrophilic glycerol "head" attached via an ester bond to two nonpolar hydrophobic fatty acid tails, which naturally form a bilayer in aqueous environments. Dispersed within the bilayer are various structural and enzymatic proteins which carry out most membrane functions. At one time, it was thought that the proteins were neatly organized along the inner and outer faces of the membrane and that this accounted for the double track

appearance of the membrane in electron micrographs. However, it is now known that while some membrane proteins are located and function on one side or another of the membrane, most proteins are partly inserted into the membrane, or possibly even traverse the membrane as channels from the outside to the inside. It is possible that proteins can move laterally along a surface of the membrane, but it is thermodynamically unlikely that proteins can be rotated within a membrane, which discounts early theories of how transport systems might work. The arrangement of proteins and lipids to form a membrane is called the **fluid mosaic model**, and is illustrated in Figure 18.

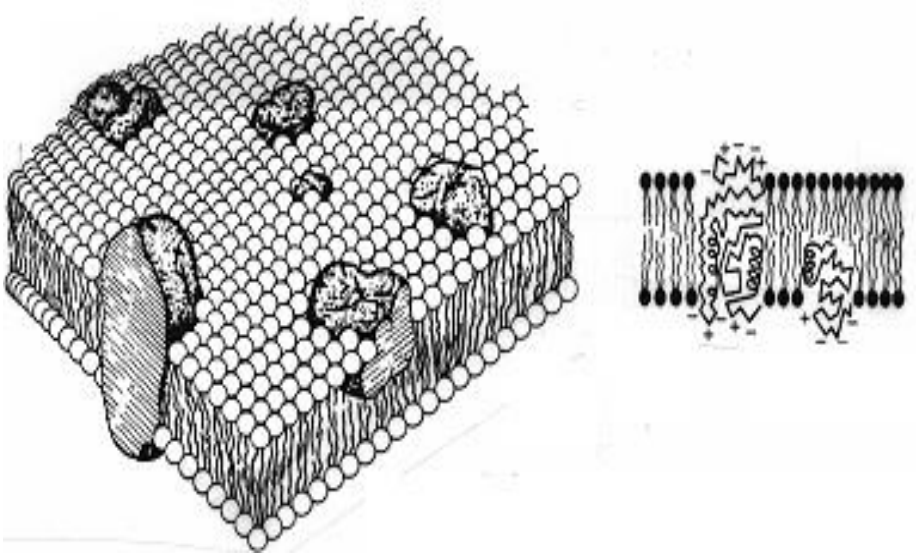


Figure 18. Fluid mosaic model of a biological membrane. In aqueous environments membrane phospholipids arrange themselves in such a way that they spontaneously form a fluid bilayer. Membrane proteins, which may be structural or functional, may be permanently or transiently associated with one side or the other of the membrane, or even be permanently built into the bilayer, while other proteins span the bilayer and may form transport channels through the membrane.

Transport Processes

The proteins that mediate the passage of solutes through membranes are referred to variously as **transport systems**, **carrier proteins**, **porters**, and **permeases**. Transport systems operate by one of three **transport processes** as described below in Figure 19. In a **uniport** process, a solute passes through the membrane

unidirectionally. In **symport** processes (also called **cotransport**) two solutes must be transported in the same direction at the same time; in **antiport** processes (also called **exchange diffusion**), one solute is transported in one direction simultaneously as a second solute is transported in the opposite direction.

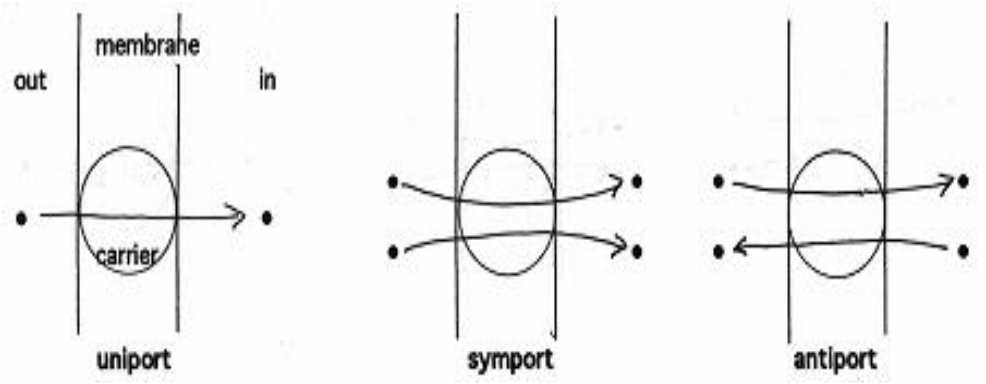


Figure 19. Transport processes in bacterial cells. Solutes enter or exit from bacterial cells by means of one of three processes: uniport, symport (also called cotransport) and antiport (also called exchange diffusion). Transport systems (Figure 20 below) operate by one or another of these processes.

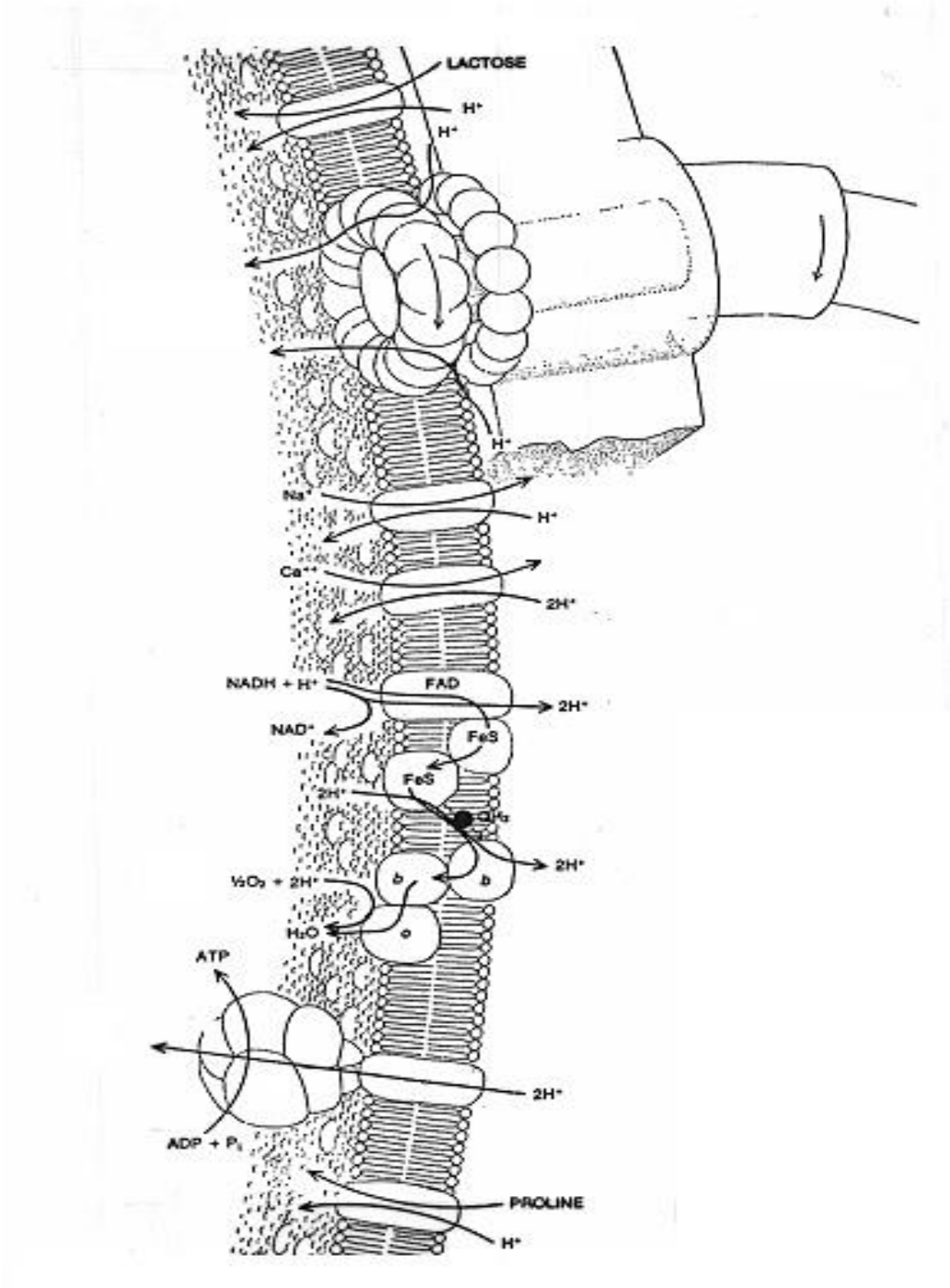


Figure 20. Schematic view of the plasma membrane of *Escherichia coli*. The S and M rings which constitute the flagellar motor are shown. The motor ring is imbedded in the phospholipid bilayer. It is powered by pmf to rotate the flagellar filament. The electron transport system is shown oxidizing NAD by removal of a pair of electrons, passing them through its sequence of carriers eventually to O2. ATPase is the transmembranous protein enzyme that utilizes protons from the outside to synthesize ATP on the inside of the membrane. Several other transmembranous proteins are

transport systems which are operating by either symport or antiport processes.

The Cytoplasm

The cytoplasmic constituents of procaryotic cells invariably include the **procaryotic chromosome** and **ribosomes**. The chromosome is typically one large circular molecule of **DNA**, more or less free in the cytoplasm. Procaryotes sometimes possess smaller extrachromosomal pieces of DNA called **plasmids**. The total DNA content of a procaryote is referred to as the cell **genome**. During cell growth and division, the procaryotic chromosome is replicated in the usual semi-conservative fashion before for distribution to progeny cells. However, the eukaryotic processes of meiosis and mitosis are absent in procaryotes. Replication and segregation of procaryotic DNA is coordinated by the membrane, possibly by mesosomes.

The distinct granular appearance of procaryotic cytoplasm is due to the presence and distribution of **ribosomes**. The ribosomes of procaryotes are smaller than cytoplasmic ribosomes of eukaryotes. procaryotic ribosomes are 70S in size, being composed of 30S and 50S subunits. The 80S ribosomes of eukaryotes are made up of 40S and 60S subunits. Ribosomes are involved in the process of translation (protein synthesis), but some details of their activities differ in eukaryotes, Bacteria and Archaea. Protein synthesis using 70S ribosomes occurs in eukaryotic mitochondria and chloroplasts, and this is taken as a major line of evidence that these organelles are descended from procaryotes.

Table 9. Inorganic ions present in a growing bacterial cell.

Ion	Function
K^+	Maintenance of ionic strength; cofactor for certain enzymes
NH_4^+	Principal form of inorganic N for assimilation
Ca^{++}	Cofactor for certain enzymes
Fe^{++}	Present in cytochromes and other metalloenzymes
Mg^{++}	Cofactor for many enzymes; stabilization of outer membrane of Gram-negative bacteria
Mn^{++}	Present in certain metalloenzymes

Co^{++}	Trace element constituent of vitamin B12 and its coenzyme derivatives and found in certain metalloenzymes
Cu^{++}	Trace element present in certain metalloenzymes
Mo^{++}	Trace element present in certain metalloenzymes
Ni^{++}	Trace element present in certain metalloenzymes
Zn^{++}	Trace element present in certain metalloenzymes
SO_4^{--}	Principal form of inorganic S for assimilation
PO_4^{---}	Principal form of P for assimilation and a participant in many metabolic reactions

Inclusions

Often contained in the cytoplasm of procaryotic cells is one or another of some type of inclusion granule. **Inclusions** are distinct granules that may occupy a substantial part of the cytoplasm. Inclusion granules are usually reserve materials of some sort. For example, carbon and energy reserves may be stored as glycogen (a polymer of glucose) or as polybetahydroxybutyric acid (a type of fat) granules. Polyphosphate inclusions are reserves of PO_4 and possibly energy; elemental sulfur (sulfur globules) are stored by some phototrophic and some lithotrophic procaryotes as reserves of energy or electrons. Some inclusion bodies are actually membranous vesicles or intrusions into the cytoplasm which contain photosynthetic pigments or enzymes.

Table 10. Some inclusions in bacterial cells.

Cytoplasmic inclusions	Where found	Composition	Function
glycogen	many bacteria e.g. *E. coli*	polyglucose	reserve carbon and energy source
polybetahydroxyutyric acid (PHB)	many bacteria e.g. *Pseudomonas*	polymerized hydroxy butyrate	reserve carbon and energy source
polyphosphate (volutin granules)	many bacteria e.g. *Corynebacterium*	linear or cyclical polymers of PO4	reserve phosphate; possibly a reserve of high energy phosphate
sulfur globules	phototrophic purple and	elemental sulfur	reserve of electrons

	green sulfur bacteria and lithotrophic colorless sulfur bacteria		(reducing source) in phototrophs; reserve energy source in lithotrophs
gas vesicles	aquatic bacteria especially cyanobacteria	protein hulls or shells inflated with gases	buoyancy (floatation) in the vertical water column
parasporal crystals	endospore-forming bacilli (genus *Bacillus*)	protein	unknown but toxic to certain insects
magnetosomes	certain aquatic bacteria	magnetite (iron oxide) Fe3O4	orienting and migrating along geo- magnetic field lines
carboxysomes	many autotrophic bacteria	enzymes for autotrophic CO2 fixation	site of CO2 fixation
phycobilisomes	cyanobacteria	phycobiliproteins	light-harvesting pigments
chlorosomes	Green bacteria	lipid and protein and bacteriochlorophyll	light-harvesting pigments and antennae

Endospores

A bacterial structure sometimes observed as an inclusion is actually a type of dormant cell called an **endospore**. Endospores are formed by a few groups of **Bacteria** as intracellular structures, but ultimately they are released as free endospores. Biologically, endospores are a fascinating type of cell. Endospores exhibit no signs of life, being described as **cryptobiotic**. They are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for hours and retain their viability), irradiation, strong acids, disinfectants, etc. They are probably the most durable cell produced in nature. Although cryptobiotic, they retain viability indefinitely such that under appropriate environmental conditions, they germinate back into vegetative cells. Endospores are formed by vegetative cells in response to environmental signals that indicate a limiting factor for vegetative growth, such as exhaustion of an essential nutrient. They germinate and become vegetative cells when the environmental stress is relieved. Hence, endospore-formation is a mechanism of survival rather than a mechanism of reproduction.

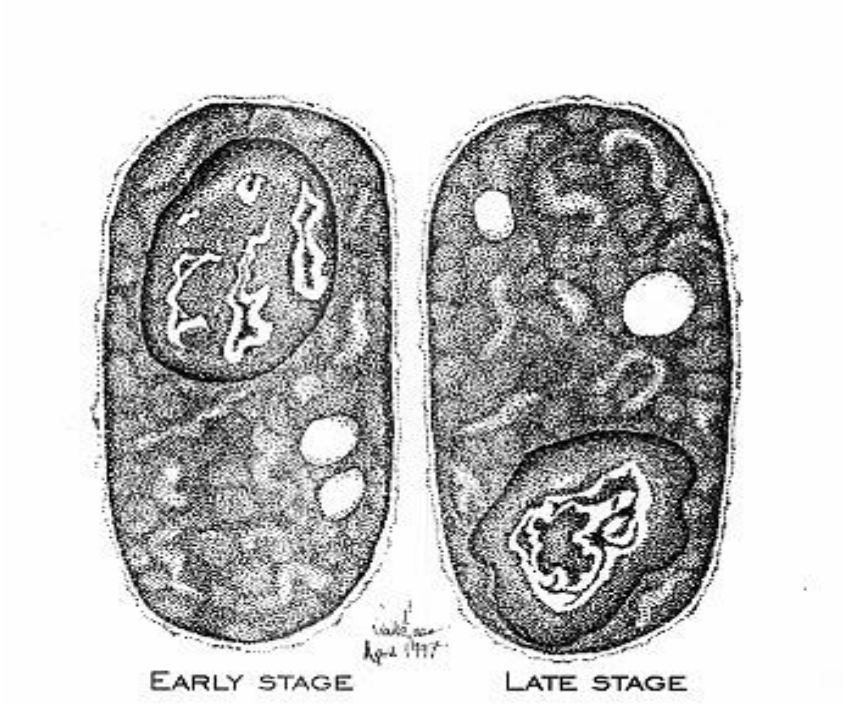


Figure 21. Early and late stages of endospore formation. Drawing by Vaike Haas, University of Wisconsin Madison. During endospore formation, a vegetative cell is converted to a heat-resistant spore. There are eight stages, O,I-VII, in the sporulation cycle of a *Bacillus* species, and the process takes about eight hours. During the early stages (Stage II,) one bacterial chromosome and a few ribosomes are partitioned off by the bacterial membrane to form a protoplast within the mother cell. By the late stages (Stage VI) the protoplast (now called a forespore) has developed a second membrane and several wall-like layers of material are deposited between the two membranes.

Table 11. Differences between endospores and vegetative cells.

Property	Vegetative cells	Endospores
Surface coats	Typical Gram-positive murein cell wall polymer	Thick spore coat, cortex, and peptidoglycan core wall
Microscopic appearance	Nonrefractile	Refractile
Calcium dipicolinic acid	Absent	Present in core
Cytoplasmic water activity	High	Very low
Enzymatic activity	Present	Absent
Macromolecular synthesis	Present	Absent

Heat resistance	Low	High
Resistance to chemicals and acids	Low	High
Radiation resistance	Low	High
Sensitivity to lysozyme	Sensitive	Resistant
Sensitivity to dyes and staining	Sensitive	Resistant

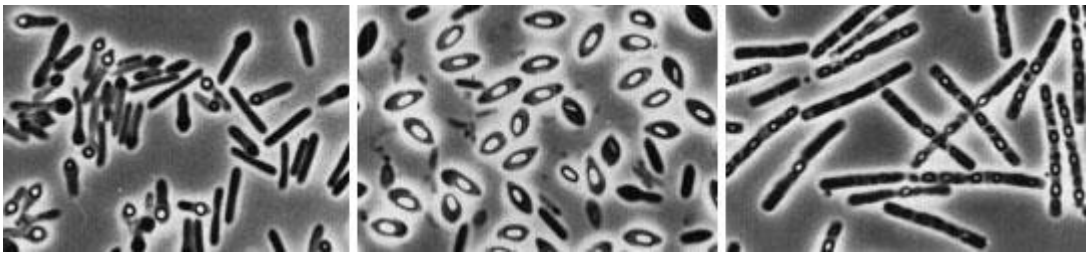


Figure 22. Bacterial endospores. Phase microscopy of sporulating bacteria demonstrates the refractility of endospores, as well as characteristic spore shapes and locations within the mother cell.

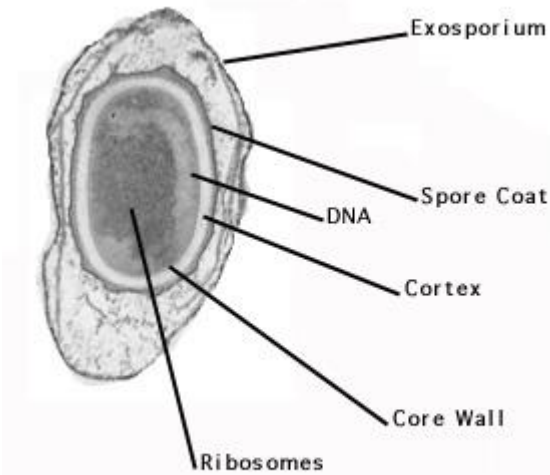


Figure 23. Electron micrograph of a bacterial endospore. The spore has a core wall of unique peptidoglycan surrounded by several layers, including the cortex, the spore coat and the exosporium. The dehydrated core contains the bacterial chromosome and a few ribosomes and enzymes to jump-start protein synthesis and metabolism during germination.

NUTRITION AND GROWTH OF BACTERIA

Nutritional Requirements of Cells

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as **nutrients** or **nutritional requirements**. In the laboratory, bacteria are grown in **culture media** which are designed to provide all the essential nutrients in solution for bacterial growth.

The Major Elements

At an elementary level, the nutritional requirements of a bacterium such as *E. coli* are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo. These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells. The general physiological functions of the elements are outlined in Table 1 below.

Table 1. Major elements, their sources and functions in bacterial cells.

Element	% of dry weight	Source	Function
Carbon	50	organic compounds or CO_2	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates	Constituent of nucleic acids, nucleotides,

		(PO_4)	phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores
Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

Trace Elements

Table 1 ignores the occurrence of trace elements in bacterial nutrition. **Trace elements** are metal ions required by certain cells in such small amounts that it is difficult to detect (measure) them, and it is not necessary to add them to culture media as nutrients. Trace elements are required in such small amounts that they are present as "contaminants" of the water or other media components. As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell. One organism's trace element may be another's required element and vice-versa, but the usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo.

Carbon and Energy Sources for Bacterial Growth

In order to grow in nature or in the laboratory, a bacterium must have an energy source, a source of carbon and other required nutrients, and a permissive range of physical conditions such as O_2 concentration, temperature, and pH. Sometimes bacteria are referred to as individuals or groups based on their patterns of growth under various chemical (nutritional) or physical conditions. For example, phototrophs

are organisms that use light as an energy source; anaerobes are organisms that grow without oxygen; thermophiles are organisms that grow at high temperatures.

All living organisms require a source of energy. Organisms that use radiant energy (light) are called **phototrophs**. Organisms that use (oxidize) an organic form of carbon are called **heterotrophs** or **chemo(hetero)trophs**. Organisms that oxidize inorganic compounds are called **lithotrophs**.

The carbon requirements of organisms must be met by organic carbon (a chemical compound with a carbon-hydrogen bond) or by CO_2. Organisms that use organic carbon are **heterotrophs** and organisms that use CO_2 as a sole source of carbon for growth are called **autotrophs**.

Thus, on the basis of carbon and energy sources for growth four major nutritional types of procaryotes may be defined (Table 2).

Table 2. Major nutritional types of procaryotes

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

Almost all eukaryotes are either photoautotrophic (e.g. plants and algae) or heterotrophic (e.g. animals, protozoa, fungi). Lithotrophy is unique to procaryotes and photoheterotrophy, common in the Purple and Green Bacteria, occurs only in a very few eukaryotic algae. Phototrophy has not been found in the Archaea, except for nonphotosynthetic light-driven ATP synthesis in the extreme halophiles.

Growth Factors

This simplified scheme for use of carbon, either organic carbon or CO_2, ignores the possibility that an organism, whether it is an autotroph or a heterotroph, may require small amounts of certain organic compounds for growth because they are essential substances that the organism is unable to synthesize from available nutrients. Such compounds are called **growth factors**.

Growth factors are required in small amounts by cells because they fulfill specific roles in biosynthesis. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells. Growth factors are organized into three categories.

1. **purines and pyrimidines**: required for synthesis of nucleic acids (DNA and RNA)

2. **amino acids**: required for the synthesis of proteins

3. **vitamins**: needed as coenzymes and functional groups of certain enzymes

Some bacteria (e.g *E. coli*) do not require any growth factors: they can synthesize all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism. Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids in order to grow. These compounds must be added in advance to culture media that are used to grow these bacteria. The growth factors are not metabolized directly as sources of carbon or energy, rather they are assimilated by cells to fulfill their specific role in metabolism. Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**. Thus, a strain of *E. coli* that requires the amino acid tryptophan in order to grow would be called a tryptophan auxotroph and would be designated *E. coli* ***trp-.***

Some vitamins that are frequently required by certain bacteria as growth factors are listed in Table 3. The function(s) of these vitamins in essential enzymatic reactions

gives a clue why, if the cell cannot make the vitamin, it must be provided exogenously in order for growth to occur.

Table 3. Common vitamins required in the nutrition of certain bacteria.

Vitamin	Coenzyme form	Function
p-Aminobenzoic acid (PABA)	-	Precursor for the biosynthesis of folic acid
Folic acid	Tetrahydrofolate	Transfer of one-carbon units and required for synthesis of thymine, purine bases, serine, methionine and pantothenate
Biotin	Biotin	Biosynthetic reactions that require CO_2 fixation
Lipoic acid	Lipoamide	Transfer of acyl groups in oxidation of keto acids
Mercaptoethane-sulfonic acid	Coenzyme M	CH_4 production by methanogens
Nicotinic acid	NAD (nicotinamide adenine dinucleotide) and NADP	Electron carrier in dehydrogenation reactions
Pantothenic acid	Coenzyme A and the Acyl Carrier Protein (ACP)	Oxidation of keto acids and acyl group carriers in metabolism
Pyridoxine (B_6)	Pyridoxal phosphate	Transamination, deamination, decarboxylation and racemation of amino acids
Riboflavin (B_2)	FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide)	Oxidoreduction reactions
Thiamine (B_1)	Thiamine pyrophosphate (TPP)	Decarboxylation of keto acids and transaminase reactions
Vitamin B_{12}	Cobalamine coupled to adenine nucleoside	Transfer of methyl groups
Vitamin K	Quinones and napthoquinones	Electron transport processes

Culture Media for the Growth of Bacteria

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional)

environment is made available as a **culture medium**, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties.

The manner in which bacteria are cultivated, and the purpose of culture media, varies widely. **Liquid media** are used for growth of pure batch cultures, while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes. The usual gelling agent for solid or **semisolid medium** is **agar**, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at 100 degrees and remains liquid until cooled to 40 degrees, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aquaeous solution.

Types of Culture Media

Culture media may be classified into several categories depending on their composition or use. A **chemically-defined (synthetic) medium** (Table 4a and 4b) is one in which the exact chemical composition is known. A **complex (undefined) medium** (Table 5a and 5b) is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals off the shelf; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. A defined medium is a **minimal medium** (Table4a) if it provides only the exact nutrients (including any growth factors) needed by the organism for growth. The use of defined minimal media requires the investigator to know the exact nutritional requirements of the organisms in question. Chemically-defined media are of value in studying the minimal

nutritional requirements of microorganisms, for enrichment cultures, and for a wide variety of physiological studies. Complex media usually provide the full range of growth factors that may be required by an organism so they may be more handily used to cultivate unknown bacteria or bacteria whose nutritional requirement are complex (i.e., organisms that require a lot of growth factors, known or unknown).

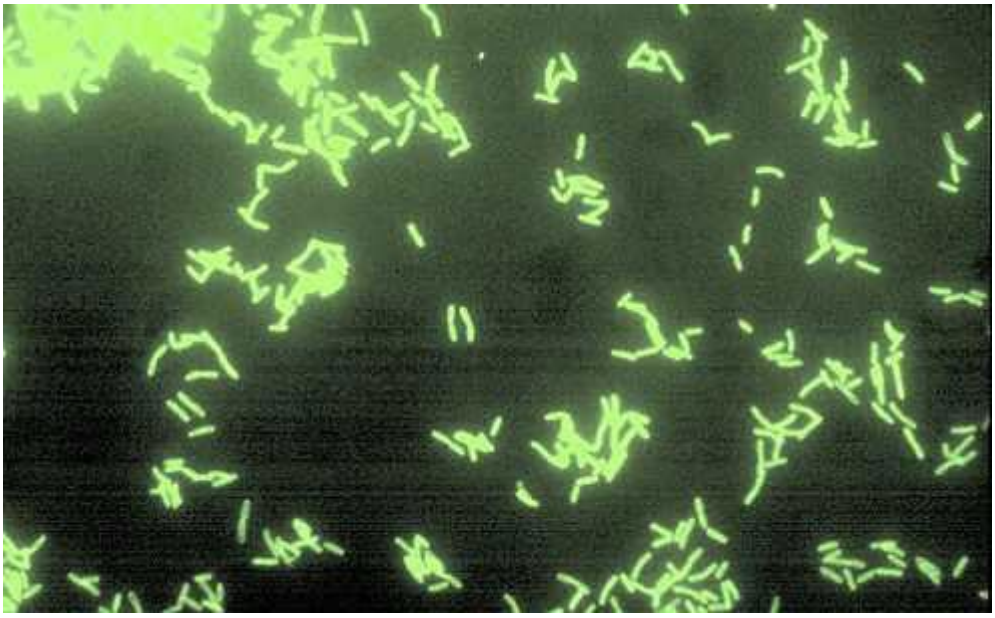


Figure 1. *Legionella pneumophila.* Direct fluorescent antibody (DFA) stain of a patient respiratory tract specimen. In spite of its natural ocurrence in water cooling towers and air conditioners, *Legionella* is a fastidious bacterium grown in the laboratory, which led to the long lag in identification of the first outbreak of Legionaire's disease in Philadelphia in 1977. Had fluoresent antibody to the bacterium been available at that time, diagnosis could have been made as quickly as the time to prepare and view this slide.

Most pathogenic bacteria of animals, which have adapted themselves to growth in animal tissues, require complex media for their growth. Blood, serum and tissue extracts are frequently added to culture media for the cultivation of pathogens. Even so, for a few fastidious pathogens such as *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the cause of leprosy, artificial culture media and conditions have not been established. This fact thwarts the the ability to do basic research on these pathogens and the diseases that they cause.

Other concepts employed in the construction of culture media are the principles of selection and enrichment. A **selective medium** is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. One can also adjust the physical conditions of a culture medium, such as pH and temperature, to render it selective for organisms that are able to grow under these certain conditions.

A culture medium may also be a **differential medium** if allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium. Thus a **selective, differential medium** for the isolation of *Staphylococcus aureus,* the most common bacterial pathogen of humans, contains a very high concentration of salt (which the staph will tolerate) that inhibits most other bacteria, mannitol as a source of fermentable sugar, and a pH indicator dye. From clinical specimens, only staph will grow. *S. aureus* is differentiated from *S. epidermidis* (a nonpathogenic component of the normal flora) on the basis of its ability to ferment mannitol. Mannitol-fermenting colonies *(S. aureus*)produce acid which reacts with the indicator dye forming a colored halo around the colonies; mannitol non-fermenters (*S. epidermidis*) use other non-fermentative substrates in the medium for growth and do not form a halo around their colonies.

An enrichment medium employs a slightly different twist. An **enrichment medium** (Table 5a and 5b) contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment. However, an enrichment medium may have selective features. An enrichment medium for nonsymbiotic nitrogen-fixing bacteria omits a source of added nitrogen to the medium. The medium is inoculated with a potential source of these bacteria (e.g. a soil sample) and incubated in the atmosphere wherein the only source of nitrogen available is N_2. A selective enrichment medium (Table 5b) for growth of the extreme halophile (*Halococcus*) contains nearly 25 percent salt [NaCl], which is required by the extreme halophile and which inhibits the growth of all other procaryotes.

Table 4a. Minimal medium for the growth of *Bacillus megaterium.* An example of a chemically-defined medium for growth of a heterotrophic bacterium.

Component	Amount	Function of component
sucrose	10.0 g	C and energy source
K_2HPO_4	2.5 g	pH buffer; P and K source
KH_2PO_4	2.5 g	pH buffer; P and K source
$(NH_4)2HPO_4$	1.0 g	pH buffer; N and P source
$MgSO_4$ $7H_2O$	0.20 g	S and Mg^{++} source
$FeSO_4$ $7H_2O$	0.01 g	Fe^{++} source
$MnSO_4$ $7H_2O$	0.007 g	Mn^{++} Source
water	985 ml	
pH 7.0		

Table 4b. Defined medium (also an enrichment medium) for the growth of *Thiobacillus thiooxidans,* a lithoautotrophic bacterium.

Component	Amount	Function of component
NH_4Cl	0.52 g	N source
KH_2PO_4	0.28 g	P and K source
$MgSO_4$ $7H_2O$	0.25 g	S and Mg^{++} source
$CaCl_2$ $2H_2O$	0.07 g	Ca^{++} source
Elemental Sulfur	1.56 g	Energy source
CO_2	5%*	C source
water	1000 ml	
pH 3.0		

* Aerate medium intermittently with air containing 5% CO_2.

Table 5a. Complex medium for the growth of fastidious bacteria.

Component	Amount	Function of component
Beef extract	1.5 g	Source of vitamins and other growth factors
Yeast extract	3.0 g	Source of vitamins and other growth factors
Peptone	6.0 g	Source of amino acids, N, S, and P
Glucose	1.0 g	C and energy source
Agar	15.0 g	Inert solidifying agent
water	1000 ml	
pH 6.6		

Table 5b. Selective enrichment medium for growth of extreme halophiles.

Component	Amount	Function of component
Casamino acids	7.5 g	Source of amino acids, N, S and P
Yeast extract	10.0 g	Source of growth factors
Trisodium citrate	3.0 g	C and energy source
KCl	2.0 g	K^+ source
$MgSO_4$ 7 H_2O	20.0 g	S and Mg^{++} source
$FeCl_2$	0.023 g	Fe^{++} source
NaCl	250 g	Na^+ source for halophiles and inhibitory to nonhalophiles
water	1000 ml	
pH 7.4		

Physical and Environmental Requirements for Microbial Growth

The procaryotes exist in nature under an enormous range of physical conditions such as O_2 concentration, Hydrogen ion concentration (pH) and temperature. The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganism, most often a procaryote, and frequently an Archaeon. Applied to all microorganisms is a vocabulary of terms used to describe their growth (ability to grow) within a range of physical conditions. A thermophile grows at high temperatures, an acidophile grows at low pH, an osmophile grows at high solute concentration, and so on. This nomenclature will be employed in this

section to describe the response of the procaryotes to a variety of physical conditions.

The Effect of Oxygen

Oxygen is a universal component of cells and is always provided in large amounts by H_2O. However, procaryotes display a wide range of responses to molecular oxygen O_2 (Table 6).

Obligate aerobes require O_2 for growth; they use O_2 as a final electron acceptor in aerobic respiration.

Obligate anaerobes (occasionally called **aerophobes**) do not need or use O_2 as a nutrient. In fact, O_2 is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic procaryotes may live by fermentation, anaerobic respiration, bacterial photosynthesis, or the novel process of methanogenesis.

Facultative anaerobes (or **facultative aerobes**) are organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions (no O_2) they grow by fermentation or anaerobic respiration, but in the presence of O_2 they switch to aerobic respiration.

Aerotolerant anaerobes are bacteria with an exclusively anaerobic (fermentative) type of metabolism but they are insensitive to the presence of O_2. They live by fermentation alone whether or not O_2 is present in their environment.

Table 6. Terms used to describe O_2 Relations of Microorganisms.

		Environment	
Group	**Aerobic**	**Anaerobic**	**O_2 Effect**
Obligate Aerobe	Growth	No growth	Required (utilized for aerobic respiration)
Microaerophile	Growth if level not too high	No growth	Required but at levels below 0.2 atm
Obligate Anaerobe	No growth	Growth Toxic	

Facultative Anaerobe (Facultative Aerobe)	Growth	Growth	Not required for growth but utilized when available
Aerotolerant Anaerobe	Growth	Growth	Not required and not utilized

The response of an organism to O_2 in its environment depends upon the occurrence and distribution of various enzymes which react with O_2 and various oxygen radicals that are invariably generated by cells in the presence of O_2. All cells contain enzymes capable of reacting with O_2. For example, oxidations of flavoproteins by O_2 invariably result in the formation of H_2O_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or $O_2^{\cdot -}$. Also, chlorophyll and other pigments in cells can react with O_2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.

In aerobes and aerotolerant anaerobes the potential for lethal accumulation of superoxide is prevented by the enzyme superoxide dismutase (Figure 1). All organisms which can live in the presence of O_2 (whether or not they utilize it in their metabolism) contain superoxide dismutase. Nearly all organisms contain the enzyme catalase, which decomposes H_2O_2. Even though certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they decompose H_2O_2 by means of peroxidase enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O. Obligate anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2. See Figure 2 below.

All photosynthetic (and some nonphotosynthetic) organisms are protected from lethal oxidations of singlet oxygen by their possession of carotenoid pigments which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state. Carotenoids are said to "quench" singlet oxygen radicals.

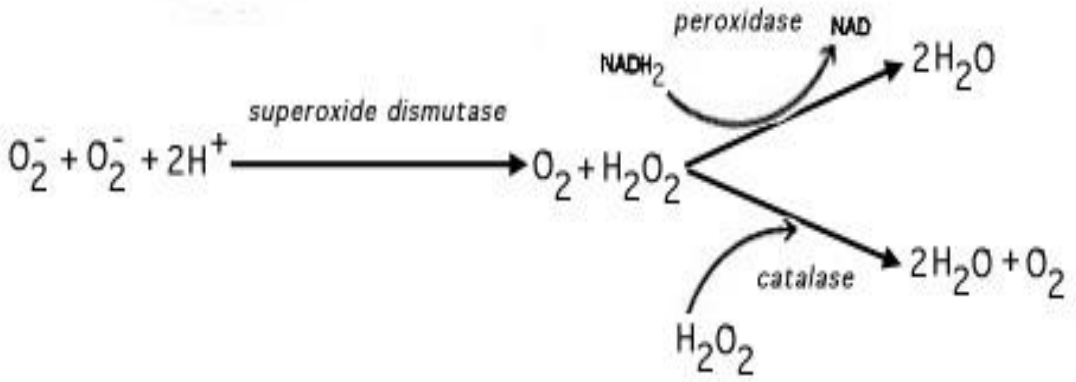


Figure 2. The action of superoxide dismutase, catalase and peroxidase. These enzymes detoxify oxygen radicals that are inevitably generated by living systems in the presence of O_2. The distribution of these enzymes in cells determines their ability to exist in the presence of O_2

Table 7. Distribution of superoxide dismutase, catalase and peroxidase in procaryotes with different O_2 tolerances.

Group	Superoxide dismutase	Catalase	Peroxidase
Obligate aerobes and most facultative anaerobes (e.g. Enterics)	+	+	-
Most aerotolerant anaerobes (e.g. Streptococci)	+	-	+
Obligate anaerobes (e.g. Clostridia, Methanogens, Bacteroides)	-	-	-

The Effect of pH on Growth

The pH, or hydrogen ion concentration, $[H^+]$, of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Appreciating that pH is measured on a logarithmic scale, the $[H^+]$ of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes! Most free-living procaryotes can grow over a range of 3 pH units, about a thousand fold change in $[H^+]$. The range of pH over which an organism grows is defined by **three cardinal points**: the **minimum pH**, below which the organism cannot grow, the **maximum pH**, above which the organism cannot grow, and the **optimum pH**, at which the organism

grows best. For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing [H^+] on the rates of enzymatic reaction (Figure 3).

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called **acidophiles**. Those which grow best at neutral pH are called **neutrophiles** and those that grow best under alkaline conditions are called **alkaliphiles**. Obligate acidophiles, such as some *Thiobacillus* species, actually require a low pH for growth since their membranes dissolve and the cells lyse at neutrality. Several genera of Archaea, including *Sulfolobus* and *Thermoplasma*, are obligate acidophiles. Among eukaryotes, many fungi are acidophiles, but the champion of growth at low pH is the eukaryotic alga *Cyanidium* which can grow at a pH of 0.

In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate **buffers** in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth. Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.

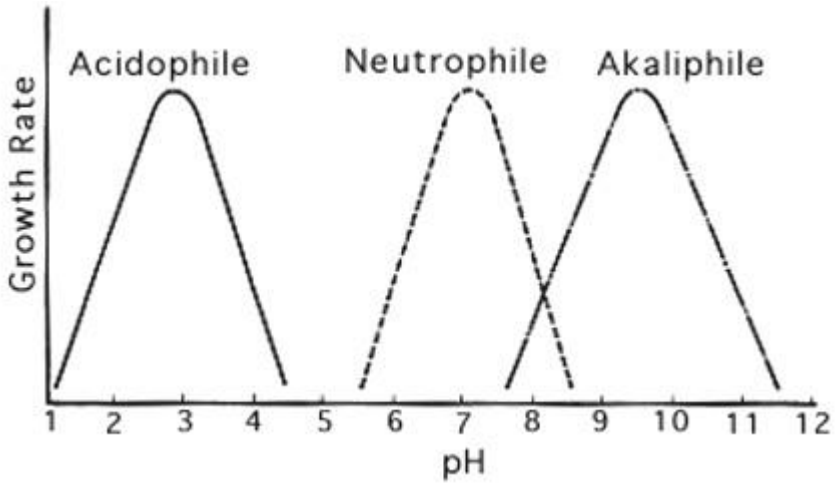


Figure 3. Growth rate vs pH for three environmental classes of procaryotes. Most free-living bacteria grow over a pH range of about three units. Note the symmetry of the curves below and above the optimum pH for growth.

Table 8. Minimum, maximum and optimum pH for growth of certain procaryotes.

Organism	Minimum pH	Optimum pH	Maximum pH
Thiobacillus thiooxidans	0.5	2.0-2.8	4.0-6.0
Sulfolobus acidocaldarius	1.0	2.0-3.0	5.0
Bacillus acidocaldarius	2.0	4.0	6.0
Zymomonas lindneri	3.5	5.5-6.0	7.5
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Thiobacillus novellus	5.7	7.0	9.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter sp	6.6	7.6-8.6	10.0

The Effect of Temperature on Growth

Microorganisms have been found growing in virtually all environments where there is liquid water, regardless of its temperature. In 1966, Professor Thomas D. Brock, then at Indiana University, made the amazing discovery in boiling hot springs of Yellowstone National Park that bacteria were not just surviving there, they were growing and flourishing.

Subsequently, procaryotes have been detected growing around black smokers and hydrothermal vents in the deep sea at temperatures at least as high as 120 degrees. Microorganisms have been found growing at very low temperatures as well. In supercooled solutions of H_2O as low as -20 degrees, certain organisms can extract water for growth, and many forms of life flourish in the icy waters of the Antarctic, as well as household refrigerators, near 0 degrees.

A particular microorganism will exhibit a range of temperature over which it can grow, defined by three cardinal points in the same manner as pH (Figure 5, cf. Figure 3). Considering the total span of temperature where liquid water exists, the procaryotes may be subdivided into several subclasses on the basis of one or another of their cardinal points for growth. For example, organisms with an optimum temperature near 37 degrees (the body temperature of warm-blooded animals) are called **mesophiles**. Organisms with an optimum T between about 45 degrees and 70 degrees are **thermophiles**. Some Archaea with an optimum T of 80 degrees or higher and a maximum T as high as 115 degrees, are now referred to as **extreme thermophiles** or **hyperthermophiles**. The cold-loving organisms are **psychrophiles** defined by their ability to grow at 0 degrees. A variant of a psychrophile (which usually has an optimum T of 10-15 degrees) is a **psychrotroph**, which grows at 0 degrees but displays an optimum T in the mesophile range, nearer room temperature. Psychrotrophs are the scourge of food storage in refrigerators since they are invariably brought in from their mesophilic habitats and continue to grow in the refrigerated environment where they spoil the food. Of course, they grow slower at 2 degrees than at 25 degrees. Think how fast milk spoils on the counter top versus in the refrigerator.

Psychrophilic bacteria are adapted to their cool environment by having largely unsaturated fatty acids in their plasma membranes. Some psychrophiles, particularly those from the Antarctic have been found to contain polyunsaturated fatty acids, which generally do not occur in procaryotes. The degree of unsaturation of a fatty acid correlates with its solidification T or thermal transition stage (i.e., the temperature at which the lipid melts or solidifies); unsaturated fatty acids remain liquid at low T but are also denatured at moderate T; saturated fatty acids, as in the membranes of thermophilic bacteria, are stable at high temperatures, but they also solidify at relatively high T. Thus, saturated fatty acids (like butter) are solid at room temperature while unsaturated fatty acids (like safflower oil) remain liquid in the refrigerator. Whether fatty acids in a membrane are in a liquid or a solid phase affects the fluidity of the membrane, which directly affects its ability to function. Psychrophiles also have enzymes that continue to function, albeit at a reduced rate,

at temperatures at or near 0 degrees. Usually, psychrophile proteins and/or membranes, which adapt them to low temperatures, do not function at the body temperatures of warm-blooded animals (37 degrees) so that they are unable to grow at even moderate temperatures.

Thermophiles are adapted to temperatures above 60 degrees in a variety of ways. Often thermophiles have a high G + C content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum T for growth. But this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means. The membrane fatty acids of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures. The membranes of hyperthermophiles, virtually all of which are Archaea, are not composed of fatty acids but of repeating subunits of the C5 compound, phytane, a branched, saturated, "isoprenoid" substance, which contributes heavily to the ability of these bacteria to live in superheated environments. The structural proteins (e.g. ribosomal proteins, transport proteins (permeases) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts. The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure, which accounts for their thermal stability.

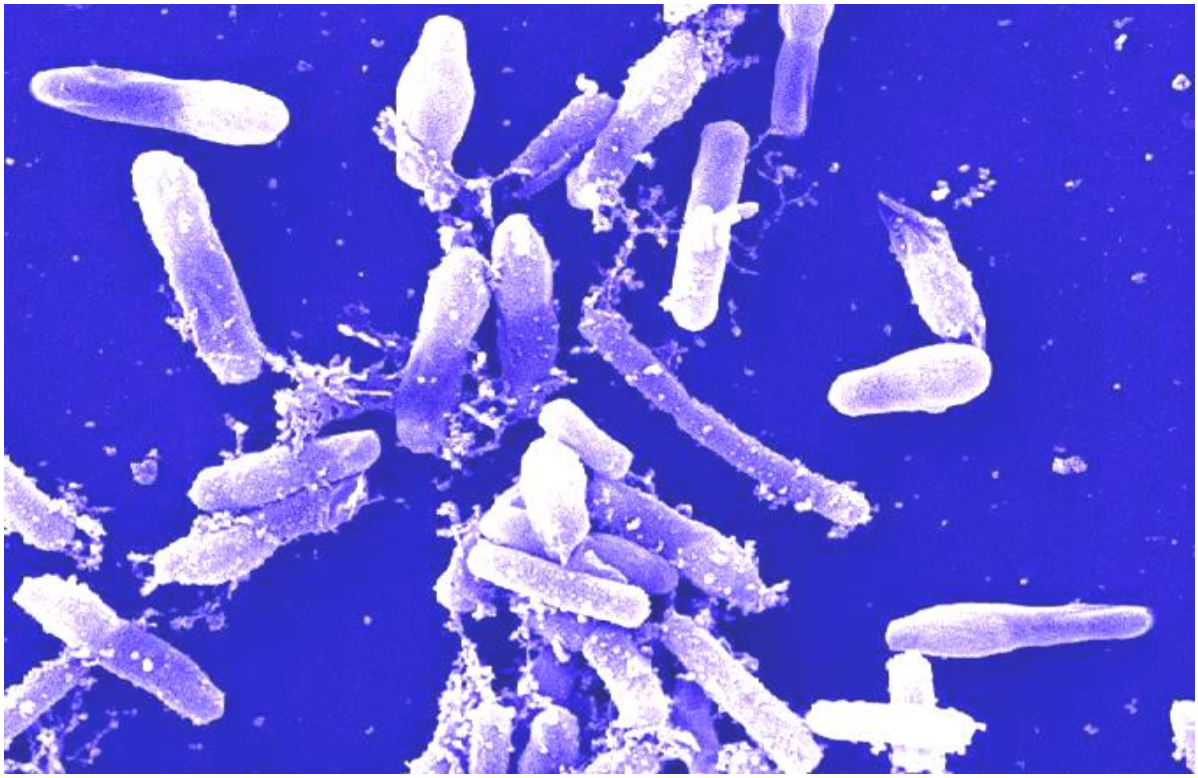


Figure 4. SEM of a thermophilic *Bacillus* species isolated from a compost pile at 55° C. The rods are 3-5 microns in length and 0.5 to 1 micron in width with terminal endospores in a slightly-swollen sporangium.

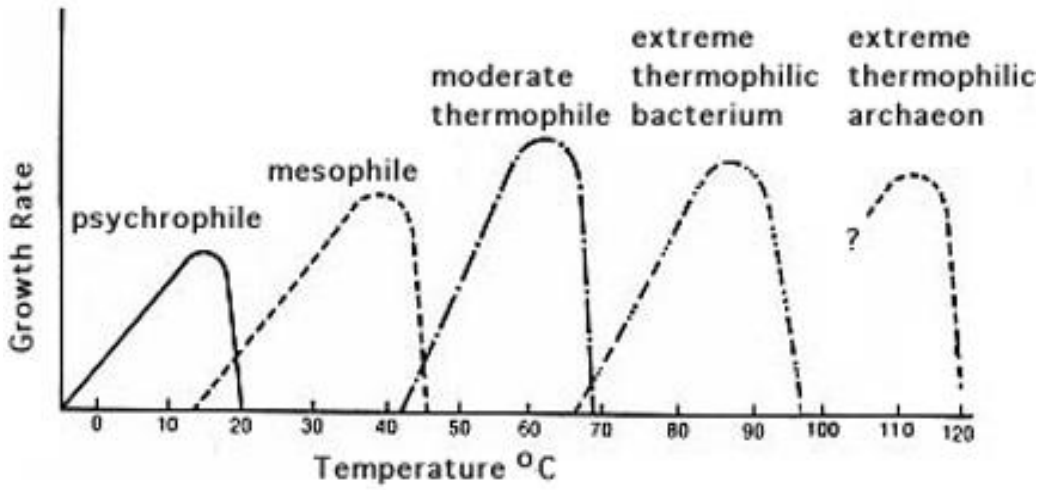


Figure 5. Growth rate vs temperature for five environmental classes of procaryotes. Most procaryotes will grow over a temperature range of about 30 degrees. The curves exhibit three cardinal points: minimum, optimum and maximum temperatures for growth. There is a steady increase in growth rate between the minimum and optimum temperatures, but slightly past the optimum a critical thermolabile cellular event occurs, and the growth rates plunge rapidly as the maximum T is approached. As expected and as predicted by

T.D. Brock, life on earth, with regard to temperature, exists wherever water remains in a liquid state. Thus, psychrophiles grow in solution wherever water is supercooled below 0 degrees; and extreme thermophilic archaea (hyperthermophiles) have been identified growing near deep-sea thermal vents at temperatures up to 120 degrees. Theoretically, the bar can be pushed to even higher temperatures.

Table 9. Terms used to describe microorganisms in relation to temperature requirements for growth.

Temperature for growth (degrees C)

Group	Minimum	Optimum	Maximum	Comments
Psychrophile	Below 0	10-15	Below 20	Grow best at relatively low T
Psychrotroph	0	15-30	Above 25	Able to grow at low T but prefer moderate T
Mesophile	10-15	30-40	Below 45	Most bacteria esp. those living in association with warm-blooded animals
Thermophile*	45	50-85	Above 100 (boiling)	Among all thermophiles is wide variation in optimum and maximum T

*For "degrees" of thermophily see text and graphs above

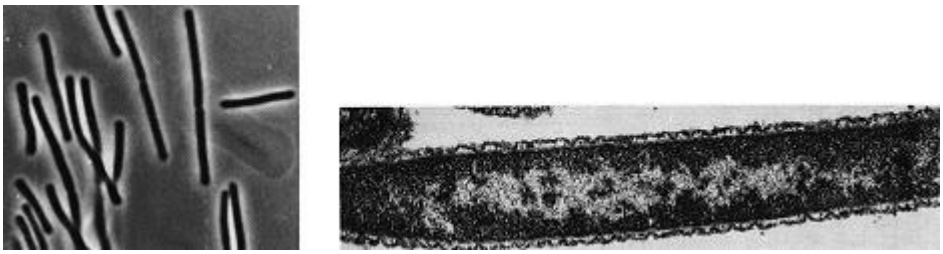


Figure 6. *Thermus aquaticus,* the thermophilic bacterium that is the source of taq polymerase. L wet mount; R electron micrograph.

Table 10. Minimum, maximum and optimum temperature for growth of certain bacteria and archaea.

Temperature for growth (degrees C)

Bacterium	Minimum	Optimum	Maximum
Listeria monocytogenes	1	30-37	45
Vibrio marinus	4	15	30
Pseudomonas maltophilia	4	35	41
Thiobacillus novellus	5	25-30	42
Staphylococcus aureus	10	30-37	45
Escherichia coli	10	37	45
Clostridium kluyveri	19	35	37
Streptococcus pyogenes	20	37	40
Streptococcus pneumoniae	25	37	42
Bacillus flavothermus	30	60	72
Thermus aquaticus	40	70-72	79
Methanococcus jannaschii	60	85	90
Sulfolobus acidocaldarius	70	75-85	90
Pyrobacterium brockii	80	102-105	115

Water Availability

Water is the solvent in which the molecules of life are dissolved, and the availability of water is therefore a critical factor that affects the growth of all cells. The availability of water for a cell depends upon its presence in the atmosphere (relative humidity) or its presence in solution or a substance (**water activity**). The water activity (A_w) of pure H_2O is 1.0 (100% water). Water activity is affected by the presence of solutes such as salts or sugars, that are dissolved in the water. The higher the solute concentration of a substance, the lower is the water activity and vice-versa. Microorganisms live over a range of A_w from 1.0 to 0.7. The A_w of human blood is 0.99; seawater = 0.98; maple syrup = 0.90; Great Salt Lake = 0.75. Water activities in agricultural soils range between 0.9 and 1.0.

The only common solute in nature that occurs over a wide concentration range is salt [NaCl], and some microorganisms are named based on their growth response to salt. Microorganisms that require some NaCl for growth are **halophiles**. **Mild halophiles** require 1-6% salt, **moderate halophiles** require 6-15% salt; **extreme halophiles** that require 15-30% NaCl for growth are found among the archaea. Bacteria that are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl, are called **halotolerant**. Although halophiles are "osmophiles" (and halotolerant organisms are "osmotolerant") the term **osmophiles** is usually reserved for organisms that are able to live in environments high in sugar. Organisms which live in dry environments (made dry by lack of water) are called **xerophiles**.

The concept of lowering water activity in order to prevent bacterial growth is the basis for preservation of foods by drying (in sunlight or by evaporation) or by addition of high concentrations of salt or sugar.

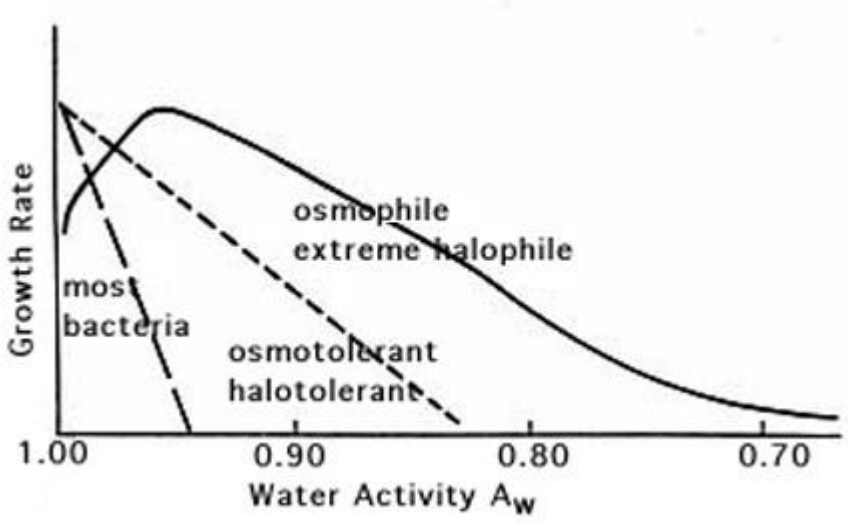


Figure 7. Growth rate vs osmolarity for different classes of procaryotes. Osmolarity is determined by solute concentration in the environment. Osmolarity is inversely related to water activity (A_w), which is more like a measure of the concentration of water (H_2O) in a solution. Increased solute concentration means increased osmolarity and decreased A_w. TFrom left to right the graph shows the growth rate of a normal (nonhalophile) such as *E. coli* or *Pseudomonas,* the growth rate of a halotolerant bacterium such as *Staphylococcus aureus,* and the growth rate of an extreme halophile such as the archaean *Halococcus.*Note that a true halophile grows best at salt concentrations where most bacteria are inhibited.

GROWTH OF BACTERIAL POPULATIONS

Measurement of Bacterial Growth

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called **binary fission**.

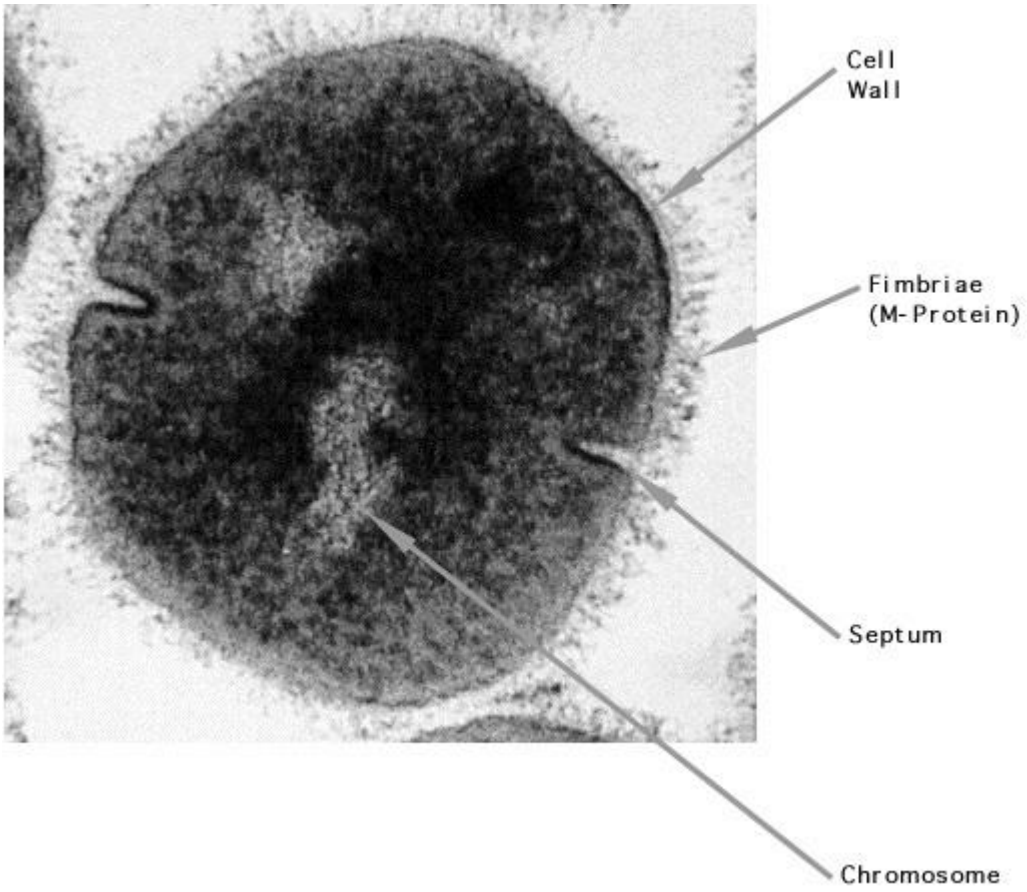


Figure 1. Bacterial growth by binary fission. Most bacteria reproduce by a relatively simple asexual process called binary fission: each cell increases in size and divides into two cells. During this process there is an orderly increase in cellular structures and components, replication and segregation of the bacterial DNA, and formation of a septum or cross wall which divides the cell into two progeny cells. The process is coordinated by the bacterial membrane perhaps by means of mesosomes. The DNA molecule is believed to be attached to a point on

the membrane where it is replicated. The two DNA molecules remain attached at points side-by-side on the membrane while new membrane material is synthesized between the two points. This draws the DNA molecules in opposite directions while new cell wall and membrane are laid down as a septum between the two chromosomal compartments. When septum formation is complete the cell splits into two progeny cells. The time interval required for a bacterial cell to divide or for a population of bacterial cells to double is called the generation time. Generation times for bacterial species growing in nature may be as short as 15 minutes or as long as several days. Electron micrograph of *Streptococcus pyogenes*.

For unicellular organisms such as the bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**.

Methods for Measurement of Cell Mass

Methods for measurement of the cell mass involve both direct and indirect techniques.

1. Direct **physical measurement** of dry weight, wet weight, or volume of cells after centrifugation.

2. Direct **chemical measurement** of some chemical component of the cells such as total N, total protein, or total DNA content.

3. Indirect **measurement of chemical activity** such as rate of O_2 production or consumption, CO_2 production or consumption, etc.

4. **Turbidity measurements** employ a variety of instruments to determine the amount of light scattered by a suspension of cells. Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity or **optical density** of a suspension of cells is directly related to cell mass or cell number, after construction and calibration of a standard curve. The method is simple and nondestructive, but the sensitivity is limited to about 10^7 cells per ml for most bacteria.

Methods for Measurement of Cell Numbers

Measuring techniques involve direct counts, visually or instrumentally, and indirect viable cell counts.

1. **Direct microscopic counts** are possible using special slides known as counting chambers. Dead cells cannot be distinguished from living ones. Only dense suspensions can be counted (>10^7 cells per ml), but samples can be concentrated by centrifugation or filtration to increase sensitivity.

A variation of the direct microscopic count has been used to observe and measure growth of bacteria in natural environments. In order to detect and prove that thermophilic bacteria were growing in boiling hot springs, T.D. Brock immersed microscope slides in the springs and withdrew them periodically for microscopic observation. The bacteria in the boiling water attached to the glass slides naturally and grew as microcolonies on the surface.

2. **Electronic counting chambers** count numbers and measure size distribution of cells. For cells the size of bacteria the suspening medium must be very clean. Such elecronic devices are more often used to count eukaryotic cells such as blood cells.

3. **Indirect viable cell counts**, also called **plate counts**, involve plating out (spreading) a sample of a culture on a nutrient agar surface. The sample or cell suspension can be diluted in a nontoxic diluent (e.g. water or saline) before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a **colony forming unit (cfu)** and the number of cfu's is related to the viable number of bacteria in the sample.

Advantages of the technique are its sensitivity (theoretically, a single cell can be detected), and it allows for inspection and positive identification of the organism counted. Disadvantages are (1) only living cells develop colonies that are counted; (2) clumps or chains of cells develop into a single colony; (3) colonies develop only from those organisms for which the cultural conditions are suitable for growth. The latter makes the technique virtually useless to characterize or count the **total**

number of bacteria in complex microbial ecosystems such as soil or the animal rumen or gastrointestinal tract. Genetic probes can be used to demonstrate the diversity and relative abundance of procaryotes in such an environment, but many species identified by genetic techniques have so far proven unculturable.

Table 1. Some Methods used to measure bacterial growth

Method	Application	Comments
Direct microscopic count	Enumeration of bacteria in milk or cellular vaccines	Cannot distinguish living from nonliving cells
Viable cell count (colony counts)	Enumeration of bacteria in milk, foods, soil, water, laboratory cultures, etc.	Very sensitive if plating conditions are optimal
Turbidity measurement	Estimations of large numbers of bacteria in clear liquid media and broths	Fast and nondestructive, but cannot detect cell densities less than 10^7 cells per ml
Measurement of total N or protein	Measurement of total cell yield from very dense cultures	only practical application is in the research laboratory
Measurement of Biochemical activity e.g. O2 uptake CO2 production, ATP production, etc.	Microbiological assays	Requires a fixed standard to relate chemical activity to cell mass and/or cell numbers
Measurement of dry weight or wet weight of cells or volume of cells after centrifugation	Measurement of total cell yield in cultures	probably more sensitive than total N or total protein measurements

The Bacterial Growth Curve

In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations). This is called **exponential growth**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in Nature.

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a **typical bacterial growth curve** (Figure 2).

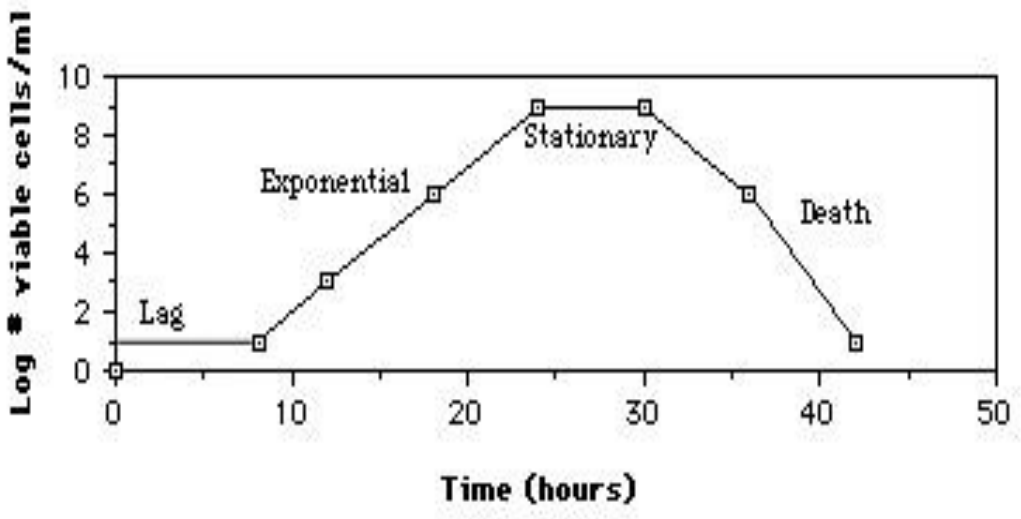


Figure 2. The typical bacterial growth curve. When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.

Four characteristic phases of the growth cycle are recognized.

1. **Lag Phase**. Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.

The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physiacal damage or shock in the transfer; time required for synthesis of essential coenzymes or

division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

2. **Exponential (log) Phase**. The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time (below) derive.

3. **Stationary Phase**. Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of three factors: 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space".

During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce **secondary metabolites**, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It si during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

4. **Death Phase**. If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. (Note, if counting by turbidimetric measurements or microoscopic counts, the death phase cannot be observed.). During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

Growth Rate and Generation Time

As mentioned above, bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours or more. The generation time for *E. coli* in the laboratory is 15-20 minutes, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 hours. For most known bacteria that can be cultured, generation times range from about 15 minutes to 1 hour. Symbionts such as *Rhizobium* tend to have longer generation times. Many lithotrophs, such as the nitrifying bacteria, also have long generation times. Some bacteria that are pathogens, such as *Mycobacterium tuberculosis* and *Treponema pallidum*, have especially long generation times, and this is thought to be an advantage in their virulence. Generation times for a few bacteria are are shown in Table 2.

Table 2. Generation times for some common bacteria under optimal conditions of growth.

Bacterium	Medium	Generation Time (minutes)
Escherichia coli	Glucose-salts	17
Bacillus megaterium	Sucrose-salts	25
Streptococcus lactis	Milk	26
Streptococcus lactis	Lactose broth	48
Staphylococcus aureus	Heart infusion broth	27-30
Lactobacillus acidophilus	Milk	66-87
Rhizobium japonicum	Mannitol-salts-yeast extract	344-461
Mycobacterium tuberculosis	Synthetic	792-932
Treponema pallidum	Rabbit testes	1980

Continuous Culture of Bacteria

The cultures so far discussed for growth of bacterial populations are called **batch cultures**. Since the nutrients are not renewed, exponential growth is limited to a few generations. Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture** (Figure 3), designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a **chemostat**, can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments.

In a chemostat, the growth chamber is connected to a reservoir of sterile medium. Once growth is initiated, fresh medium is continuously supplied from the reservoir. The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacteria grow (cells are formed) at the same rate that bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients.

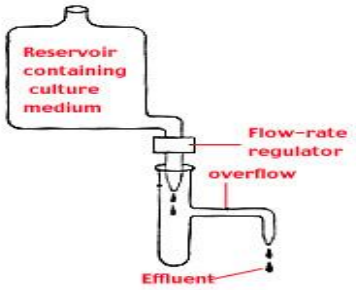


Figure 3. Schematic diagram of a chemostat, a device for the continuous culture of bacteria. The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.

Synchronous Growth of Bacteria

Studying the growth of bacterial populations in batch or continuous cultures does not permit any conclusions about the growth behavior of individual cells, because the distribution of cell size (and hence cell age) among the members of the population is completely random. Information about the growth behavior of individual bacteria can, however, be obtained by the study of **synchronous cultures**. Synchronized cultures must be composed of cells which are all at the same stage of the **bacterial cell cycle**. Measurements made on synchronized cultures are equivalent to measurements made on individual cells.

A number of clever techniques have been devised to obtain bacterial populations at the same stage in the cell cycle. Some techniques involve manipulation of environmental parameters which induces the population to start or stop growth at the same point in the cell cycle, while others are physical methods for selection of cells that have just completed the process of binary fission. Theoretically, the smallest cells in a bacterial population are those that have just completed the process of cell division. Synchronous growth of a population of bacterial cells is illustrated in Figure 5. Synchronous cultures rapidly lose synchrony because not all cells in the population divide at exactly the same size, age or time.

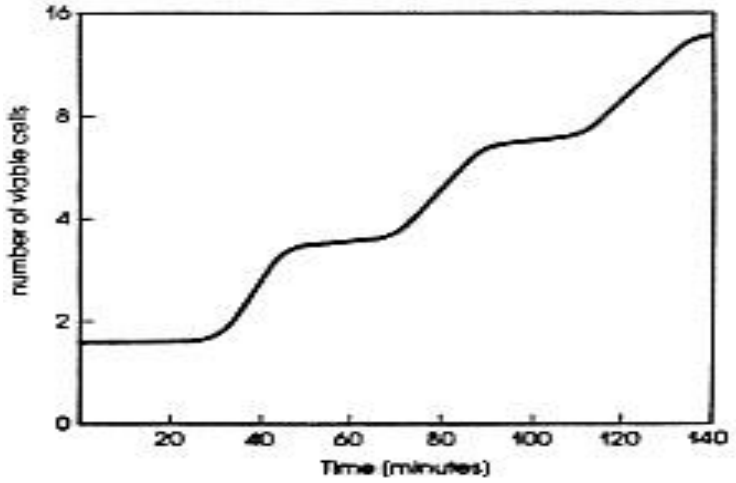


Figure 4. The synchronous growth of a bacterial population. By careful selection of cells that have just divided, a bacterial population can be synchronized in the bacterial cell division cycle. Synchrony can be maintained for only a few generations.

THE CONTROL OF MICROBIAL GROWTH

Introduction

The control of microbial growth is necessary in many practical situations, and significant advances in agriculture, medicine, and food science have been made through study of this area of microbiology.

"Control of growth", as used here, means to prevent growth of microorganisms. This control is affected in two basic ways: (1) by killing microorganisms or (2) by inhibiting the growth of microorganisms. Control of growth usually involves the use of physical or chemical agents which either kill or prevent the growth of microorganisms. Agents which kill cells are called **cidal** agents; agents which inhibit the growth of cells (without killing them) are referred to as **static** agents. Thus the term **bactericidal** refers to killing bacteria and **bacteriostatic** refers to inhibiting the growth of bacterial cells. A **bactericide** kills bacteria, a **fungicide** kills fungi, and so on.

Sterilization is the complete destruction or elimination of all viable organisms (in or on an object being sterilized). There are no degrees of sterilization: an object is

either sterile or not. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

Methods of Sterilization

Heat: most important and widely used. For sterilization always consider type of heat, time of application and temperature to ensure destruction of all microorganisms. Endospores of bacteria are considered the most thermoduric of all cells so their destruction guarantees sterility.

Incineration: burns organisms and physically destroys them. Used for needles , inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

Boiling: 100^{o} for 30 minutes. Kills everything except some endospores (Actually, for the purposes of purifying drinking water 100^{o} for five minutes is probably adequate though there have been some reports that Giardia cysts can survive this process). To kill endospores, and therefore **sterilize** the solution, very long or **intermittent boiling** is required.

Autoclaving (steam under pressure or pressure cooker): 121^{o} for 15 minutes (15#/in^{2} pressure). Good for sterilizing almost anything, but heat-labile substances will be denatured or destroyed.

Dry heat (hot air oven): 160^{o}/2hours or 170^{o}/1hour. Used for glassware, metal, and objects that won't melt.

The protocol and recommendations for the use of heat to control microbial growth are given in Table 1.

Table 1. Recommended use of heat to control bacterial growth

Treatment	Temperature	Effectiveness
Incineration	>500^{o}	Vaporizes organic material on nonflammable surfaces but may destroy many substances in the process

Boiling	100°	30 minutes of boiling kills microbial pathogens and vegetative forms of bacteria but may not kill bacterial endospores
Intermittent boiling	100°	Three 30-minute intervals of boiling, followed by periods of cooling kills bacterial endospores
Autoclave and pressure cooker (steam under pressure)	121°/15 minutes at 15# pressure	kills all forms of life including bacterial endospores. The substance being sterilized must be maintained at the effective T for the full time
Dry heat (hot air oven)	160°/2 hours	For materials that must remain dry and which are not destroyed at T between 121° and 170° Good for glassware, metal, not plastic or rubber items
Dry heat (hot air oven)	170°/1 hour	Same as above. Note increasing T by 10 degrees shortens the sterilizing time by 50 percent
Pasteurization (batch method)	63°/30 minutes	kills most vegetative bacterial cells including pathogens such as streptococci, staphylococci and Mycobacterium tuberculosis
Pasteurization (flash method)	72°/15 seconds	Effect on bacterial cells similar to batch method; for milk, this method is more conducive to industry and has fewer undesirable effects on quality or taste

Irradiation: usually destroys or distorts nucleic acids. Ultraviolet light is usually used (commonly used to sterilize the surfaces of objects), although x-rays and microwaves are possibly useful.

Filtration: involvres the physical removal (exclusion) of all cells in a liquid or gas, especially important to sterilize solutions which would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.)

Chemical and gas: (formaldehyde, glutaraldehyde, ethylene oxide) toxic chemicals kill all forms of life in a specialized gas chamber.

Control of Microbial Growth by Physical Agents

Applications of Heat The lethal **temperature** varies in microorganisms. The **time** required to kill depends on the number of organisms, species, nature of the product

being heated, pH, and temperature. Whenever heat is used to control microbial growth inevitably **both time and temperature are considered**.

Sterilization (boiling, autoclaving, hot air oven) kills all microorganisms with heat; commonly employed in canning, bottling, and other sterile packaging procedures.

Pasteurization is the use of mild heat to reduce the number of microorganisms in a product or food. In the case of pasteurization of milk the time and temperature depend on killing potential pathogens that are transmitted in milk, i.e., staphylococci, streptococci, Brucella abortus and Mycobacterium tuberculosis. For pasteurzation of milk: batch nethod: 63°/30minutes; flash method: 71°/15 seconds.

Low temperature (refrigeration and freezing): Most organisms grow very little or not at all at 0o. Store perishable foods at low temperatues to slow rate of growth and consequent spoilage (e.g. milk). Low temperatures are not bactericidal. Psychrotrophs, rather than true psychrophiles, are the usual cause of food spoilage in refrigerated foods.

Drying (removal of H_2O): Most microorganisms cannot grow at reduced water activity (Aw < 0.90). Often used to preserve foods (e.g. fruits, grains, etc.). Methods involve removal of water from product by heat, evaporation, freeze-drying, addition of salt or sugar.

Irradiation (microwave, UV, x-ray): destroys microorganisms as described under "sterilization". Many spoilage organisms are easily killed by irradiation. In some parts of Europe, fruits and vegetables are irradiated to increase their shelf life up to 500 percent. The practice has not been accepted in the U.S.

Control of microbial growth by chemical agents

Antimicrobial agents are chemicals that kill or inhibit the growth microorganisms. Antimicrobial agents include chemical preservatives and antiseptics, as well as drugs used in the treatment of infectious diseases of plants and animals. Antimicrobial agents may be of natural or synthetic origin, and they may have a static or cidal effect on microorganisms.

Types of antimicrobial agents

Antiseptics: microbicidal agents harmless enough to be applied to the skin and mucous membrane; should not be taken internaslly. Examples: mercurials, silver nitrate, iodine solution, alcohols, detergents.

Disinfectants: Agents that kill microorganisms, but not necessarily their spores,not safe for application to living tissues; they are used on inanimate objects such as tables, floors, utensils, etc. Examples: chlorine, hypochlorites, chlorine compounds, lye, copper sulfate, quaternary ammonium compounds.

Note: disinfectants and antiseptics are distinguished on the basis of whether they are safe for application to mucous membranes. Often, safety depends on the concentration of the compound. For example, sodium hypochlorite (chlorine), as added to water is safe for drinking, but "chlorox" (5% hypochlorite), an excellent disinfectant, is hardly safe to drink.

Common antiseptics and disinfectants and their uses are summarized in Table 2.

Table 2. Common antiseptics and disinfectants

Chemical	Action	Uses
Ethanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Isopropanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH_2, SH and COOH groups	Disinfectant, kills endospores
Tincture of Iodine (2% I2 in 70% alcohol)	Inactivates proteins	Antiseptic used on skin
Chlorine (Cl_2) gas	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	Precipitates proteins	General antiseptic and used in the eyes of newborns

Mercuric chloride	Inactivates proteins by reacting with sulfide groups	Disinfectant, although occasionally used as an antiseptic on skin
Detergents (e.g. quaternary ammonium compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carboloic acid, lysol, hexylresorcinol, hexachlorophene)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations
Ethylene oxide gas	Alkylating agent	Disinfectant used to sterilize heat-sensitive objects such as rubber and plastics

Preservatives: static agents used to inhibit the growth of microorganisms, most often in foods. If eaten they should be nontoxic. Examples; calcium propionate, sodium benzoate, formaldehyde, nitrate, sulfur dioxide. Table 3 is a list of common preservative and their uses.

Table 3. Common food preservatives and their uses

Preservative	Effective Concentration	Uses
Propionic acid and propionates	0.32%	Antifungal agent in breads, cake, Swiss cheeses
Sorbic acid and sorbates	0.2%	Antifungal agent in cheeses, jellies, syrups, cakes
Benzoic acid and benzoates	0.1%	Antifungal agent in margarine, cider, relishes, soft drinks
Sodium diacetate	0.32%	Antifungal agent in breads
Lactic acid	unknown	Antimicrobial agent in cheeses, buttermilk, yogurt and pickled foods
Sulfur dioxide, sulfites	200-300 ppm	Antimicrobial agent in dried fruits, grapes, molasses
Sodium nitrite	200 ppm	Antibacterial agent in cured meats, fish
Sodium chloride	unknown	Prevents microbial spoilage of meats, fish, etc.
Sugar	unknown	Prevents microbial spoilage of preserves, jams,

		syrups, jellies, etc.
Wood smoke	unknown	Prevents microbial spoilage of meats, fish, etc.

Chemotherapeutic agents: antimicrobial agents of synthetic origin useful in the treatment of microbial or viral disease. Examples: sulfonilamides, isoniazid, ethambutol, AZT, chloramphenicol. Note that the microbiologist's definition of a chemotherapeutic agent requires that the agent be used for antimicrobial purposes and so excludes synthetic agents used for therapy against diseases that are not of microbial origin.

Antibiotics: antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms. This is the microbiologist's definition. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell) which has the effect to kill or inhibit the growth of other types cells. Since most clinically-useful antibiotics are produced by microorganisms and are used to kill or inhibit infectious Bacteria, we will follow the classic definition.

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Most of these microorganisms form some type of a spore or other dormant cell, and there is thought to be some relationship (besides temporal) between antibiotic production and the processes of sporulation. Among the molds, the notable antibiotic producers are Penicillium and Cephalosporium , which are the main source of the beta-lactam antibiotics (penicillin and its relatives). In the Bacteria, the Actinomycetes, notably Streptomyces species, produce a variety of types of antibiotics including the aminoglycosides (e.g. streptomycin), macrolides (e.g. erythromycin), and the tetracyclines. Endospore-forming Bacillus species produce polypeptide antibiotics such as polymyxin and bacitracin. The table below (Table 4) is a summary of the classes of antibiotics and their properties including their biological sources.

Table 4. Classes of antibiotics and their properties

Chemical class	Examples	Biological	Spectrum	Mode of action

		source	(effective against)	
Beta-lactams (penicillins and cephalosporins)	Penicillin G, Cephalothin	Penicillium notatum and Cephalosporium species	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Semisynthetic penicillin	Ampicillin, Amoxycillin		Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Clavulanic Acid	Clavamox is clavulanic acid plus amoxycillin	Streptomyces clavuligerus	Gram-positive and Gram-negative bacteria	Suicide inhibitor of beta-lactamases
Monobactams	Aztreonam	Chromobacter violaceum	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Carboxypenems	Imipenem	Streptomyces cattleya	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Aminoglycosides	Streptomycin	Streptomyces griseus	Gram-positive and Gram-negative bacteria	Inhibit translation (protein synthesis)
	Gentamicin	Micromonospora species	Gram-positive and Gram-negative bacteria esp. Pseudomonas	Inhibit translation (protein synthesis)
Glycopeptides	Vancomycin	Streptomyces orientales	Gram-positive bacteria, esp. Staphylococcus aureus	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly

Lincomycins	Clindamycin	Streptomyces lincolnensis	Gram-positive and Gram-negative bacteria esp. anaerobic Bacteroides	Inhibits translation (protein synthesis)
Macrolides	Erythromycin	Streptomyces erythreus	Gram-positive bacteria, Gram-negative bacteria not enterics, Neisseria, Legionella, Mycoplasma	Inhibits translation (protein synthesis)
Polypeptides	Polymyxin	Bacillus polymyxa	Gram-negative bacteria	Damages cytoplasmic membranes
	Bacitracin	Bacillus subtilis	Gram-positive bacteria	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Polyenes	Amphotericin	Streptomyces nodosus	Fungi	Inactivate membranes containing sterols
	Nystatin	Streptomyces noursei	Fungi (Candida)	Inactivate membranes containing sterols
Rifamycins	Rifampicin	Streptomyces mediterranei	Gram-positive and Gram-negative bacteria, Mycobacterium tuberculosis	Inhibits transcription (eubacterial RNA polymerase)
Tetracyclines	Tetracycline	Streptomyces species	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)

			Ehrlichia, Borellia	
Chloramphenicol	Chloramphenicol	Streptomyces venezuelae	Gram-positive and Gram-negative bacteria	Inhibits translation (protein synthesis)

Antimicrobial Agents Used in the Treatment of Infectious Disease

The modern era of antimicrobial chemotherapy began in 1929 with Fleming's discovery of the powerful bactericidal substance penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity. In the early 1940's, spurred partially by the need for antibacterial agents in WW II, penicillin was isolated, purified and injected into experimental animals, where it was found to not only cure infections but also to possess incredibly low toxicity for the animals. This fact ushered into being the age of antibiotic chemotherapy and an intense search for similar antimicrobial agents of low toxicity to animals that might prove useful in the treatment of infectious disease. The rapid isolation of streptomycin, chloramphenicol and tetracycline soon followed, and by the 1950's, these and several other antibiotics were in clinical usage.

The most important property of a clinically-useful antimicrobial agent, especially from the patient's point of view, is its **selective toxicity**, i.e., that the agent acts in some way that inhibits or kills bacterial pathogens but has little or no toxic effect on the animal taking the drug This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic are is expressed as its **spectrum of action**. Antibiotics effective against procaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be **broad spectrum** . If effective mainly against Gram-positive or Gram-negative bacteria, they are **narrow spectrum** . If effective against a single organism or disease, they are referred to as **limited spectrum**.

Kinds of Antimicrobial Agents and their Primary Modes of Action

1. Cell wall synthesis inhibitors Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan. Generally they exert their selective toxicity against eubacteria because human cells lack cell walls.

Beta lactam antibiotics Chemically, these antibiotics contain a 4-membered beta lactam ring. They are the products of two groups of fungi, Penicillium and Cephalosporium molds, and are correspondingly represented by the penicillins and cephalosporins. The beta lactam antibiotics inhibit the last step in peptidoglycan synthesis, the final cross-linking between between peptide side chains, mediated by bacterial carboxypeptidase and transpeptidase enzymes . Beta lactam antibiotics are normally bactericidal and require that cells be actively growing in order to exert their toxicity.

Natural penicillins, such as **Penicillin G** or **Penicillin V**, are produced by fermentation of Penicillium chrysogenum. They are effective against streptococcus, gonococcus and staphylococcus, except where resistance has developed. They are considered narrow spectrum since they are not effective against Gram-negative rods.

Semisynthetic penicillins first appeared in 1959. A mold produces the main part oif the molecule (6-aminopenicillanic acid) which can be modified chemically by the addition of side shains. Many of these compounds have been developed to have distinct benefits or advantages over penicillin G, such as increased spectrum of activity (effectiveness against Gram-negative rods), resistance to penicillinase, effectiveness when administered orally, etc. **Amoxycillin** and **Ampicillin** have broadened spectra against Gram-negatives and are effective orally; **Methicillin** is penicillinase-resistant.

Clavulanic acid is a chemical sometimes added to a semisynthetic penicillin preparation. Thus, **amoxycillin** plus **clavulanate is clavamox** or **augmentin**. The clavulanate is not an antimicrobial agent. It inhibits beta lactamase enzymes and has given extended life to penicillinase-sensitive beta lactams.

Although nontoxic, penicillins occasionally cause death when administered to persons who are allergic to them. In the U.S. there are 300 - 500 deaths annually due to penicillin allergy. In allergic individuals the beta lactam molecule attaches to a serum protein which initiates an IgE-mediated inflammatory response.

Cephalolsporins are beta lactam antibiotics with a similar mode of action to penicillins that are produced by species of Cephalosporium. The have a low toxicity and a somewhat broader spectrum than natural penicillins. They are often used as penicillin substitutes, against Gram-negative bacteria, and in surgical prophylaxis. They are subject to degradation by some bacterial beta-lactamases, but they tend to be resistant to beta-lactamases from S. aureus.

Bacitracin is a polypeptide antibiotic produced by *Bacillus* species. It prevents cell wall growth by inhibiting the release of the muropeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane Teichoic acid synthesis, which requires the same carrier, is also inhibited. Bacitracin has a high toxicity which precludes its systemic use. It is present in many topical antibiotic preparations, and since it is not absorbed by the gut, it is given to "sterilize" the bowel prior to surgery.

2. Cell membrane inhibitors disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital to bacteria, and compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in eubacterial and eukaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically. The only antibacterial antibiotic of clinical importance that acts by this mechanism is **Polymyxin**, produced by Bacillus polymyxis. Polymyxin is effective mainly against Gram-negative bacteria and is usually limited to topical usage. Polymyxins bind to membrane phospholipids and thereby interfere with membrane function. Polymyxin is occasionally given for urinary tract infections caused by Pseudomonas that are gentamicin, carbenicillin and tobramycin resistant. The balance between effectiveness and damage to the kidney and other organs is

dangerously close, and the drug should only be given under close supervision in the hospital.

3. Protein synthesis inhibitors Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of translation. Their attack is always at one of the events occurring on the ribosome and rather than the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 70S (as opposed to 80S) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotics with this mode of action are the **tetracyclines**, **chloramphenicol**, the **macrolides** (e.g. erythromycin) and the aminoglycosides (e.g. streptomycin).

The **aminoglycosides** are products of *Streptomyces* species and are represented by streptomycin, kanamycin, tobramycin and gentamicin. These antibiotics exert their activity by binding to bacterial ribosomes and preventing the initiation of protein synthesis. Aminoglycosides have been used against a wide variety of bacterial infections caused by Gram-positive and Gram-negative bacteria. **Streptomycin** has been used extensively as a primary drug in the treatment of tuberculosis. **Gentamicin** is active against many strains of Gram-positive and Gram-negative bacteria, including some strains of Pseudomonas aeruginosa. **Kanamycin** (a complex of three antibiotics, A, B and C) is active at low concentrations against many Gram-positive bacteria, including penicillin-resistant staphylococci. Gentamicin and **Tobramycin** are mainstays for treatment of Pseudomonas infections. An unfortunate side effect of aminoglycosides has tended to restrict their usage: prolonged use is known to impair kidney function and cause damage to the auditory nerves leading to deafness.

The **tetracyclines** consist of eight related antibiotics which are all natural products of *Streptomyces*, although some can now be produced semisynthetically. **Tetracycline**, **chlortetracycline** and **doxycycline** are the best known. The tetracyclines are broad-spectrum antibiotics with a wide range of activity against both Gram-positive and Gram-negative bacteria. The tetracyclines act by blocking the binding of aminoacyl tRNA to the A site on the ribosome. Tetracyclines inhibit protein

synthesis on isolated 70S or 80S (eukaryotic) ribosomes, and in both cases, their effect is on the small ribosomal subunit. However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline which is harmless to animal tissues can halt protein synthesis in invading bacteria.

The tetracyclines have a remarkably low toxicity and minimal side effects when taken by animals. The combination of their broad spectrum and low toxicity has led to their overuse and misuse by the medical community and the wide-spread development of resistance has reduced their effectiveness. Nonetheless, tetracyclines still have some important uses, such as in the treatment of Lyme disease.

Chloramphenicol has a broad spectrum of activity but it exerts a bacteriostatic effect. It is effective against intracellular parasites such as the rickettsiae. Unfortunately, aplastic anemia, which is dose related develops in a small proportion (1/50,000) of patients. Chloramphenicol was originally discovered and purified from the fermentation of a Streptomyces, but currently it is produced entirely by chemical synthesis. Chloramphenicol inhibits the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis.

Chloramphenicol is entirely selective for 70S ribosomes and does not affect 80S ribosomes. Its unfortunate toxicity towards the small proportion of patients who receive it is in no way related to its effect on bacterial protein synthesis. However, since mitochondria probably originated from procaryotic cells and have 70S ribosomes, they are subject to inhibition by some of the protein synthesis inhibitors including chloroamphenicol. This likely explains the toxicity of chloramphenicol. The eukaryotic cells most likely to be inhibited by chloramphenicol are those undergoing rapid multiplication, thereby rapidly synthesizing mitochondria. Such cells include the blood forming cells of the bone marrow, the inhibition of which could present as aplastic anemia. Chloramphenicol was once a highly prescribed antibiotic and a

number of deaths from anemia occurred before its use was curtailed. Now it is seldom used in human medicine except in life-threatening situations (e.g. typhoid fever).

The **Macrolides** are a family of antibiotics whose structures contain large lactone rings linked through glycoside bonds with amino sugars. The most important members of the group are **erythromycin** and **oleandomycin**. Erythromycin is active against most Gram-positive bacteria, *Neisseria*, *Legionella* and *Haemophilus*, but not against the *Enterobacteriaceae*. Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit. Binding inhibits elongation of the protein by peptidyl transferase or prevents translocation of the ribosome or both. Macrolides are bacteriostatic for most bacteria but are cidal for a few Gram-positive bacteria.

4. Effects on Nucleic Acids Some chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. Either case, of course, can block the growth of cells. The majority of these drugs are unselective, however, and affect animal cells and bacterial cells alike and therefore have no therapeutic application. Two nucleic acid synthesis inhibitors which have selective activity against procaryotes and some medical utility are nalidixic acid and rifamycins.

Nalidixic acid is a synthetic chemotherapeutic agent which has activity mainly against Gram-negative bacteria. Nalidixic acid belongs to a group of compounds called **quinolones**. Nalidixic acid is a bactericidal agent that binds to the DNA gyrase enzyme (topoisomerase) which is essential for DNA replication and allows supercoils to be relaxed and reformed. Binding of the drug inhibits DNA gyrase activity.

Some quinolones penetrate macrophages and neutrophils better than most antibiotics and are thus useful in treatment of infections caused by intracellular parasites. However, the main use of nalidixic acid is in treatment of lower urinary tract infections (UTI). The compound is unusual in that it is effective against several types of Gram-negative bacteria such as *E. coli, Enterobacter aerogenes, K. pneumoniae* and species which are common causes of UTI. It is not usually effective

against *Pseudomonas aeruginosa*, and Gram-positive bacteria are resistant. However, a fluoroquinolone, Ciprofloxacin (Cipro) was recently recommended as the drug of choice for prophylaxis and treatment of anthrax.

The **rifamycins** are also the products of *Streptomyces*. **Rifampicin** is a semisynthetic derivative of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis*) and some Gram-negative bacteria. Rifampicin acts quite specifically on eubacterial RNA polymerase and is inactive towards RNA polymerase from animal cells or towards DNA polymerase. The antibiotic binds to the beta subunit of the polymerase and apparently blocks the entry of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis. It has been found to have greater bactericidal effect against M .tuberculosis than other anti-tuberculosis drugs, and it has largely replaced isoniazid as one of the front-line drugs used to treat the disease, especially when isoniazid resistance is indicated. It is effective orally and penetrates well into the cerebrospinal fluid and is therefore useful for treatment of tuberculosis meningitis and meningitis caused by Neisseria meningitidis.

5. Competitive Inhibitors The competitive inhibitors are mostly all synthetic chemotherapeutic agents. Most are "growth factor analogs" which are structurally similar to a bacterial growth factor but which do not fulfill its metabolic function in the cell. Some are bacteriostatic and some are bactericidal.

Sulfonamides were introduced as chemotherapeutic agents by Domagk in 1935, who showed that one of these compounds (prontosil) had the effect of curing mice with infections caused by beta-hemolytic streptococci. Chemical modifications of the compound sulfanilamide gave compounds with even higher and broader antibacterial activity. The resulting sulfonamides have broadly similar antibacterial activity, but differ widely in their pharmacological actions. Bacteria which are almost always sensitive to the sulfonamides include *Streptococcus pneumoniae*, beta-hemolytic streptococci and *E. coli*. The sulfonamides have been extremely useful in the treatment of uncomplicated UTI caused by E. coli, and in the treatment of meningococcal meningitis (because they cross the blood-brain barrier).

The sulfonamides (e.g. **Gantrisin**) and **Trimethoprim** are inhibitors of the bacterial enzymes required for the synthesis of tetrahydofolic acid (THF), the vitamin form of folic acid essential for 1-carbon transfer reactions. Sulfonamides are structurally similar to para aminobenzoic acid (PABA), the substrate for the first enzyme in the THF pathway, and they competitively inhibit that step . Trimethoprim is structurally similar to dihydrofolate (DHF) and competitively inhibits the second step in THF synthesis mediated by the DHF reductase. Animal cells do not synthesize their own folic acid but obtain it in a preformed fashion as a vitamin. Since animals do not make folic acid, they are not affected by these drugs, which achieve their selective toxicity for bacteria on this basis.

Three additional synthetic chemotherapeutic agents have been used in the treatment of tuberculosis: **isoniazid (INH)**, **paraaminosalicylic acid (PAS)**, and **ethambutol**. The usual strategy in the treatment of tuberculosis has been to administer a single antibiotic (historically streptomycin, but now, most commonly, rifampicin is given) in conjunction with INH and ethambutol. Since the tubercle bacillus rapidly develops resistance to the antibiotic, ethambutol and INH are given to prevent outgrowth of a resistant strain. It must also be pointed out that the tubercle bacillus rapidly develops resistance to ethambutol and INH if either drug is used alone. Ethambutol inhibits incorporation of mycolic acids into the mycobacterial cell wall. Isoniazid has been reported to inhibit mycolic acid synthesis in mycobacteria and since it is an analog of pyridoxine (Vitamin B6) it may inhibit pyridoxine catalyzed reactions as well. Isoniazid is activated by a mycobacterial peroxidase enzyme and destroys several targets in the cell. PAS is an anti-folate. PAS was once a primary anti-tuberculosis drug, but now it is a secondary agent, having been largely replaced by ethambutol.

Bacterial resistance to antibiotics

Penicillin became generally available for treatment of bacterial infections, especially those caused by staphylococci and streptococci, about 1946. Initially, the antibiotic was effective against all sorts of infections caused by these two Gram-positive bacteria. Resistance to penicillin in some strains of staphylococci was recognized

almost immediately. (Resistance to penicillin today occurs in as many as 80% of all strains of *Staphylococcus aureus*). Surprisingly, *Streptococcus pyogenes* (Group A strep) have never fully developed resistance to penicillin and it remains a reasonable choice antibiotic for many types of streptococcal infections. Natural penicillins have never been effective against most Gram-negative pathogens (e.g. *Salmonella, Shigella, Bordetella pertussis, Yersinia pestis, Pseudomonas*) with the notable exception of *Neisseria gonorrhoeae*. Gram-negative bacteria are inherently resistant because their vulnerable cell wall is protected by an outer membrane that prevents permeation of the penicillin molecule.

The period of the late 1940s and early 1950s saw the discovery and introduction of streptomycin, chloramphenicol, and tetracycline, and the age of antibiotic chemotherapy came into full being. These antibiotics were effective against the full array of bacterial pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites, and the tuberculosis bacillus. However, by 1953, during a *Shigella* outbreak in Japan, a strain of the dysentery bacillus was isolated which was multiple drug resistant, exhibiting resistance to chloramphenicol, tetracycline, streptomycin, and the sulfanilamides. There was also evidence mounting that bacteria could pass genes for multiple drug resistance between strains and even between species. It was also apparent that Mycobacterium tuberculosis was capable of rapid development of resistance to streptomycin which had become a mainstay in tuberculosis therapy.

By the 1960's it became apparent that some bacterial pathogens were developing resistance to antibiotic-after-antibiotic, at a rate faster than new antibiotics could be brought to market. A more conservative approach to the use of antibiotics has not been fully accepted by the medical and agricultural communities, and the problems of emerging multiple-drug resistant pathogens still loom. The most important pathogens to emerge in multiple drug resistant forms so far have been *Mycobacterium tuberculosis* and *Staphylococcus aureus*.

The basis of bacterial resistance to antibiotics

Inherent (Natural) Resistance Bacteria may be inherently resistant to an antibiotic. For example, a streptomycete has some gene that is responsible for resistance to its own antibiotic; or a Gram-negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic.

Acquired Resistance Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously-sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria: (1) mutation and selection (sometimes referred to as vertical evolution); (2) exchange of genes between strains and species (sometimes called horizontal evolution).

Vertical evolution is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild type (non mutants) are killed and the resistant mutant is allowed to grow and flourish. The mutation rate formost bacterial genes is approximately 10^{-8}. This means that if a bacterial population doubles from 10^8 cells to 2 x 10^8 cells, there is likely to be a mutant present for any given gene. Since bacteria grow to reach population densities far in excess of 10^9 cells, such a mutant could develop from a single generation during 15 minutes of growth.

Horizontal evolution is the acquisition of genes for resistance from another organism. For example, a streptomycete has a gene for resistance to streptomycin (its own antibiotic), but somehow that gene escapes and gets into *E. coli* or *Shigella*. Or, more likely, Some bacterium develops genetic resistance through the process of mutation and selection and then donates these genesto some other bacterium through one of several processes for genetic exchange that exist in bacteria.

Bacteria are able to exchange genes in nature by three processes: conjugation, transduction and transformation. **Conjugation** involves cell-to-cell contact as DNA

crosses a sex pilus from donor to recipient. During **transduction**, a virus transfers the genes between mating bacteria. In **transformation**, DNA is acquired directly from the environment, having been released from another cell. Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as plasmids between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids (called resistance transfer factors, or RTFs), they are able to spread drug resistance to other strains and species during genetic exchange processes.

The combined effects of fast growth rates, high concentrations of cells, genetic processes of mutation and selection, and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in the bacteria. For these reasons bacterial adaptation (resistance) to the antibiotic environment seems to take place very rapidly in evolutionary time: bacteria evolve fast!

The medical problem of bacterial drug resistance

Obviously, if a bacterial pathogen is able to develop or acquire resistance to an antibiotic, then that substance becomes useless in the treatment of infectious disease caused by that pathogen (unless the resistance can somehow be overcome with secondary measures). So as pathogens develop resistance, we must find new (different) antibiotics to fill the place of the old ones in treatment regimes. Hence, natural penicillins have become useless against staphylococci and must be replaced by other antibiotics; tetracycline, having been so widely used and misused for decades, has become worthless for many of the infections that once designated it as a "wonder drug".

Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is a parallel problem to find new antibiotics to fight new diseases. In the past two decades, many "new" bacterial diseases have been discovered (Legionnaire's disease, gastric ulcers, Lyme disease,

toxic shock syndrome, "skin-eating" streptococci). We are only now able to examine patterns of susceptibility and resistance to antibiotics among new pathogens that cause these diseases. Broad patterns of resistance exist in these pathogens, and it seems likely that we will soon need new antibiotics to replace the handful that are effective now against these bacteria, especially as resistance begins to emerge among them in the selective environment antibiotic chemotherapy.

IMPORTANT GROUPS OF PROCARYOTES

BACTERIA

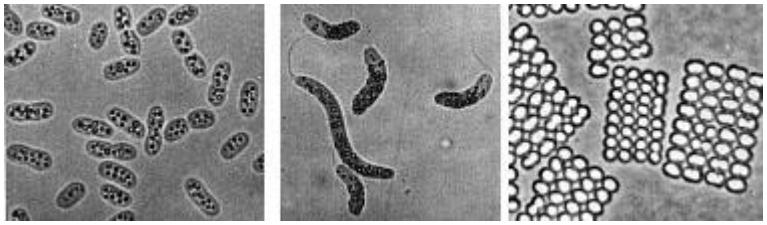
Phylogenetic analysis of the **Bacteria** has demonstrated the existence of at least 13 distinct groups, but many groups consist of members that are phenotypically and physiologically unrelated, and sometimes phylogenetically unrelated. The current edition of Bergey's Manual of Systematic Bacteriology (2001) recognizes 23 distinct phyla of Bacteria (Phylum is the highest taxon in a Domain), but there may still be great variation in phenotype among members. Below we discuss the major groups of Bacteria based on morphology, physiology, or ecology, and often use informal, but familiar, terms to identify them.

Photosynthetic purple and green bacteria. These bacteria conduct **anoxygenic photosynthesis**, also called **bacterial photosynthesis**. Bacterial photosynthesis differs from plant-type (oxygenic) photosynthesis in several ways. Bacterial photosynthesis does not produce O_2; in fact, it only occurs under anaerobic conditions. Bacterial photosynthesis utilizes a type of chlorophyll other than chlorophyll *a,* and only one photosystem, photosystem I. The electron donor for bacterial photosynthesis is never H_2O but may be H_2, H_2S or S^o, or certain organic compounds. The light-absorbing pigments of the purple and green bacteria consist of bacterial chlorophylls and carotenoids. Phycobilins, characteristic of the cyanobacteria, are not found. Many purple and green sulfur bacteria store elemental sulfur as a reserve material that can be further oxidized to SO_4 as a photosynthetic electron donor.

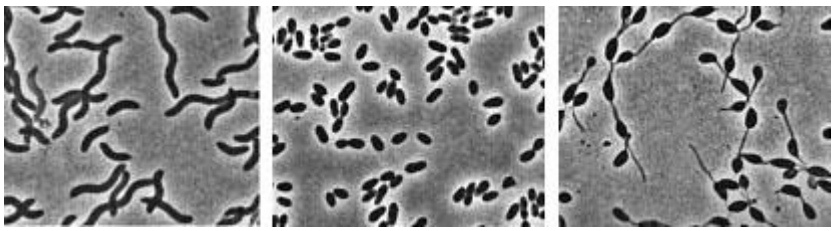
The **purple and green bacteria** may use H_2S during photosynthesis in the same manner that cyanobacteria or algae or plants use H_2O as an electron donor for autotrophic CO_2 reduction (the "dark reaction" of photosynthesis). Or they may utilize organic compounds as electron donors for photosynthesis. For example, *Rhodobacter* can use light as an energy source while oxidizing succinate or butyrate in order to obtain electrons for CO_2 fixation.

The bacterium that became an endosymbiont of eucaryotes and evolved into mitochondria is thought to be a relative of the purple nonsulfur bacteria. This conclusion is based on similar metabolic features of mitochondria and purple nonsulfur bacteria and on comparisons of the base sequences in their 16S rRNAs.

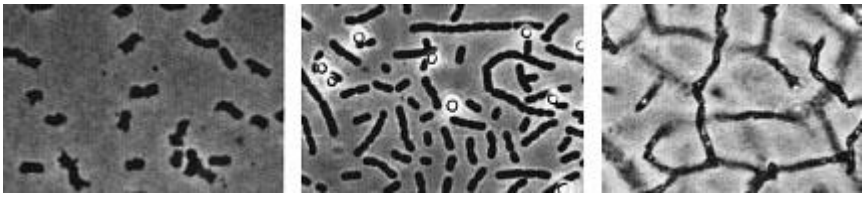
Figure 1. Photomicrographs (phase contrast and and ordinary illumination) of various photosynthetic bacteria. Magnifications are about 1400X. The purple and green bacteria exhibit a full range of procaryotic morphologies, as these photomicrographs illustrate. Diversity among their phylogenetic relationships is also noted.



A. Purple sulfur bacteria (L to R): *Chromatium vinosum, Thiospirillum jenense, Thiopedia rosea*.



B. Purple nonsulfur bacteria (L to R): *Rhodospirillum rubrum, Rhodobacter sphaeroides, Rhodomicrobium vannielii.* The purple nonsulfur bacteria are in the Alphaproteobacteria, which also includes *Rhizobium, Agrobacterium* and the Rickettsias. The latter bacteria represent a direct lineage to mitochondria.



C. Green sulfur bacteria (L to R): *Chlorobium limicola, Prosthecochloris aestuarii, Pelodictyon clathratiforme.* The Green sulfur bacteria represent a distinct phylogenetic lineage and cluster in their own phylum represented by *Chlorobium* .

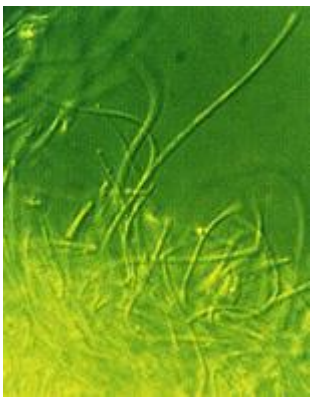


Figure 2. Green nonsulfur bacterium, *Chloroflexus* (T.D. Brock). *Chloroflexus* also represents a phylogenetically distinct group of green bacteria. *Chloroflexus* is a thermophilic, filamentous gliding bacterium.

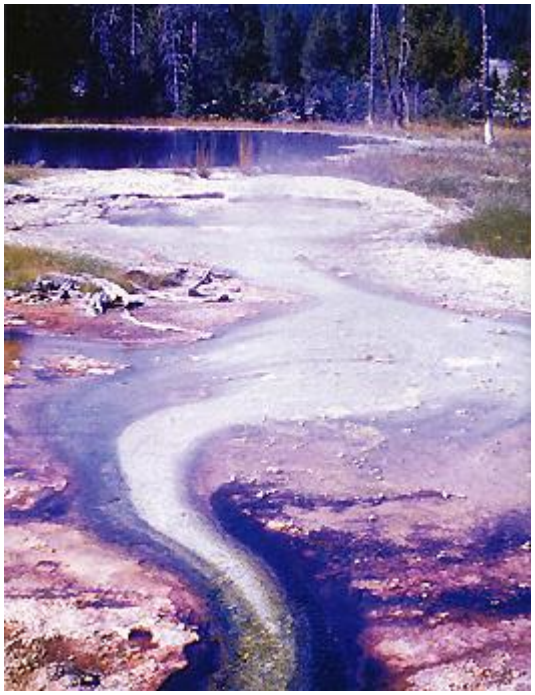


Figure 3. Photosynthetic procaryotes growing in a hot spring run-off channel (T.D. Brock). The white area of the channel is too hot for photosynthetic life, but as the water cools along a gradient, the colored phototrophic bacteria colonize and

ultimately construct the colored microbial mats composed of a consortium of photosynthetic microorganisms.

Cyanobacteria. The cyanobacteria deserve special emphasis because of their great ecological importance in the global carbon, oxygen and nitrogen cycles, as well as their evolutionary significance in relationship to plants. Photosynthetic cyanobacteria have chlorophyll *a* and carotenoids in addition to some unusual accessory pigments named **phycobilins**. The blue pigment, **phycocyanin** and the red one, **phycoerythrin**, absorb wavelengths of light for photosynthesis that are missed by chlorophyll and the carotenoids. Within the cytoplasm of cyanobacteria are numerous layers of membranes, often parallel to one another. These membranes are photosynthetic thylakoids that resemble those found in chloroplasts, which, in fact, correspond in size to the entire cyanobacterial cell. The main storage product of the cyanobacteria is glycogen, and glycogen inclusions may be seen in the cytoplasm of the cells. Cyanobacteria are thought to have given rise to eucaryotic chloroplasts during the evolutionary events of endosymbiosis. In biochemical detail, cyanobacteria are especially similar to the chloroplasts of red algae (*Rhodophyta*).

Most cyanobacteria have a mucilaginous sheath, or coating, which is often deeply pigmented, particularly in species that occur in terrestrial habitats. The colors of the sheaths in different species include light gold, yellow, brown, red, green, blue, violet, and blue-black. It is these pigments that impart color to individual cells and colonies as well as to "blooms" of cyanobacteria in aquatic environments

Figure 4. Some common cyanobacteria L to R: *Oscillatoria,* a filamentous species common in fresh water and hot springs; *Nostoc,* a sheathed communal species; *Anabaena,* a nitrogen-fixing species. The small cell with an opaque surface (third from right) in the anabaena filament is a heterocyst, a specialized cell for nitrogen fixation. The large bright

cell in the filament is a type of spore called an akinete; *Synechococcus,* a unicelluar species in marine habitats and hot springs. *Synechococcus* is among the most important photosynthetic bacteria in the marine environment, estimated to account for about 25 percent of the primary production that occurs in typical marine habitats.

Although thousands of cyanobacteria have been observed, only about 200 species have been identified as distinct, free-living, nonsymbiotic procaryotes. Relative to other oxygenic phototrophs, cyanobacteria often grow under fairly extreme environmental conditions such as high temperature and salinity . They are the only oxygenic phototrophs present in many hot springs of the Yellowstone ecosystem; and in frigid lakes and oceans of Antarctica, they form luxuriant mats 2 to 4 centimeters thick in water beneath more than five meters of permanent ice. However, cyanobacteria are absent in acidic waters where their eucaryotic counterparts, the algae, may be abundant.

Layered chalk deposits called **stromatolites**, which exhibit a continuous geologic record covering 2.7 billion years, are produced when colonies of cyanobacteria bind calcium-rich sediments. Today, stromatolites are formed in only a few places, such as shallow pools in hot dry climates. The abundance of cyanobacteria in the fossil record is evidence of the early development of the cyanobacteria and their important role in elevating the level of free oxygen in the atmosphere of the early Earth.

Cyanobacteria often form filaments and may grow in large masses or "tufts" one meter or more in length. Some are unicellular, a few form branched filaments, and a few form irregular plates or irregular colonies. Cyanobacterial cells usually divide by binary fission, and the resulting progeny cells may separate to form new colonies. In addition, filaments may break into fragments, called **hormogonia**, which separate and develop into new colonies. As in other filamentous or colonial bacteria, the cells of cyanobacteria may joined by their walls or by mucilaginous sheaths, but each cell is an independent unit of life.

As true Bacteria, cyanobacteria contain peptidoglycan or murein in their cell walls. Most cyanobacteria have a Gram-negative type cell wall that consists of an outer membrane component, even though they may show a distant phylogenetic

relationship with certain Gram-positive bacteria. Some of the filamentous cyanobacteria are motile by means gliding or rotating around a longitudinal axis. Short segments (hormogonia) may break off from a cyanobacterial colony and glide away from their parent colony at rates as rapid as 10 micrometers per second. The mechanism for this movement is unexplained but may be connected to the extrusion of slime (mucilage) through small pores in their cell wall, together with contractile waves in one of the surface layers of the wall.

Cyanobacteria are found in most aerobic environments where water and light are available for growth. Mainly they live in fresh water and marine habitats. Those inhabiting the surface layers of water are part of a complex microbial community called **plankton**. Planktonic cyanobacteria usually contain cytoplasmic inclusions called **gas vesicles** which are hollow protein structures filled with various gases. The vesicles can be inflated or deflated with gases allowing the organisms to maintain buoyancy and to float at certain levels in the water. Thus, the cyanobacteria can regulate their position in the water column to meet their optimal needs for photosynthesis, oxygen, and light-shielding. When numerous cyanobacteria become unable to regulate their gas vesicles properly (for example, because of extreme fluctuations of temperature or oxygen supply), they may float to the surface of a body of water and form visible "blooms". A planktonic species related to *Oscillatoria* gives rise to the redness (and the name) of the Red Sea.

The cyanobacteria have very few harmful effects on plants or animals. They may be a nuisance if they bloom in large numbers and then die and decay in bodies of fresh water that are used for drinking or recreational purposes. Many cyanobacteria are responsible for the earthy odors and flavors of fresh waters, including drinking waters, due to the production of compounds called **geosmins.** Some cyanobacteria that form blooms secrete poisonous substances that are toxic for animals that ingest large amounts of the contaminated water.

Many marine cyanobacteria occur in limestone (calcium carbonate) or lime-rich substrates, such as coral algae and the shells of mullosks. Some fresh water species,

particularly those that grow in hot springs, often deposit thick layers of lime in their colonies.

Some cyanobacteria can fix nitrogen. In filamentous cyanobacteria, nitrogen fixation often occurs in **heterocysts**, which are specialized, enlarged cells, usually distributed along the length of a filament or at the end of a filament. Heterocysts have intercellular connections to adjacent vegetative cells, and there is continuous movement of the products of nitrogen fixation moving from heterocysts to vegetative cells, and the products of photosynthesis moving from vegetative cells to heterocysts. Heterocysts are low in phycobilin pigments and have only photosystem I. They lack the oxygen-evolving photosystem II. Furthermore, they are surrounded in a thickened, specialized glycolipid cell wall that slows the rate of diffusion of O_2 into the cell. Any O_2 that diffuses into the heterocyst is rapidly reduced by hydrogen, a byproduct of N_2 fixation, or is expelled through the wall of the heterocyst. The process of nitrogen fixation, specifically the enzyme nitrogenase, only functions in anaerobic conditions so the organism must maintain these oxygen-free compartments in order for N_2fixation to occur.

In addition to the heterocysts, some cyanobacteria form resistant spores called **akinetes** enlarged cells around which thickened outer walls develop. Akinetes are resistant to heat, freezing and drought (desiccation) and thus allow the cyanobacteria to survive unfavorable environmental conditions. They are functionally analogous to bacterial endospores, but they bear little resemblance and lack the extraordinary resistance properties of endospores.

A few cyanobacteria are symbionts of liverworts, ferns, cycads, flagellated protozoa, and algae, sometimes occurring as endosymbionts of the eucaryotic cells. In the case of the water fern, *Azolla,* the cyanobacterial endophyte (a species of *Anabaena)* fixes nitrogen that becomes available to the plant. In addition, it is often the case that the photosynthetic partners of **lichens** are cyanobacteria.

The planktonic cyanobacteria fix an enormous amount of CO_2 during photosynthesis, and as "primary producers" they are the basis of the food chain in marine

environments. Their type of photosynthesis, which utilizes photosystem II, generates a substantial amount of oxygen present in the earth's atmosphere. Since many cyanobacteria can fix N_2 under certain conditions, they are one of the most significant free-living nitrogen-fixing procaryotes. Cyanobacteria carried out plant-type (oxygenic) photosynthesis for at least a billion and a half years before the emergence of plants, and cyanobacteria are believed to be the evolutionary forerunners of modern-day plant and algal chloroplasts. A group of phototrophic procaryotes, called **prochlorophytes** contain chlorophyll *a* and *b* but do **not** contain phycobilins. Prochlorophytes, therefore, resemble both cyanobacteria (because they are procaryotic and contain chlorophyll *a*) and the plant chloroplast (because they contain chlorophyll *b* instead of phycobilins). *Prochloron,*the first prochlorophyte discovered, is phenotypically very similar to certain plant chloroplasts and is the leading candidate for the type of bacterium that might have undergone endosymbiotic events that led to the development of the plant chloroplast.

Spirochetes are a phylogenetically distinct group of Bacteria which have a unique cell morphology and mode of motility. Spirochetes are very thin, flexible, spiral-shaped procaryotes that move by means of structures called **axial filaments** or **endoflagella**. The flagellar filaments are contained within a sheath between the cell wall peptidoglycan and an outer membrane. The filaments flex or rotate within their sheath which causes the cells to bend, flex and rotate during movement. Most spirochetes are free living (in muds and sediments), or live in associations with animals (e.g. in the oral cavity or GI tract). A few are pathogens of animals (e.g. leptospirosis in dogs, Syphilis in humans and Lyme disease in dogs and humans).

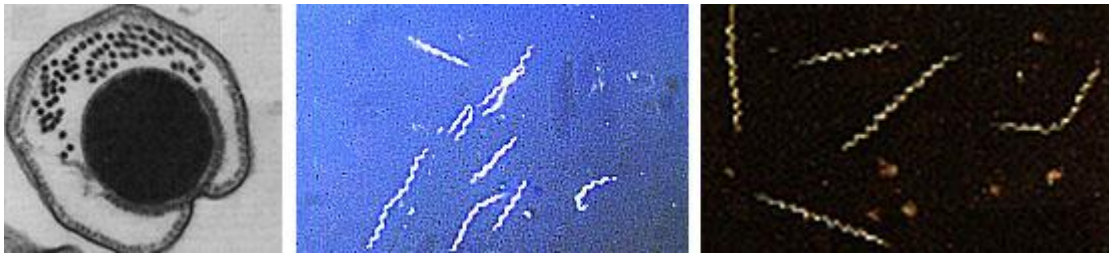


Figure 5. Spirochetes: A. Cross section of a spirochete showing the location of endoflagella between the inner membrane and outer sheath; B. *Borrelia burgdorferi,* the agent of Lyme disease; C. *Treponema pallidum,* the spirochete that causes syphilis.

Other Spiral-Shape and and Curved Bacteria. The main thing that unifies this group of bacteria is their spiral or vibrioid (curved) shape, although they are all classified among the Proteobacteria. Nontheless, in certain environments, their characteristic shape can instantly inform an observer of their identity. Bacteria referred to as "**spirilla**" are Gram-negative aerobic heterotrophic bacteria with a helical or spiral shape. Their metabolism is usually respiratory and never fermentative. Unlike spirochetes, they have a rigid cell wall and are motile by means of ordinary polar flagella. Spirilla are inhabitants of microaerophilic aquatic environments. Most spirilla require or prefer that oxygen in their environment be present in an amount that is well below atmospheric concentration. The *Rhodospirillaceae* are found in the Alpha group of Proteobacteria; *Spirillaceae* and *Oceanospirillaceae* are Gammaproteobacteria.

As inhabitants of marine and fresh waters many spirilla are endowed with some interesting properties. *Magnetospirillum* contains **magnetosomes** and exhibits the property of **magnetotaxis** (movement in relationship to the magnetic field of the earth). *Oceanospirillum* lives in marine habitats and is able to grow at NaCl concentrations as high as 9 percent. *Azospirillum* is a nitrogen-fixing bacterium that enters into a mutualistic symbiosis with certain tropical grasses and grain crops. Spirilla are thought to play a significant role in recycling of organic matter, particularly in aquatic environments.

Two pathogens of humans are found among the spiril forms in the Epsilon group of Proteobacteria. *Campylobacter jejuni* is an important cause of bacterial diarrhea, especially in children. The bacterium is transmitted via contaminated food, usually undercooked poultry or shellfish, or untreated drinking water. *Helicobacter pylori* is able to colonize the gastric mucosal cells of humans, i.e., the lining of the stomach, and it has been well established as the cause of peptic ulcers.

Bacteria with a curved rod or comma shape are referred to as "**vibrios**". Like the spiral forms, vibrios are very common bacteria in aquatic environments. They are found among the Gammaproteobacteria and have structural and metabolic properties that overlap with both the enterics and the pseudomonads. In Bergey's Manual

(2001) *Vibrionaceae* is a family on the level with *Enterobacteriaceae*. Vibrios are facultative like enterics, but they have polar flagella, are oxidase-positive, and dissimilate sugars in the same manner as the pseudomonads. In aquatic habitats they overlap with the *Pseudomonadaceae* in their ecology, although *Pseudomonas* species favor fresh water and vibrios prefer salt water. The genus *Vibrio* contains an important pathogen of humans, *Vibrio cholerae,* the cause of **Asiatic cholera**. Cholera is an intestinal disease with a pathology related to diarrheal diseases caused by the enteric bacteria.

Five species of marine vibrios exhibit the property of **bioluminescence**, the ability to emit light of a blue-green color. These bacteria may be found as saprophytes of dead fish or as symbionts of living fish and invertebrates in marine environments. Some grow in special organs of the fish and emit light for the benefit of the fish (to attract prey, or as a mating signal) in return for a protected habitat and supply of nutrients. The reaction leading to light emission, catalyzed by the enzyme **luciferase**, has been found to be the same in all procaryotes, and differs from light emission by eucaryotes such as the fire fly. Luciferase diverts electrons from the normal respiratory electron transport chain and causes formation of an excited peroxide that leads to emission of light.

The small vibrioid bacterium, *Bdellovibrio,*is a tiny curved rod that is a parasite of other Gram-negative bacteria, including *E. coli*. It preys on other bacteria by entering into the periplasmic space and obtaining nutrients from the cytoplasm of its host cell while undergoing an odd type of reproductive cycle. *Bdellovibrio*is a member of the Deltaproteobacteria.

The **Myxobacteria** are a group of **fruiting gliding bacteria** that comprise a unique order of Deltaproteobacteria. They exhibit a unique type of gliding motility. The vegetative cells move (glide) about together as a swarm, and then they aggregate together to form a multicellular fruiting body in which development and spore formation takes place. They exhibit the most complex behavioral patterns and life cycles of all known procaryotes. Myxobacteria are inhabitants of the soil. They

have a eucaryotic counterpart in nature in the *Myxomycetes*, or slime molds, and the two types of organisms are an example of parallel or **convergent evolution**, having adopted similar life styles in the soil environment.

The vegetative cells of myxobacteria are typical Gram-negative rods that glide across a substrate such as a decaying leaf or piece of animal dung, or colonies of other bacteria. They obtain nutrients from the substrate as they glide across it and they secrete a slime track which other myxobacterial cells preferentially follow. If their nutrients become exhausted, the cells signal to one another to aggregate and form a swarm of myxobacteria which eventually differentiate into a multicellular **fruiting body** that contains **myxospores**, a type of dormant cell descended from a differentiated vegetative cell. In the case of *Stigmatella*, the myxospores are packed into secondary structures called **cysts**, which develop at the tips of the fruiting body (Figure 6). The bright-colored fruiting bodies of myxobacteria, containing millions of cells and spores, can often be seen with the unaided eye on dung pellets and decaying vegetation in the soil.

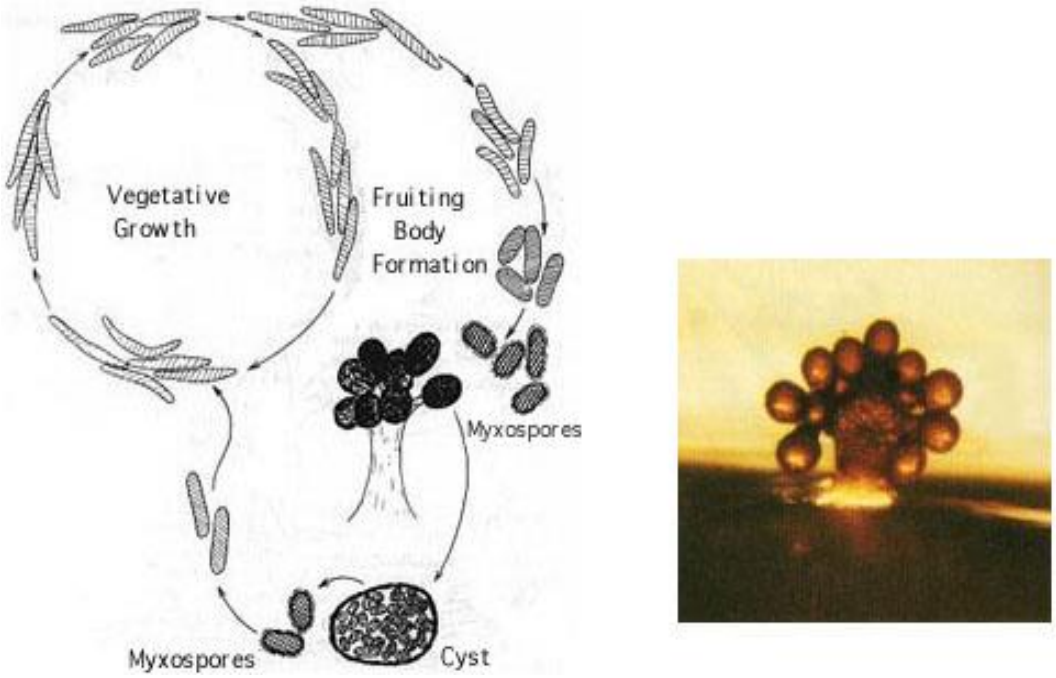


Figure 6. *Stigmatella aurantiaca*, a fruiting myxobacterium: L. Life Cycle R. Fruiting Body.

Lithotrophs. Lithotrophy, a type of metabolism that requires inorganic compounds as sources of energy. This metabolism is firmly established in both the Archaea and the Bacteria. The methanogens utilize H_2 as an energy source, and many extreme thermophiles use H_2S or elemental sulfur as a source of energy for growth. Lithotrophic Bacteria are typically Gram-negative species that utilize inorganic substrates including H_2, NH_3, NO_2, H_2S, S, Fe^{++}, and CO. Ecologically, the most important lithotrophic Bacteria are the **nitrifying bacteria**, *Nitrosomonas* and *Nitrobacter* that together convert NH_3 to NO_2, and NO_2 to NO_3, and the **colorless sulfur bacteria,** such as *Thiobacillus,* that oxidize H_2S to S and S to SO_4. Most lithotrophic bacteria are autotrophs, and in some cases, they may play an important role in primary production of organic material in nature. Lithotrophic metabolism does not extend to eucaryotes (unless a nucleated cell harbors lithotrophic endosymbiotic bacteria), and these bacteria are important in the biogeochemical cycles of the elements.

Figure 7. Lithotroph Habitats. A. Stream in Northern Wisconsin near Hayward is a good source of iron bacteria (John Lindquist). B. Bacteriologist J.C. Ensign of the University of Wisconsin observing growth of iron bacteria in a run-off channel from the Chocolate Pots along the Gibbon River, in Yellowstone National Park (K.Todar). C. An acid hot spring at the Norris Geyser Basin in Yellowstone is rich in iron and sulfur (T.D. Brock). D. A black smoker chimney in the deep sea emits iron sulfides at very high temperatures (270 to 380 degrees C).

Pseudomonads. "Pseudomonad" is an informal term for bacteria which morphologically and physiologically resemble members of the genus *Pseudomonas,* a

very diverse group of Gram-negative rods with a strictly-respiratory mode of metabolism. The term is usually applied to bacteria in the genera *Pseudomonas,* and *Xanthomonas,* which are Alphaproteobacteria, and to plant and animal pathogens such as *Burkholderia, Ralstonia* and *Acidovorax,* which are Betaproteobacteria. But many other related bacteria share their definitive characterictics, i.e., Gram-negative aerobic rods. The morphology and habitat of many pseudomonads sufficiently overlaps with the enterics (below) that microbiologists must quickly learn how to differentiate these two types of Gram-negative motile rods. Pseudomonads move by polar flagella; enterics such as *E. coli* swim by means of peritrichous flagella. Enterics ferment sugars such as glucose; pseudomonads generally do not ferment sugars. And most pseudomonads have an unusual cytochrome in their respiratory electron transport chain that can be detected in colonies by a colorimetric test called the **oxidase test**. Pseudomonads are typically oxidase- positive.

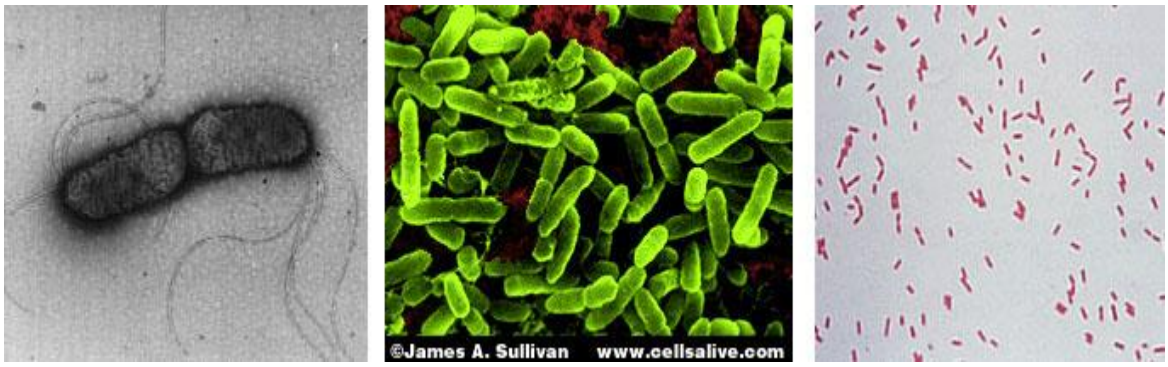


Figure 8. Profile of a pseudomonad: Gram-negative rods motile by polar flagella. A. Electron micrograph, negative stain. B. Scanning electron micrograph. C. Gram stain.

Most pseudomonads are free-living organisms in soil and water; they play an important role in decomposition, biodegradation, and the C and N cycles. The phrase "no naturally-occurring organic compound cannot be degraded by some microorganism" must have been coined to apply to members of the genus *Pseudomonas,* known for their ability to degrade hundreds of different organic compounds including insecticides, pesticides, herbicides, plastics, petroleum substances, hydrocarbons and other of the most refractory molecules in nature. However, they are usually unable to degrade biopolymers in their environment, such as cellulose and lignen, and their role in anaerobic decomposition is minimal.

There are about 150 species of *Pseudomonas*, but, especially among the plant pathogens, there are many strains and biovars among the species. These bacteria are frequently found as part of the normal flora of plants, but they are one of the most important bacterial pathogens of plants, as well. *Pseudomonas syringae* and *Xanthomonas* species cause a wide variety of plant diseases as discussed below. One strain of *Pseudomonas* that lives on the surfaces of plants can act as an "ice nucleus" which causes ice formation and inflicts frost damage on plants at one or two degrees *above* the conventional freezing temperature of water (0 degrees C). One *Pseudomonas* species is an important pathogen of humans, *Pseudomonas aeruginosa,* the quintessential opportunistic pathogen, which is a leading cause of hospital-acquired infections.

Among some interesting or important ecologic relatives of the pseudomonads are *Rhizobium*and *Bradyrhizobium,* species that fix nitrogen in association with leguminous plants, and related *Agrobacterium* species that cause tumors ("galls") in plants. These bacteria are discussed later in this article because of their special relationships with plants. Relatives of the pseudomonads also include the **methanotrophs** that can oxidize methane and other one-carbon compounds, the **azotobacters**, which are very prevalent free-living (nonsymbiotic) nitrogen-fixing bacteria.

Enterics. Enteric bacteria are Gram-negative rods with facultative anaerobic metabolism that live in the intestinal tracts of animals. This group consists of *Escherichia coli* and its relatives, the members of the family *Enterobacteriaceae.* Enteric bacteria are related phenotypically to several other genera of bacteria such as *Pseudomonas* and *Alcaligenes,* but are physiologically quite unrelated. Generally, a distinction can be made on the ability to ferment glucose: enteric bacteria all ferment glucose to acid end products while similar Gram-negative bacteria cannot ferment glucose. Because they are consistent members of the normal flora of humans, and because of their medical importance, an extremely large number of enteric bacteria have been isolated and characterized.

Escherichia coli is, of course, the type species of the enterics. *E. coli* is such a regular inhabitant of the intestine of humans that it is used by public health authorities as an indicator of fecal pollution of drinking water supplies, swimming beaches, foods, etc. *E. coli* is the most studied of all organisms in biology because of its occurrence, and the ease and speed of growing the bacteria in the laboratory. It has been used in hundreds of thousands of experiments in cell biology, physiology, and genetics, and was among the first cells for which the entire chromosomal DNA base sequence was determined. In spite of the knowledge gained about the molecular biology and physiology of *E. coli,* surprisingly little is known about its ecology, for example why it consistently associates with humans, how it helps its host, how it harms its host, etc. A few strains of *E. coli* are pathogenic (one is notorious, strain 0157:H7, that keeps turning up in raw hamburger headed for a fast-food restaurants). Pathogenic strains of *E. coli* cause **intestinal tract infections** (usually acute and uncomplicated, except in the very young), uncomplicated **urinary tract infections** and **neonatal meningitis.**

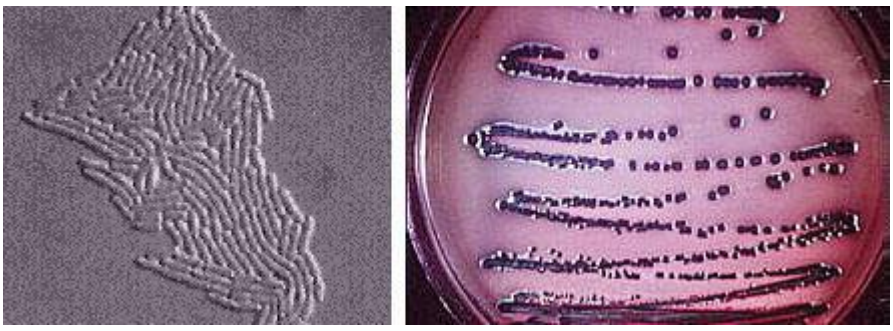


Figure 9. Left: *Escherichia coli* cells. Right: *E. coli* colonies on EMB Agar.

The enteric group also includes some other intestinal pathogens of humans such as *Shigella dysenteriae*, cause of **bacillary dysentery**, and *Salmonella typhimurium*, cause of **gastroenteritis**. *Salmonella typhi*, which infects via the intestinal route, causes **typhoid feve**r. Some bacteria that don't have an intestinal habitat resemble *E. coli* in enough ways to warrant inclusion in the enteric group. This includes *Proteus,* a common saprophyte of decaying organic matter, *Yersinia pestis,* which causes **bubonic plague**, and *Erwinia,* an important pathogen of plants.

Gram-negative pathogens. The Gram negative bacteria that are important pathogens of humans are found sattered throughout the Proteobacteria. In the Alphaproteobacteria, one finds the Rickettsias, a group of obligate intracellular parasites which are the cause of **typhus** and **Rocky Mountain Spotted fever**. In the Beta group, the agents of **whooping cough (pertussis)** (*Bordetella pertussis),* gonorrhea (*Neisseria gonorrhoeae*), and meningococcal meningitis (*Neisseria meningitidis*) are found. Among the Gamma group, *Pseudomonas aeruginosa,* the enterics, and *Vibrio cholerae* have already been mentioned. Likewise, the agents of Legionaires' pneumonia (*Legionella pneumophilia),* and childhood meningitis *(Haemophilus influenzae)* are Gammaproteobacteria. *Campylobacter* and *Helicobacter* are Epsilonproteobacteria. Most of these bacteria are discussed elsewhere in this article and/or in separate chapters which deal with their pathogenicity for humans.

Nitrogen-fixing organisms. This is a diverse group of procaryotes, reaching into phylogenetically distinct groups of Archaea and Bacteria. Members are unified only on the basis of their metabolic ability to "fix" nitrogen. **Nitrogen fixation** is the reduction of N_2 (atmospheric nitrogen) to NH_3 (ammonia). It is a complicated enzymatic process mediated by the enzyme **nitrogenase**. Nitrogenase is found only in procaryotes and is second only to RUBP carboxylase (the enzyme responsible for CO_2 fixation) as the most abundant enzyme on Earth.

The conversion of nitrogen gas (which constitutes about 80 percent of the atmosphere) to ammonia introduces nitrogen into the biological nitrogen cycle. Living cells obtain their nitrogen in many forms, but usually from ammonia (NH_3) or nitrates (NO_3), and never from N_2. Nitrogenase extracts N_2 from the atmosphere and reduces it to NH_3 in a reaction that requires substantial reducing power (electrons) and energy (ATP). The NH_3 is immediately assimilated into amino acids and proteins by subsequent cellular reactions. Thus, nitrogen from the atmosphere is fixed into living (organic) material.

Although a widespread trait in procaryotes, nitrogen fixation occurs in only a few select genera. Outstanding among them are the symbiotic bacteria *Rhizobium* and

Bradyrhizobium which form nodules on the roots of legumes. In this symbiosis the bacterium invades the root of the plant and fixes nitrogen which it shares with the plant. The plant provides a favorable habitat for the bacterium and supplies it with nutrients and energy for efficient nitrogen fixation. *Rhizobium* and *Bradyrhizobium* are Gram-negative aerobes related to the pseudomonads (above). An unrelated bacterium, an actinomycete (below), enters into a similar type of symbiosis with plants. The actinomycete, *Frankia,* forms nodules on the roots of several types of trees and shrubs, including alders (*Alnus*), wax myrtles (*Myrica*) and mountain lilacs (*Ceanothus*). They, too, fix nitrogen which is provided to their host in a useful form. This fact allows alder species to be "pioneer plants" (among the first to colonize) in newly-forming nitrogen-deficient soils. Still other bacteria live in regular symbiotic associations with plants on roots or leaves and fix nitrogen for their hosts, but they do not cause tissue hyperplasia or the formation of nodules.

Cyanobacteria are likewise very important in nitrogen fixation. Cyanobacteria provide fixed nitrogen, in addition to fixed carbon, for their symbiotic partners which make up lichens. This enhances the capacity for lichens to colonize bare areas where fixed nitrogen is in short supply. In some parts of Asia, rice can be grown in the same paddies continuously without the addition of fertilizers because of the presence of nitrogen fixing cyanobacteria. The cyanobacteria, especially *Anabaena*, occur in association with the small floating water fern *Azolla,* which forms masses on the paddies. Because of the nearly obligate association of *Azolla* with *Anabaena,* paddies covered with *Azolla* remain rich in fixed nitrogen.

In addition to symbiotic nitrogen-fixing bacteria, there are various free-living nitrogen-fixing procaryotes in both soil and aquatic habitats. Cyanobacteria may be able to fix nitrogen in virtually all habitats that they occupy. Clostridia and some methanogens fix nitrogen in anaerobic soils and sediments, including thermophilic environments. A common soil bacterium, *Azotobacter* is a vigorous nitrogen fixer, as is *Rhodospirillum*, a purple sulfur bacterium. Even *Klebsiella*, an enteric bacterium closely related to *E. coli,* fixes nitrogen. There is great scientific interest, of course, in knowing how one might move the genes for nitrogen fixation from a procaryote into a eucaryote such as corn or some other crop plant. The genetically engineered plant

might lose its growth requirement for costly ammonium or nitrate fertilizers and grow in nitrogen deficient soils.

Besides nitrogen fixation, bacteria play other essential roles in the processes of the nitrogen cycle. For example, saprophytic bacteria, decompose proteins releasing NH_3 in the process of **ammoniafication**. NH_3 is oxidized by lithotrophic *Nitrosomonas* species to NO_2 which is subsequently oxidized by *Nitrobacter* to NO_3. The overall conversion of NH_3 to NO_3 is called **nitrification**. NO_3 can be assimilated by cells as a source of nitrogen (**assimilatory nitrate reduction**), or certain bacteria can reduce NO_3 during a process called **anaerobic respiration**, wherein nitrate is used in place of oxygen as a terminal electron acceptor for a process analogous to aerobic respiration. In the case of anaerobic respiration, NO_3 is first reduced to NO_2, which is subsequently reduced to N_2O or N_2 or NH_3 (all gases). This process is called **denitrification** and it occurs in anaerobic environments where nitrates are present. If denitrification occurs in crop soils it may not be beneficial to agriculture if it converts utilizable forms of nitrogen (as in nitrate fertilizers) to nitrogen gases that will be lost into the atmosphere. One rationale for tilling the soil is to keep it aerobic in order to discourage denitrification processes in *Pseudomonas* and *Bacillus* which are ubiquitous inhabitants.

The **pyogenic cocci** are spherical bacteria which cause various suppurative (pus-producing) infections in animals. Included are the Gram-positive cocci *Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus pneumoniae,* and the Gram-negative cocci, *Neisseria gonorrhoeae* and *N. meningitidis.* These bacteria are leading pathogens of humans. It is estimated that they produce at least a third of all the bacterial infections of humans, including strep throat, pneumonia, food poisoning, various skin diseases and severe types of septic shock, gonorrhea and meningitis. *Staphylococcus aureus* is arguably the most successful of all bacterial pathogens because it has a very wide range of virulence determinants (so it can produce a wide range of infections) and it often occurs as normal flora of humans (on skin, nasal membranes and the GI tract), which ensures that it is readily transmitted from one individual to another. In terms of their phylogeny, physiology

and genetics, these genera of bacteria are quite unrelated to one another. They share a common ecology, however, as parasites of humans.

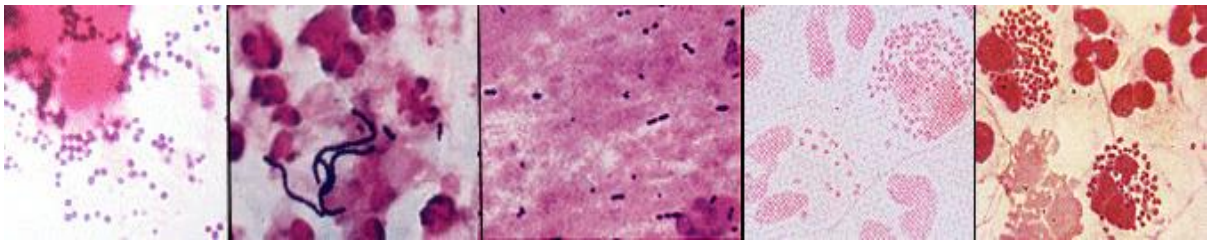


Figure 10. Gallery of pyogenic cocci, Gram stains of clinical specimens (pus), L to R: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*. The large cells with lobed nuclei are neutrophils. Pus is the outcome of the battle between phagocytes (neutrophils) and the invading cocci. As the bacteria are ingested and killed by the neutrophils, the neutrophils eventually lyse (rupture) and release their own components, plus the digested products of bacterial cells, which are the make-up of pus. As a defense against phagocytes the staphylococci and streptococci produce toxins that kill the neutrophils before they are able to ingest the bacteria. This contributes to the pus, and therefore these bacteria are "pyogenic" during their pathogenic invasions.

Two species of Staphylococcus live in association with humans: *Staphylococcus epidermidis* which lives normally on the skin and mucous membranes, and *Staphylococcus aureus* which may occur normally at various locales, but in particular on the nasal membranes (nares). *S. epidermidis* is rarely a pathogen and probably benefits its host by producing acids on the skin that retard the growth of dermatophytic fungi. *Staphylococcus aureus* always has the potential to cause disease and so is considered a pathogen. Different strains of *S. aureus* differ in the range of diseases they can cause, including boils and pimples, **wound infections**, **pneumonia, osteomyelitis**, **septicemia**, **food intoxication**, and **toxic shock syndrome**. *S. aureus* is the leading cause of **nosocomial (hospital-acquired) infections** by Gram-positive bacteria. Also, it is notoriously resistant to penicillin and many other antibiotics. Recently, a strain of *S. aureus* has been reported that is resistant to **EVERY** known antibiotic in clinical usage, which is a grim reminder that the clock is ticking on the lifetime of the usefulness of current antibiotics in treatment of infectious disease.

Streptococcus pypgenes, more specifically the **Beta-hemolytic Group A Streptococci**, like *S. aureus*, causes an array of suppurative diseases and toxinoses (diseases due to the production of a bacterial toxin), in addition to some autoimmune or allergic diseases. *S. pyogenes* is rarely found as normal flora (<1%), but it is the main streptococcal pathogen for man, most often causing tonsillitis or **strep throat**. Streptococci also invade the skin to cause localized infections and lesions, and produce toxins that cause **scarlet fever** and toxic shock. Sometimes, as a result of an acute streptococcal infection, anomalous immune responses are started that lead to diseases like **rheumatic fever** and **glomerulonephritis**, which are called **post-streptococcal sequelae**. Unlike the staphylococci, the streptococci have not developed widespread resistance to penicillin and the other beta lactam antibiotics, so that the beta lactams remain drugs of choice for the treatment of acute streptococcal infections.

Streptococcus pneumoniae is the most frequent cause of bacterial **lobar pneumonia** in humans. It is also a frequent cause of **otitis media** (infection of the middle ear) and **meningitis**. The bacterium colonizes the nasopharynx and from there gains access to the lung or to the eustachian tube. If the bacteria descend into the lung they can impede engulfment by alveolar macrophages if they possess a capsule which somehow prevents the engulfment process. Thus, encapsulated strains are able to invade the lung and are virulent (cause disease) and noncapsulated strains, which are readily removed by phagocytes, are nonvirulent.

The *Neisseriaceae* comprise a family of Gram-negative BetaProteobacteria with metabolic characteristics similar to pseudomonads. The neisseriae are small, Gram-negative cocci usually seen in pairs with flattened adjacent sides. Most neisseriae are normal flora or harmless commensals of mammals living on mucous membranes. In humans they are common residents of the throat and upper respiratory tract. Two species are primary pathogens of humans, *Neisseria gonorrhoeae* and *Neisseria meningitidis,* the bacterial causes of gonorrhea and meningicoccal meningitis.

Neisseria gonorrhoeae is the second leading cause of sexually-transmitted disease in the U.S., causing over three million cases of **gonorrhea** annually. Sometimes, in

females, the disease may be unrecognized or asymptomatic such that an infected mother can give birth and unknowingly transmit the bacterium to the infant during its passage through the birth canal. The bacterium is able to colonize and infect the newborn eye resulting **neonatal ophthalmia**, which may produce blindness. For this reason (as well as to control Chlamydia which may also be present), an antimicrobial agent is usually added to the neonate eye at the time of birth.

Neisseria meningitidis is one bacterial cause of meningitis, an inflammation of the meninges of the brain and spinal cord. Other bacteria that cause meningitis include *Haemophilus influenzae*, *Staphylococcus aureus* and *Escherichia coli*. **Meningococcal meningitis** differs from other causes in that it is often responsible for epidemics of meningitis. It occurs most often in children aged 6 to 11 months, but it also occurs in older children and in adults. Meningococcal meningitis can be a rapidly fatal disease, and untreated meningitis has a mortality rate near 50 percent. However, early intervention with antibiotics is highly effective, and with treatment most individuals recover without permanent damage to the nervous system.

Lactic acid bacteria are Gram-positive, nonsporeforming rods and cocci which produce lactic acid as a sole or major end product of fermentation. They are important in the food industry as fermentation organisms in the production of cheese, yogurt, buttermilk, sour cream, pickles, sauerkraut, sausage and other foods. Important genera are *Streptococcus* and *Lactobacillus*. Some species are normal flora of the human body (found in the oral cavity, GI tract and vagina); some streptococci are pathogens of humans (see pyogenic cocci above). Certain oral lactic acid bacteria are responsible for the formation of dental plaque and the initiation of dental caries (cavities).

Endospore-forming bacteria produce a unique resting cell called an **endospore**. They are Gram-positive and usually rod-shaped, but there are exceptions. The two important genera are *Bacillus*, the members of which are aerobic sporeformers in the soils, and *Clostridium,* whose species are anaerobic sporeformers of soils, sediments and the intestinal tracts of animals.

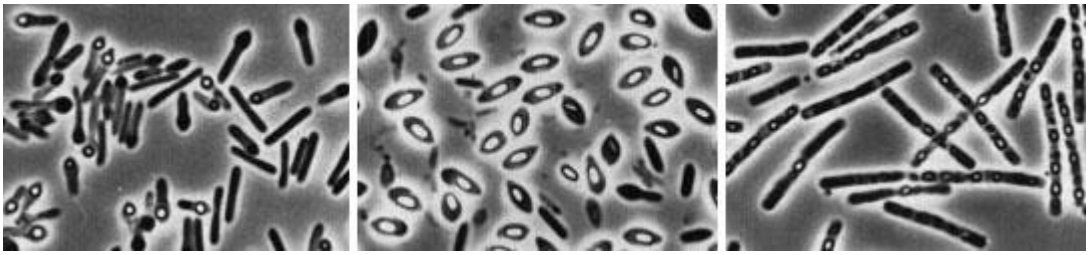


Figure 11. Endospore-forming bacilli (phase contrast illumination). Endospores are dehydrated, refractile cells appearing as points of bright light under phase microcsopy. Endospore-forming bacteria are characterized by the location (position) of the endospore in the mother cell (sporangium) before its release. The spore may be central, terminal or subterminal, and the sporangium may or may not be swollen to accomodate the spore.

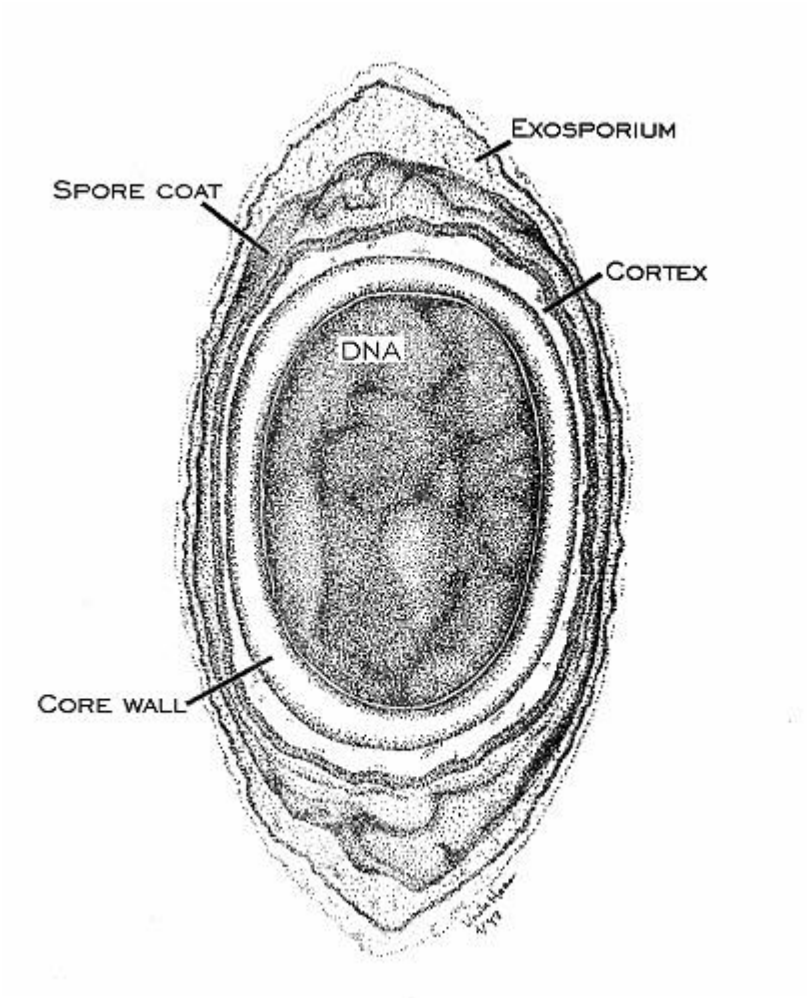


Figure 12. Anatomy of an endospore, cross section drawing by Viake Haas. Endospores differ from the vegetative cells that form them in a variety of ways. Several new surface layers develop outside the core (cell) wall, including the cortex and spore coat. The cytoplasm is dehydrated and contains only the cell genome and a few ribosomes and

enzymes. The endospore is cryptobiotic (exhibits no signs of life) and is remarkably resistant to environmental stress such as heat (boiling), acid, irradiation, chemicals and disinfectants. Some endospores have remained dormant for 25 million years preserved in amber, only to be shaken back into life when extricated and introduced into a favorable environment.

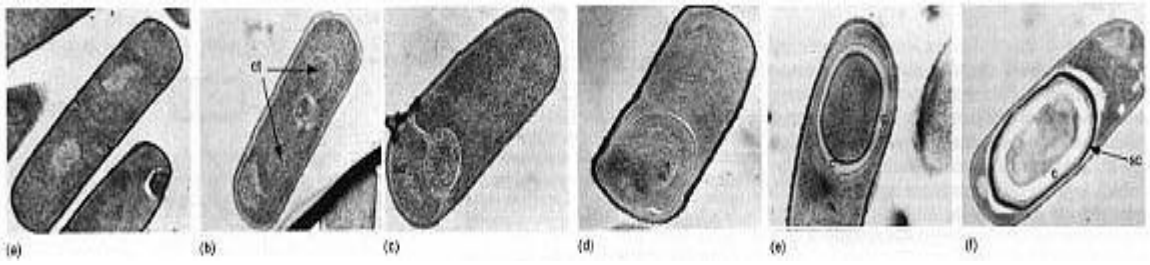


Figure 13. The sequential steps in the process of endospore formation in *Bacillus subtilis.*

Some sporeformers are pathogens of animals, usually due to the production of powerful toxins. *Bacillus anthracis* causes **anthrax**, a disease of domestic animals (cattle, sheep, etc.) which may be transmitted to humans. *Bacillus cereus* is becoming inceasingly recognized as an agent of food poisoning. *Clostridium botulinum* causes **botulism** a form of food-poisoning, and *Clostridium tetani* causes **tetanus**.

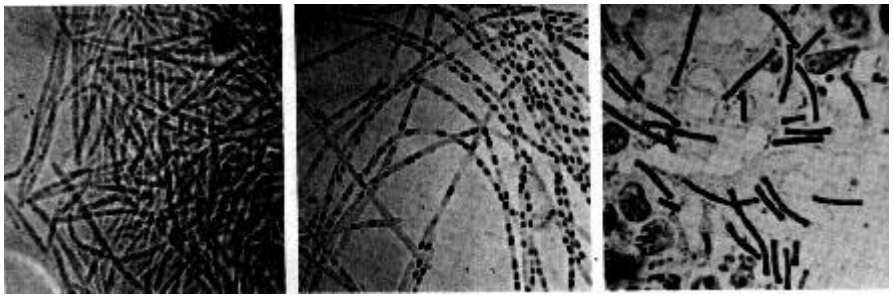


Figure 14. Robert Koch's original photomicrographs of *Bacillus anthracis.* In 1876, Koch established by careful microscopy that the bacterium was always present in the blood of animals that died of anthrax. He took a small amount of blood from such an animal and injected it into a healthy mouse, which subsequently became diseased and died. He took blood from that mouse and injected it into another healthy mouse. After repeating this several times he was able to recover the original anthrax organism from the dead mouse, demonstrating for the first time that a specific bacterium is the cause of a specific disease. In so doing, he established Koch's Postulates, which still today supply the microbiological standard to demonstrate that a specific microbe is the cause of a specific disease.

In association with the process of sporulation, some *Bacillus* species form a crystalline protein inclusion called **parasporal crystals**. The protein crystal and the

spore (actually the spore coat) are toxic to lepidopteran insects (certain moths and caterpillars) if ingested. The crystals and spores of *Bacillus thuringiensis* are marketed as "Bt" a natural insecticide for use on garden or crop plants. Another species of *Bacillus*, *B. cereus*, produces an antibiotic that inhibits growth of *Phytophthera,*, a fungus that attacks alfalfa seedling roots causing a "damping off" disease. The bacteria, growing in association with the roots of the seedlings, can protect the plant from disease.

Also, apparently in association with the sporulation process, some *Bacillus* species produce clinically-useful antibiotics. *Bacillus* antibiotics such as polymyxin and bacitracin are usually polypeptide molecules that contain unusual amino acids.

جامعة جنوب الوادى

كلية العلوم بقنا

الرؤيـــة

كلية العلوم بقنا تقدم خدمات تعليمية وبحثية ومجتمعية متميزة

الرســـالة

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PROF. DR. WESAM M. A. SALEM

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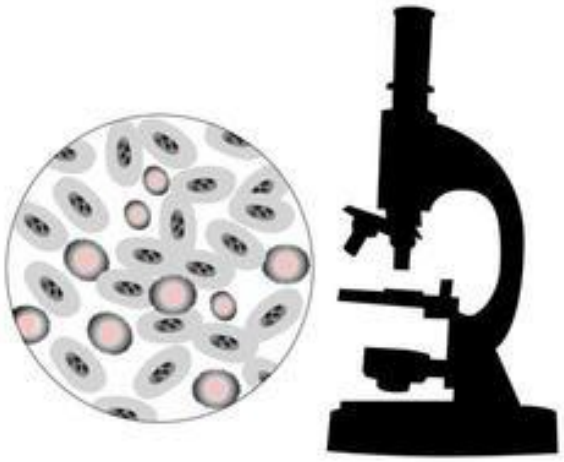


Part (1) Laboratory basics

LABORATORY SKILLS

A student successfully completing basic microbiology will demonstrate the ability to:

1. Use a bright-field light microscope to view and interpret slides, including:

a. correctly setting up and focusing the microscope

b. proper handling, cleaning and storage of the microscope

c. correct use of all lenses

d. recording microscopic observations

2. Properly prepare slides for microbiological examination, including:

a. cleaning and disposal of slides

b. preparing smears from solid and liquid cultures

c. performing wet- mount and/ or hanging drop preparations

d. performing Gram stains

3. Properly use aseptic techniques for the transfer and handling of microorganisms and instruments, including:

a. sterilizing and maintaining sterility of transfer instruments

b. performing aseptic transfer

c. obtaining microbial samples

4. Use appropriate microbiological media and test systems, including:

a. isolating colonies and/ or plaques

b. maintaining pure cultures

c. using biochemical test media

d. accurately recording macroscopic observations

5. Estimate the number of microorganisms in a sample using serial dilution techniques, including:

a. correctly choosing and using pipettes and pipetting devices

b. correctly spreading diluted samples for counting

c. extrapolating plate counts to obtain correct CFU in the starting sample

6. Use standard microbiology laboratory equipment correctly, including:

a. using the standard metric system for weights, lengths, diameters, and volumes

b. lighting and adjusting a laboratory burner

c. using an incubator

Laboratory Thinking Skills

A student successfully completing basic microbiology will demonstrate an increased skill level in:

1. Cognitive processes, including:

a. formulating a clear, answerable question

b. developing a testable hypothesis

c. predicting expected results

d. following an experimental protocol

2. Analysis skills, including:

a. collecting and organizing data in a systematic fashion

b. presenting data in an appropriate form (graphs, tables, figures, or descriptive paragraphs)

c. drawing appropriate conclusions based on the results

3. Communications skills, including:

a. discussing and presenting laboratory results or findings in the laboratory

4. Interpersonal and citizenry skills, including:

a. working effectively in groups or teams so that the task, results, and analysis are shared

b. effectively managing time and tasks to be done simultaneously, by individuals and within a group

c. integrating knowledge and making informed judgments about microbiology in everyday life

LABORATORY RULES AND GENERAL SAFETY

A student successfully completing basic microbiology will demonstrate the ability to explain and practice safe:

1. <u>Microbiological procedures</u>, including:

a. reporting all spills and broken glassware to the instructor and receiving instructions for cleanup

b. methods for aseptic transfer

c. minimizing or containing the production of aerosols and describing the hazards associated with aerosols

d. washing hands prior to and following laboratories and at any time contamination is suspected

e. never eating or drinking in the laboratory

f. disinfecting lab benches prior to and at the end of each lab session

g. identification and proper disposal of different types of waste

h. never applying cosmetics, including contact lenses, or placing objects (fingers, pencils) in the mouth or touching the face

i. understands the safety rules of the laboratory

j. good lab practice, including returning materials to proper locations, proper care and handling of equipment, and keeping the bench top clear of extraneous materials

2. <u>Protective procedures</u>, including:

a. tying long hair back, wearing personal protective equipment (eye protection, coats, closed shoes; glasses may be preferred to contact lenses), and using such equipment in appropriate situations

b. always using appropriate pipetting devices and understanding that mouth pipetting is forbidden

3. <u>Emergency procedures</u>, including:

a. locating and properly using emergency equipment (first aid kits, fire extinguishers, chemical safety showers, and emergency numbers)

b. reporting all injuries immediately to the instructor

c. following proper steps in the event of an emergency

LABORATORY PROTOCOL

Scheduling

Each laboratory session will begin with a short discussion to brief you on the availability of materials and procedures. Since the preliminary instructions start promptly at the beginning of the period, it is **extremely important that you are not late to class.**

Personal Items

When you first enter the lab, place all personal items such as jackets, bags, and books in some out of the way place for storage. Don't stack them on your desktop. Desk space is minimal and must be reserved for essential equipment and your laboratory manual. The storage place may be a drawer, locker, coatrack, or perimeter counter. Your instructor will indicate where they should be placed.

Attire

A lab coat must be worn at all times in the laboratory. It will protect your clothing from accidental contamination and stains in the lab. When leaving the laboratory, remove the coat or apron. In addition, long hair must be secured in a ponytail to prevent injury from Bunsen burners and contamination of culture material.

Terminology

Various terms such as sterilization, disinfection, germicides, sepsis, and aseptic techniques will be used here. To be sure that you understand exactly what they mean, the following definitions are provided.

Sterilization is a process in which all living microorganisms, including viruses, are destroyed. The organisms may be killed with steam, dry heat,

or incineration. If we say an article is sterile, we understand that it is completely free of all living microorganisms. Generally speaking, when we refer to sterilization as it pertains here to laboratory safety, we think, primarily, in terms of steam sterilization with the autoclave. The ultimate method of sterilization is to burn up the infectious agents or **incinerate** them. All biological wastes must ultimately be incinerated for disposal.

Disinfection is a process in which vegetative, nonsporing microorganisms are destroyed. Agents that cause disinfection are called **disinfectants** or **germicides.** Such agents are used only on inanimate objects because they are toxic to human and animal tissues.

Sepsis is defined as the growth (multiplication) of microorganisms in tissues of the body. The term **asepsis** refers to any procedure that prevents the entrance of infectious agents into sterile tissues, thus preventing infection. **Aseptic techniques** refer to those practices that are used by microbiologists to exclude all organisms from contaminating media or contacting living tissues. **Antiseptics** are chemical agents (often dilute disinfectants) that can be safely applied externally to human tissues to destroy or inhibit vegetative bacteria.

Accidental spills

All accidental spills, whether chemical or biological, must be reported immediately to your instructor. Although the majority of microorganisms used in this laboratory are nonpathogens, some pathogens will be encountered. It is for this reason that we must treat all accidental biological spills as if pathogens were involved.

Chemical spills are just as important to report because some agents used in this laboratory may be carcinogenic; others are poisonous; and some can cause dermal damage such as blistering and depigmentation.

Decontamination Procedure Once your instructor is notified of an accidental spill, the following steps will take place:

1. Any clothing that is contaminated should be placed in an autoclavable plastic bag and autoclaved.
2. Paper towels, soaked in a suitable germicide, such as 5% bleach, are placed over the spill.
3. Additional germicide should be poured around the edges of the spill to prevent further aerosolization.
4. After approximately 20 minutes, the paper towels should be scraped up off the floor with an autoclavable squeegee into an autoclavable dust pan.
5. The contents of the dust pan are transferred to an autoclavable plastic bag, which may itself be placed in a stainless steel bucket or pan for transport to an autoclave.
6. All materials, including the squeegee and dustpan, are autoclaved.

Pipette Handling

Success in this experiment depends considerably on proper pipetting techniques. Pipettes may be available to you in metal cannisters or in individual envelopes; they may be disposable or reusable. In the distant past pipetting by mouth was routine practice. However, the hazards are obvious, and today it must be avoided. Your instructor will indicate the techniques that will prevail in this laboratory. If this is the first time that you have used sterile pipettes, keep the following points that are shown in the following figure.

Pipette-handling techniques

ASEPTIC PROCEDURES

The proper handling of materials in the Bacteriology lab requires special skills that you must master. It's all about aseptic technique. The procedures discussed above entitled **Laboratory Protocol** outline some of the specifics to be observed to ensure that you understand what is required in maintaining an aseptic environment when handling cultures of microorganisms. In this exercise you will have an opportunity to actually work with cultures and different kinds of media to develop those skills that are required to maintain asepsis. Aseptic transfer of a culture from one culture vessel to another is successful only if no contaminating microorganisms are introduced in the process. A transfer may involve the transport of organisms from an isolated colony on a plate of solid medium to a broth tube, or inoculating various media (solid or liquid) from a broth culture for various types of tests.

When you start handling bacterial cultures, you will learn the specifics of aseptic techniques. The general procedure is as follows:

Hand Washing Before you start working in the lab, wash your hands with a liquid detergent and dry them with paper toweling. At the end of the period, before leaving the laboratory, wash them again.

Tabletop Disinfection. The first chore of the day will be to sponge down your desktop with a disinfectant. This process removes any dust that may be present and minimizes the chances of bacterial contamination of cultures that you are about to handle. Your instructor will indicate where the bottles of disinfectant and sponges are located. At the end of the period before leaving the laboratory, perform the same procedure to protect students that may occupy your desk in the next class.

Work Area Disinfection. The work area is first treated with a disinfectant to kill any microorganisms that may be present. This step destroys

vegetative cells and viruses; endospores, however, are not destroyed in this brief application of disinfectant.

Bunsen Burner Usage. When using a Bunsen burner to flame loops, needles, and test tubes, follow the instructed procedures. Inoculating loops and needles should be heated until they are red- hot. Before they are introduced into cultures, they must be allowed to cool down sufficiently to prevent killing organisms that are to be transferred. If your burner has a pilot on it and you plan to use the burner only intermittently, use it. If your burner lacks a pilot, turn off the burner when it is not being used. Excessive unnecessary use of Bunsen burners in a small laboratory can actually raise the temperature of the room. More important is the fact that unattended burner flames are a constant hazard to hair, clothing, and skin.

The proper handling of test tubes, while transferring bacteria from one tube to another, requires a certain amount of skill. Test tube caps must never be placed down on the desktop while you are making inoculations. Techniques that enable you to make transfers properly must be mastered.

Pipetting. Transferring solutions or cultures by pipette must always be performed with a mechanical suction device. Under no circumstances is pipetting by mouth allowed in this laboratory.

Disposal of Cultures and Broken Glass. The following rules apply to culture and broken glass disposal:

1. Petri dishes must be placed in a plastic bag to be autoclaved.
2. Unneeded test tube cultures must be placed in a wire basket to be autoclaved.
3. Used pipettes must be placed in a plastic bag for autoclaving.
4. Broken glass should be swept up into a dustpan and placed in a container reserved for broken glass. Don't try to pick up the glass fragments with your fingers.
5. Contaminated material must never be placed in a wastebasket.

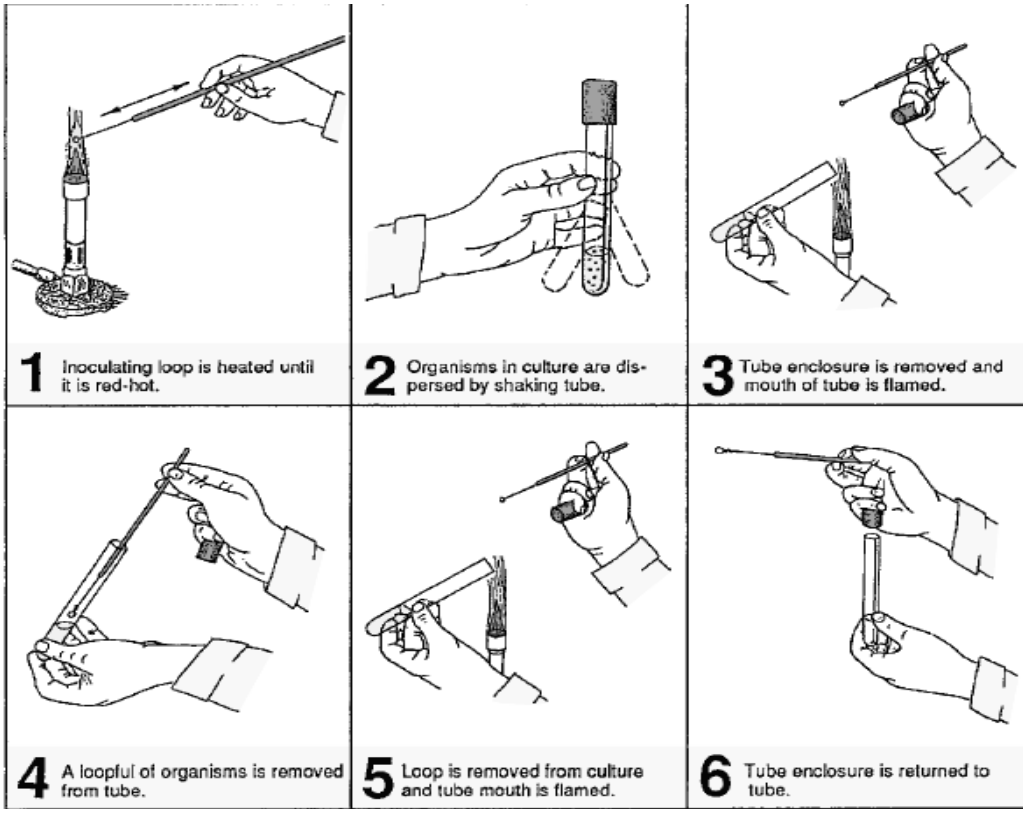
Loops and Needles. The transport of organisms will be performed with an inoculating loop or needle. To sterilize the loop or needle prior to picking up the organisms, heat must be applied with a Bunsen burner flame, rendering them glowing red- hot.

Culture Tube Flaming. Before inserting the cooled loop or needle into a tube of culture, the tube cap is removed and the mouth of the culture tube flamed. Once the organisms have been removed from the tube, the tube mouth must be flamed again before returning the cap to the tube.

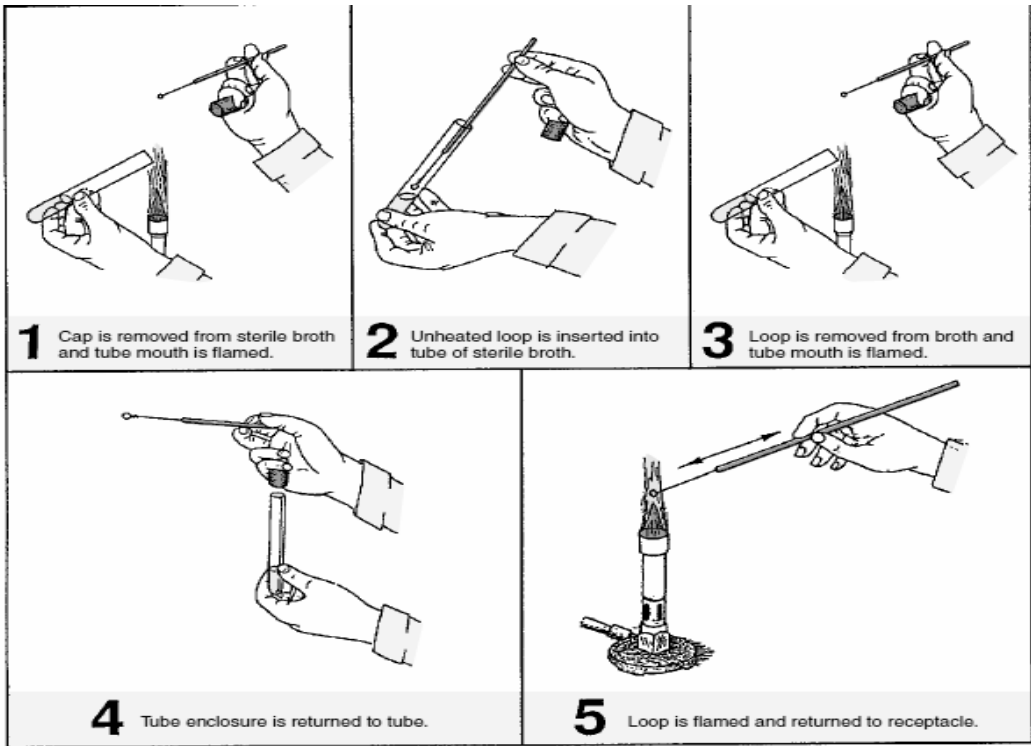
Liquid Medium Inoculation. If a tube of liquid medium is to be inoculated, the tube mouth must be flamed before inserting the loop into the tube. To disperse the organisms on the loop, the loop should be twisted back and forth in the medium. If an inoculating needle is used for stabbing a solid medium, the needle is inserted deep into the medium. Once the inoculation is completed, the loop or needle is removed from the tube, flamed as before, and returned to a receptacle. These tools should never be placed on the tabletop. The inoculated tube is also flamed before placing the cap on the tube.

Petri Plate Inoculation. To inoculate a Petri plate, no heat is applied to the plate and a loop is used for the transfer. When streaking the surface of the medium, the cover should be held diagonally over the plate bottom to prevent air contamination of the medium.

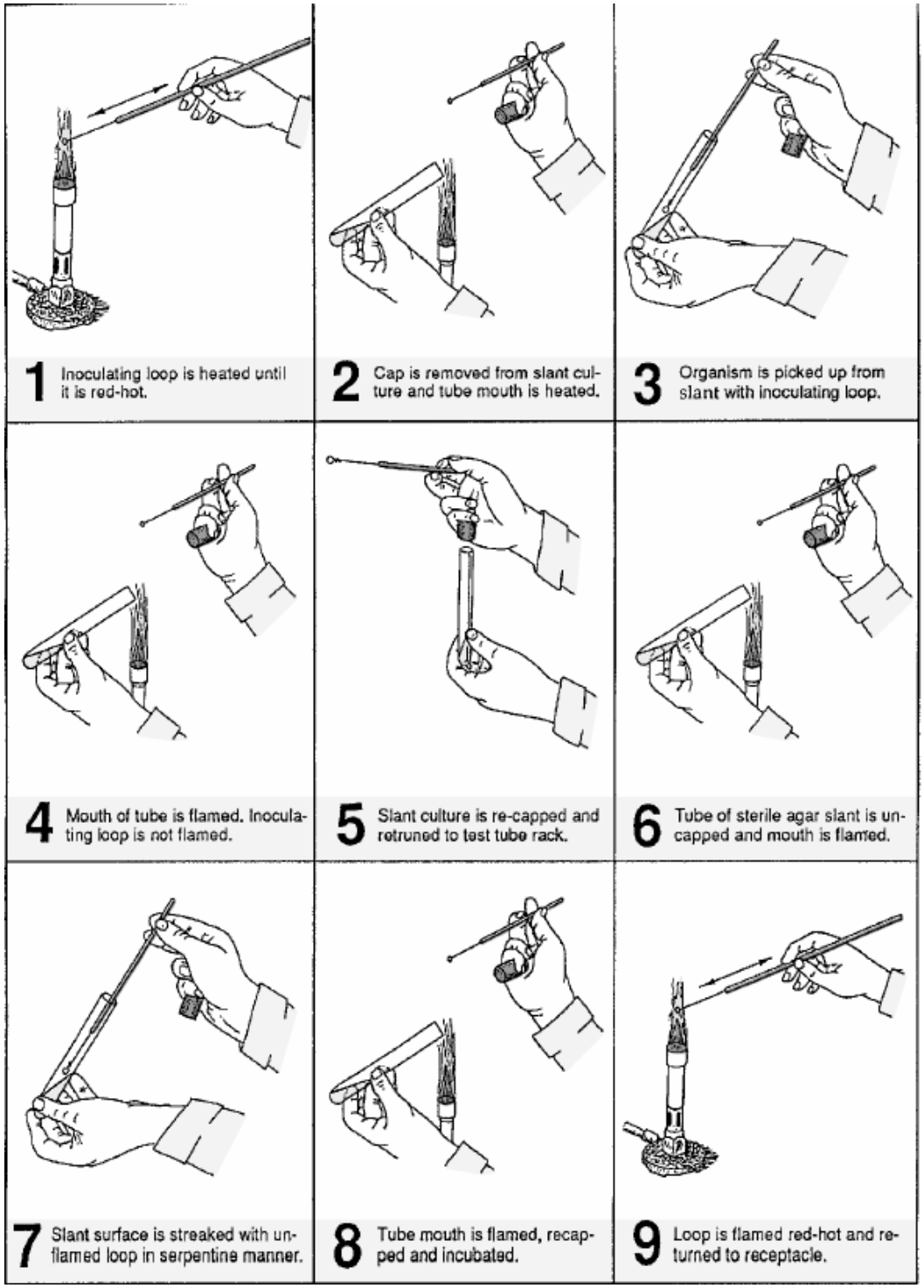
Final Disinfection. When all work is finished, the work area is treated with disinfectant to ensure that any microorganisms deposited during any of the procedures are eliminated. The following Figures illustrate some of these procedures.



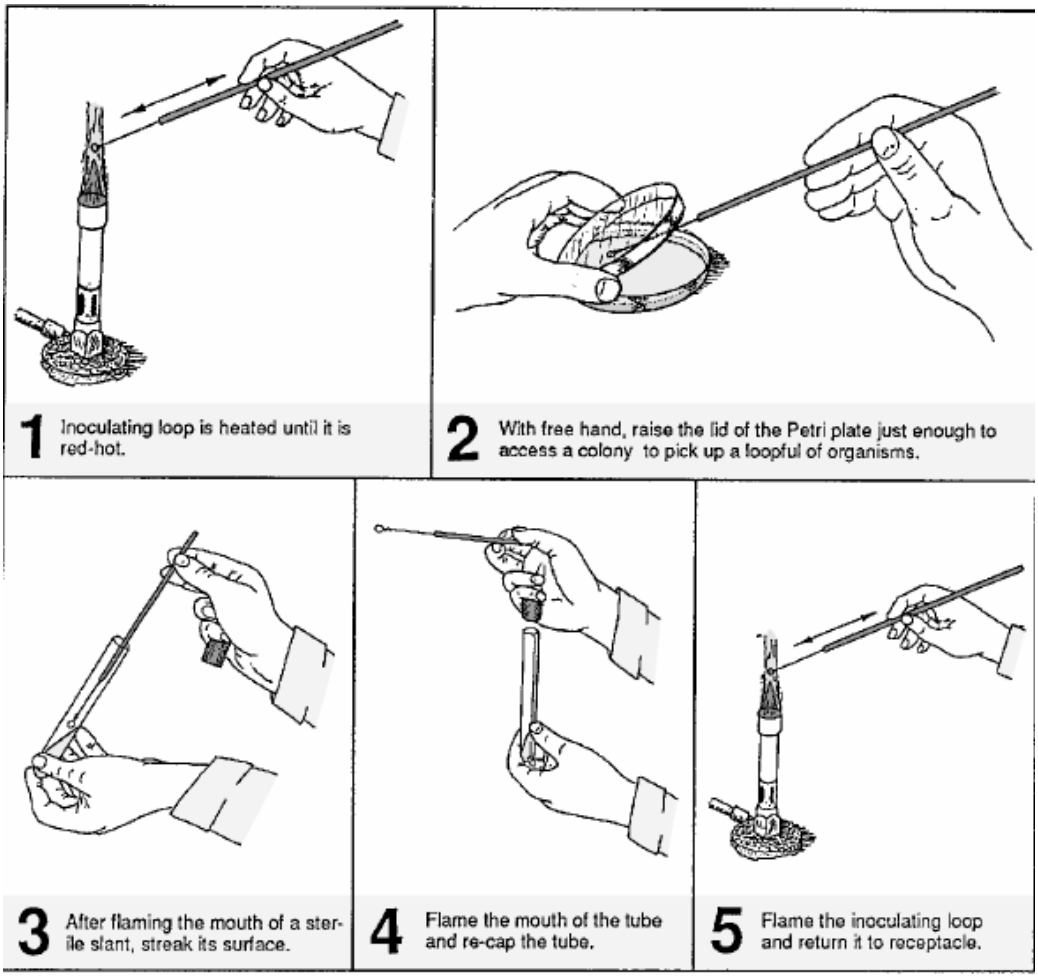
Procedure for removing organisms from a broth culture with inoculating loop



Procedure for inoculating a nutrient broth



Procedure for inoculating a nutrient agar slant from a slant culture



Procedure for inoculating a nutrient agar slant from an agar plate

ADDITIONAL IMPORTANT REGULATIONS

1. Don’t remove cultures, reagents, or other materials from the laboratory unless you have been granted specific permission.

2. Don’t smoke or eat food in the laboratory.

3. Make it a habit to keep your hands away from your mouth. Obviously, labels are never moistened with the tongue; use tap water or self-adhesive labels instead.

4. Always clean up after yourself. Gram stained slides that have no further use to you should be washed and dried and returned to a slide box. Coverslips should be cleaned, dried, and returned. Staining trays should be rinsed out and returned to their storage place.

5. Return all bulk reagent bottles to places of storage.

6. Return inoculating loops and needles to your storage container. Be sure that they are not upside down.

7. If you have borrowed something from someone, return it.

8. Do not leave any items on your desk at the end of the period.

9. Do not disturb another class at any time. Wait until the class is dismissed.

10. Treat all instruments, especially microscopes, with extreme care. If you don’t understand how a piece of equipment functions, ask your instructor.

11. Work cooperatively with other students in group assigned experiments, but do your own analyses of experimental results.

MICROSCOPY

Microscopes in a college laboratory represent a considerable investment and require special care to prevent damage to the lenses and mechanicals. The fact that a laboratory microscope may be used by several different individuals during the day and moved around from one place to another results in a much greater chance for damage and wear to occur than if the instrument were used by only one individual. The complexity of some of the more expensive microscopes also requires that certain adjustments be made periodically. Knowing how to make these adjustments to get the equipment to perform properly is very important. An attempt is made in the following exercises to provide the necessary assistance in getting the most out of the equipment.

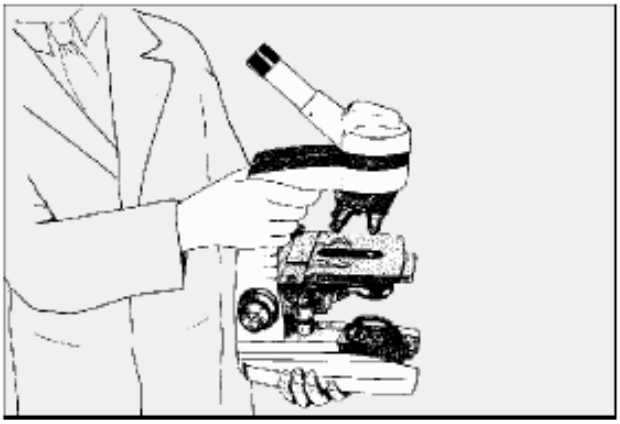
Brightfield Microscopy

A microscope that allows light rays to pass directly through to the eye without being deflected by an intervening opaque plate in the condenser is called a **brightfield microscope.** All brightfield microscopes have certain things in common, yet they differ somewhat in mechanical operation.

CARE OF THE INSTRUMENT

Microscopes represent considerable investment and can be damaged rather easily if certain precautions are not observed. The following suggestions cover most hazards.

Transport When carrying your microscope from one part of the room to another, use both hands when holding the instrument, as illustrated in the following figure. If it is carried with only one hand and allowed to dangle at your side, there is always the danger of collision with furniture or some other object. And, incidentally, under no circumstances should one attempt to carry two microscopes at one time.



The microscope should be held firmly with both hands while carrying it.

Clutter Keep your workstation uncluttered while doing microscopy. Keep unnecessary books, lunches, and other unneeded objects away from your work area. A clear work area promotes efficiency and results in fewer accidents.

Electric Cord Microscopes have been known to tumble off of tabletops when students have entangled a foot in a dangling electric cord. Don't let the light cord on your microscope dangle in such a way as to hazard foot entanglement.

Lens Care At the beginning of each laboratory period check the lenses to make sure they are clean. At the end of each lab session be sure to wipe any immersion oil off the immersion lens if it has been used.

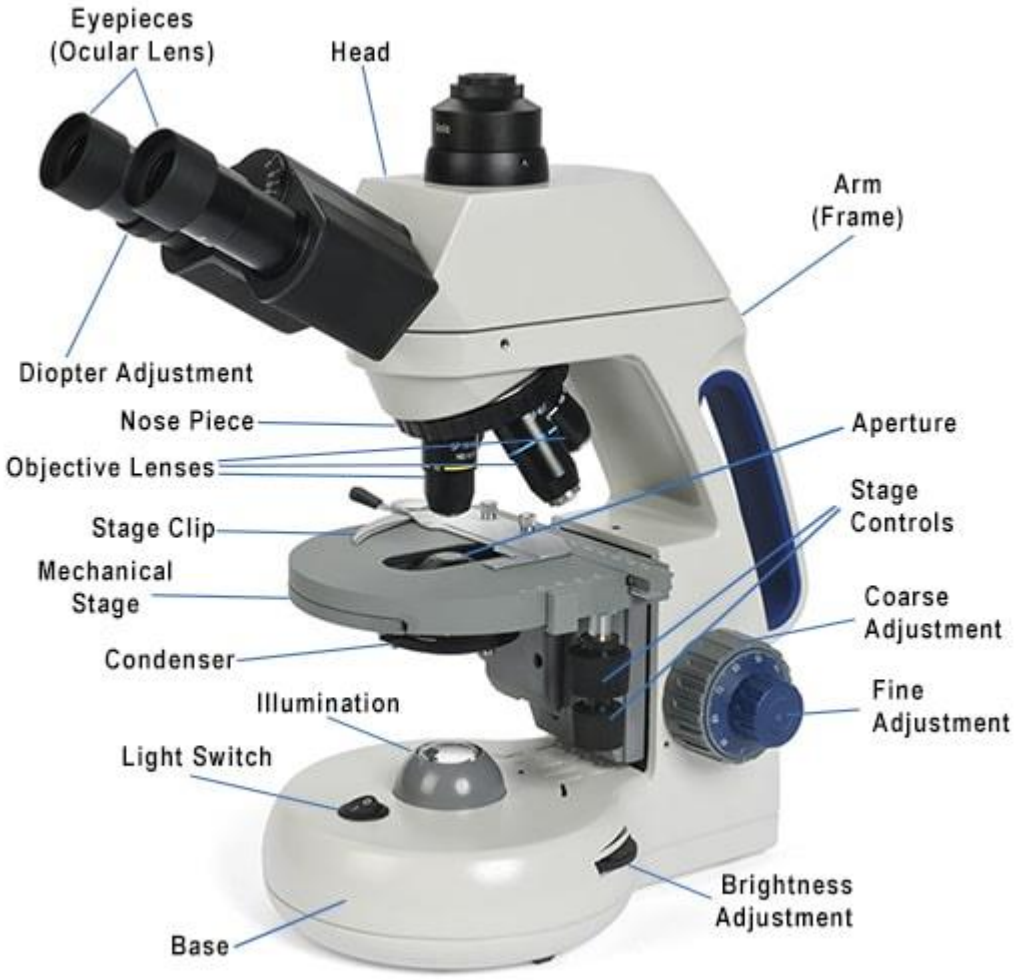
Dust Protection In most laboratories dustcovers are used to protect the instruments during storage. If one is available, place it over the microscope at the end of the period.

COMPONENTS

The principal parts of the instrument are illustrated in the following figure.

Framework All microscopes have a basic frame structure, which includes the **arm** and **base.** To this framework all other parts are attached. On many of the older microscopes the base is not rigidly attached to the arm; instead, a pivot point is present that enables one to tilt the arm backward to adjust the eyepoint height.

Stage The horizontal platform that supports the microscope slide is called the stage. Note that it has a clamping device, the **mechanical stage,** which is used for holding and moving the slide around on the stage. Note, also, the location of the **mechanical stage control** in figure

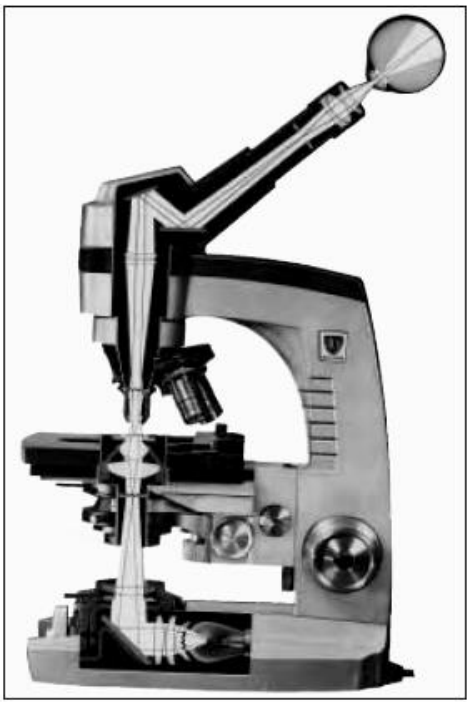


The compound microscope

Light Source In the base of most microscopes is positioned some kind of light source. Ideally, the lamp should have a **voltage control** to vary the intensity of light. The microscope in the above figure has a knurled wheel on its base to regulate the voltage supplied to the light bulb.

Most microscopes have some provision for reducing light intensity with a **neutral density filter.** Such a filter is often needed to reduce the intensity of light below the lower limit allowed by the voltage control.

Lens Systems All microscopes have three lens systems: the oculars, the objectives, and the condenser. The following Figure illustrates the light path through these three systems.



The light pathway of a microscope

The **ocular,** or eyepiece, is a complex piece, located at the top of the instrument, that consists of two or more internal lenses and usually has a magnification of 10X. Sometimes the microscope has two oculars (binocular) and others are monocular (as in the above figure).

Three or more **objectives** are usually present. Note that they are attached to a rotatable **nosepiece,** which makes it possible to move them into position over a slide. Objectives on most laboratory microscopes have magnifications of 10X, 45X, and 100X, designated as **low power, high-**

dry, and **oil immersion,** respectively. Some microscopes will have a fourth objective for rapid scanning of microscopic fields that is only 4X.

The third lens system is the **condenser,** which is located under the stage. It collects and directs the light from the lamp to the slide being studied. The condenser can be moved up and down by a knob under the stage. A **diaphragm** within the condenser regulates the amount of light that reaches the slide. Microscopes that lack a voltage control on the light source rely entirely on the diaphragm for controlling light intensity.

Focusing Knobs The concentrically arranged **coarse adjustment** and **fine adjustment knobs** on the side of the microscope are used for bringing objects into focus when studying an object on a slide. On some microscopes these knobs are not positioned concentrically. As the magnification of the lens increases, the distance between the objective lens and slide, called working distance, decreases, whereas the numerical aperture of the objective lens increases (see the following figure).

Ocular Adjustments On binocular microscopes one must be able to change the distance between the oculars and to make diopter changes for eye differences. On most microscopes the interocular distance is changed by simply pulling apart or pushing together the oculars.

RESOLUTION

The resolution limit, or **resolving power,** of a microscope lens system is a function of its numerical aperture, the wavelength of light, and the design of the condenser. The optimum resolution of the best microscopes with oil immersion lenses is around 0.2 µm. This means that two small objects that are 0.2 µm apart will be seen as separate entities; objects closer than that will be seen as a single object. To get the maximum amount of resolution from a lens system, the following factors must be taken into consideration:

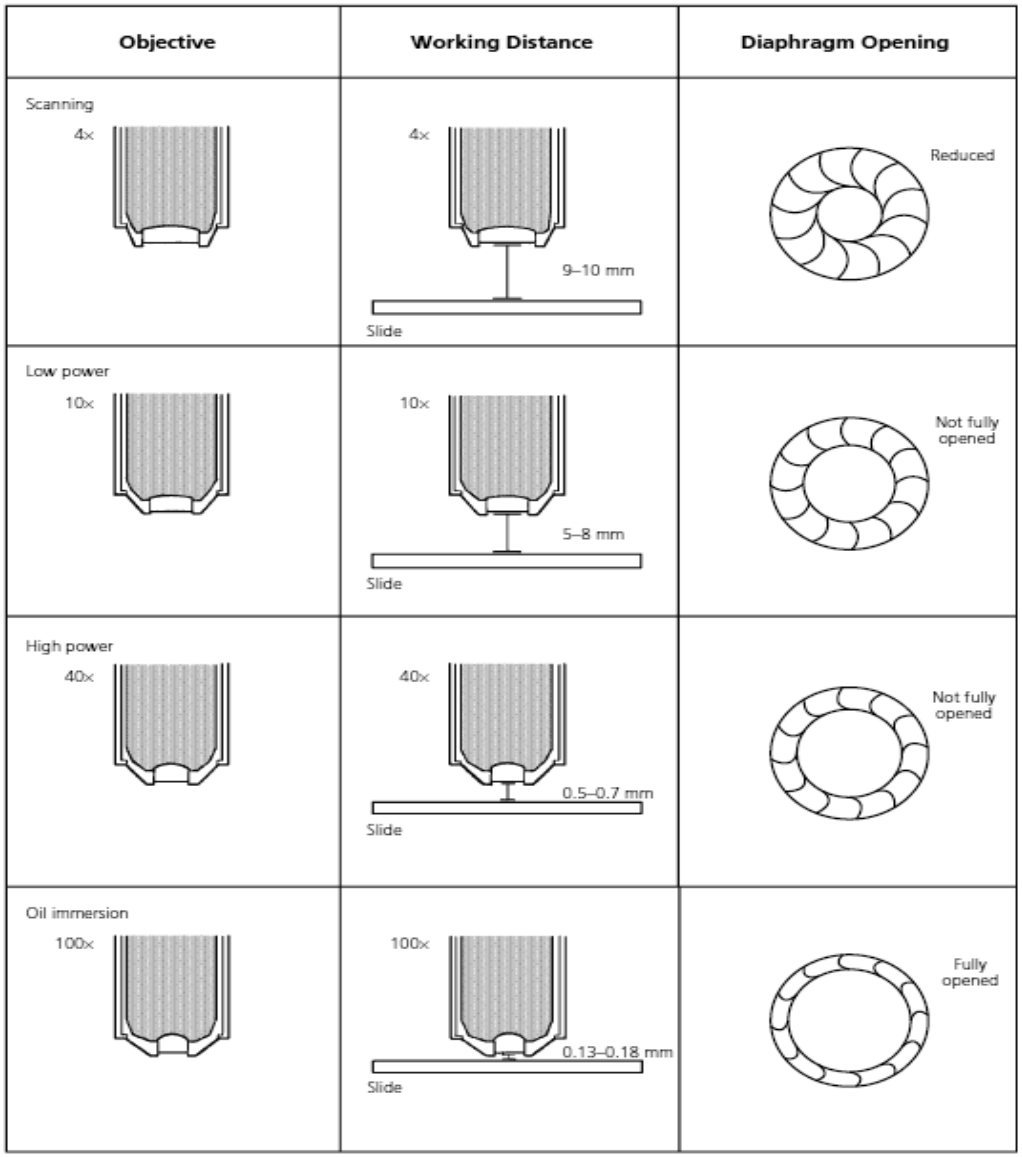
• A **blue filter** should be in place over the light source because the short wavelength of blue light provides maximum resolution.

• The **condenser** should be kept at its highest position where it allows a maximum amount of light to enter the objective.

• The **diaphragm** should not be stopped down too much. Although stopping down improves contrast, it reduces the numerical aperture.

• **Immersion oil** should be used between the slide and the 100X objective.

Of significance is the fact that increasing magnification won't increase the resolution.



Relationship between working distance, objective, and diaphragm opening

LENS CARE

Keeping the lenses of your microscope clean is a constant concern. Unless all lenses are kept free of dust, oil, and other contaminants, they are unable to achieve the degree of resolution that is intended.

Consider the following suggestions for cleaning the various lens components:

Cleaning Tissues Only lint- free, optically safe tissues should be used to clean lenses. Tissues free of abrasive grit fall in this category. Booklets of lens tissue are most widely used for this purpose. Although several types of boxed tissues are also safe, use only the type of tissue that is recommended by your instructor.

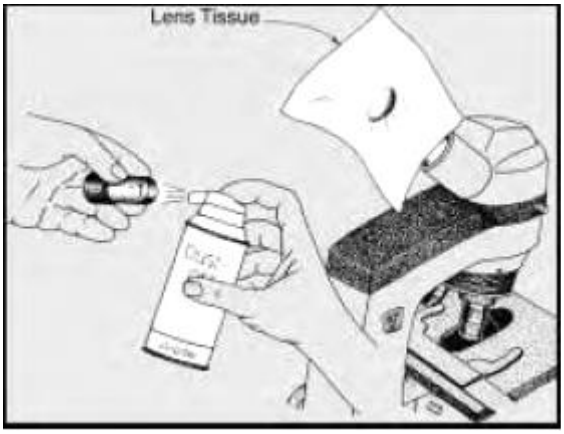
Solvents Various liquids can be used for cleaning microscope lenses. Green soap with warm water works very well. Xylene is universally acceptable. Alcohol and acetone are also recommended, but often with some reservations. Acetone is a powerful solvent that could possibly dissolve the lens mounting cement in some objective lenses if it were used too liberally. When it is used it should be used sparingly. Your instructor will inform you as to what solvents can be used on the lenses of your microscope.

Oculars The best way to determine if your eyepiece is clean is to rotate it between the thumb and forefinger as you look through the microscope. A rotating pattern will be evidence of dirt. If cleaning the top lens of the ocular with lens tissue fails to remove the debris, one should try cleaning the lower lens with lens tissue and blowing off any excess lint with an air syringe or gas cannister.

Whenever the ocular is removed from the microscope, it is imperative that a piece of lens tissue be placed over the open end of the microscope as illustrated in the following figure.

Objectives Objective lenses often become soiled by materials from slides or fingers. A piece of lens tissue moistened with green soap and water, or

one of the acceptable solvents mentioned above, will usually remove whatever is on the lens. Sometimes a cotton swab with a solvent will work better than lens tissue. At any time that the image on the slide is unclear or cloudy, assume at once that the objective you are using is soiled.



When oculars are removed for cleaning, cover the ocular opening with lens tissue. A blast from an air syringe or gas cannister removes dust and lint.

Condenser Dust often accumulates on the top surface of the condenser; thus, wiping it off occasionally with lens tissue is desirable.

PROCEDURES

If your microscope has three objectives you have three magnification options: (1) low- power, or 100X total (10x 10)

magnification, (2) high- dry magnification, which is 450X total with a 45X objective, and (3) 1000X total magnification with a 100X oil immersion objective. Note that the total magnification seen through an objective is calculated by simply multiplying the power of the ocular by the power of the objective.

Whether you use the low- power objective or the oil immersion objective will depend on how much magnification is necessary. Generally speaking, however, it is best to start with the low- power objective and progress to the higher magnifications as your study progresses. Consider the following

suggestions for setting up your microscope and making microscopic observations.

Low- Power Examination The main reason for starting with the low-power objective is to enable you to explore the slide to look for the object you are planning to study. Once you have found what you are looking for, you can proceed to higher magnifications. Use the following steps when exploring a slide with the low- power objective:

1. Position the slide on the stage with the material to be studied on the upper surface of the slide. The slide must be held in place by the mechanical stage retainer lever.

2. Turn on the light source, using a minimum amount of voltage. If necessary, reposition the slide so that the stained material on the slide is in the exact center of the light source.

3. Check the condenser to see that it has been raised to its highest point.

4. If the low- power objective is not directly over the center of the stage, rotate it into position. Be sure that as you rotate the objective into position it clicks into its locked position.

5. Turn the coarse adjustment knob to lower the objective until it stops. A built- in stop should prevent the objective from touching the slide. Be careful in this step because some microscopes has no built- in stop for lenses and this may cause permanent damage to the lens.

6. While looking down through the ocular (or oculars), bring the object into focus by turning the fine adjustment focusing knob. Don't readjust the coarse adjustment knob. If you are using a binocular microscope it will also be necessary to adjust the interocular distance and diopter adjustment to match your eyes.

7. Manipulate the diaphragm lever to reduce or increase the light intensity to produce the clearest, sharpest image. Note that as you close down the diaphragm to reduce the light intensity, the contrast improves and the

depth of field increases. Stopping down the diaphragm when using the low- power objective does not decrease resolution.

8. Once an image is visible, move the slide about to search out what you are looking for. The slide is moved by turning the knobs that move the mechanical stage.

9. Check the cleanliness of the ocular, using the procedure outlined earlier.

10. Once you have identified the structures to be studied and wish to increase the magnification, you may proceed to either high- dry or oil immersion magnification. However, before changing objectives, be sure to center the object you wish to observe.

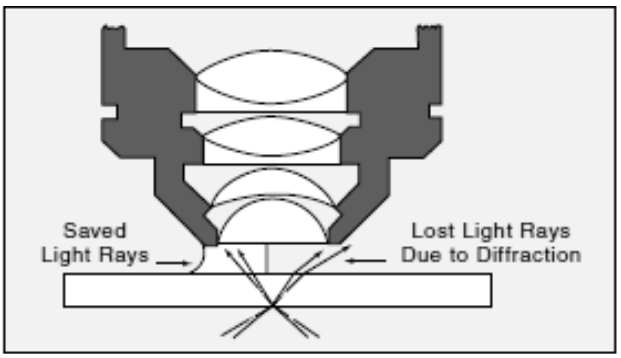
High- Dry Examination To proceed from low power to high- dry magnification, all that is necessary is to rotate the high- dry objective into position and open up the diaphragm somewhat. It may be necessary to make a minor adjustment with the fine adjustment knob to sharpen up the image, but the coarse adjustment knob should not be touched.

If a microscope is of good quality, only minor focusing adjustments are needed when changing from low power to high- dry because all the objectives will be **parfocalized.** Non parfocalized microscopes do require considerable refocusing when changing objectives. High- dry objectives should be used only on slides that have cover glasses; without them, images are usually unclear. When increasing the lighting, be sure to open up the diaphragm first instead of increasing the voltage on your lamp; reason: lamp life is greatly extended when used at low voltage. If the field is not bright enough after opening the diaphragm, feel free to increase the voltage. A final point: Keep the condenser at its highest point.

Oil Immersion Techniques The oil immersion lens derives its name from the fact that a special mineral oil is interposed between the lens and the microscope slide. The oil is used because it has the same refractive index as glass, which prevents the loss of light due to the bending of light rays as

they pass through air. The use of oil in this way enhances the resolving power of the microscope. The following Figure reveals this phenomenon.

With parfocalized objectives one can go to oil immersion from either low power or high- dry. On some microscopes, however, going from low power to high power and then to oil immersion is better.



Immersion oil, having the same refractive index as glass, prevents light loss due to diffraction.

Once the microscope has been brought into focus at one magnification, the oil immersion lens can be rotated into position without fear of striking the slide. Before rotating the oil immersion lens into position, however, a drop of immersion oil must be placed on the slide. An oil immersion lens should never be used without oil. Incidentally, if the oil appears cloudy it should be discarded.

When using the oil immersion lens it is best to open the diaphragm as much as possible. Stopping down the diaphragm tends to limit the resolving power of the optics. In addition, the condenser must be kept at its highest point. If different colored filters are available for the lamp housing, it is best to use blue or greenish filters to enhance the resolving power. Since the oil immersion lens will be used extensively in all bacteriological studies, it is of paramount importance that you learn how to use this lens properly. Using this lens takes a little practice due to the difficulties usually encountered in manipulating the lighting.

At the end of the laboratory period remove all immersion oil from the lens tip with lens tissue.

PUTTING IT AWAY

When you take a microscope from the cabinet at the beginning of the period, you expect it to be clean and in proper working condition. The next person to use the instrument after you have used it will expect the same consideration. A few moments of care at the end of the period will ensure these conditions. Check over this list of items at the end of each period before you return the microscope to the cabinet.

1. Remove the slide from the stage.

2. If immersion oil has been used, wipe it off the lens and stage with lens tissue. (Do not wipe oil off slides you wish to keep. Simply put them into a slide box and let the oil drain off.)

3. Rotate the low- power objective into position.

4. If the microscope has been inclined, return it to an erect position.

5. If the microscope has a built- in movable lamp, raise the lamp to its highest position.

6. If the microscope has a long attached electric cord, wrap it around the base or leave it as it is for the staff to do the job.

7. Adjust the mechanical stage so that it does not project too far on either side.

8. Replace the dustcover (if any).

9. If the microscope has a separate transformer, return it to its designated place.

10. Return the microscope to its correct place in the cabinet.

Part (2) Laboratory Exercises

MICROSCOPE SLIDE TECHNIQUES

(BACTERIAL MORPHOLOGY)

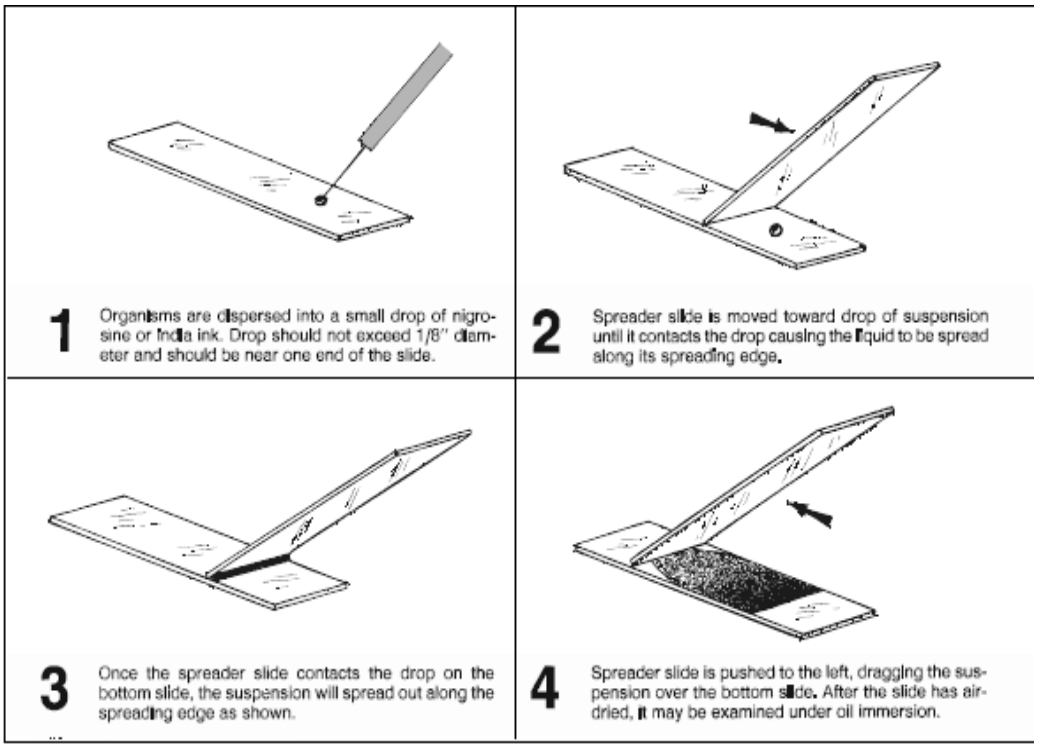
These exercises are intended to serve two important functions: (1) to help you to develop the necessary skills in making slides and (2) to introduce you to the morphology of bacteria.

Although the title of each exercise pertains to a specific technique, the organisms chosen for each method have been carefully selected so that you can learn to recognize certain morphological features. For example, in Gram staining you will observe the differences between cocci and bacilli, as well as learn how to execute the staining routine. Although one is seldom able to make species identification on the basis of morphological characteristics alone, it is a very significant starting point. This fact will become increasingly clear with subsequent experiments. Although the steps in the various staining procedures may seem relatively simple, student success is often quite unpredictable. Unless your instructor suggests a variation in the procedure, try to follow the procedures exactly as stated.

NEGATIVE STAINING

The simplest way to make a slide of bacteria is to prepare a wet mount. Although this method will quickly produce a slide, finding the bacteria on the slide may be difficult, especially for a beginner. The problem one encounters is that bacteria are quite colorless and transparent. Unless the diaphragm is carefully adjusted, the beginner usually has considerable difficulty bringing the organisms into focus. A better way to observe bacteria for the first time is to prepare a slide by a process called **negative,** or **background staining.** This method consists of mixing the microorganisms in a small amount of nigrosine or india ink and spreading the mixture over the surface of a slide. (Incidentally, nigrosine is far

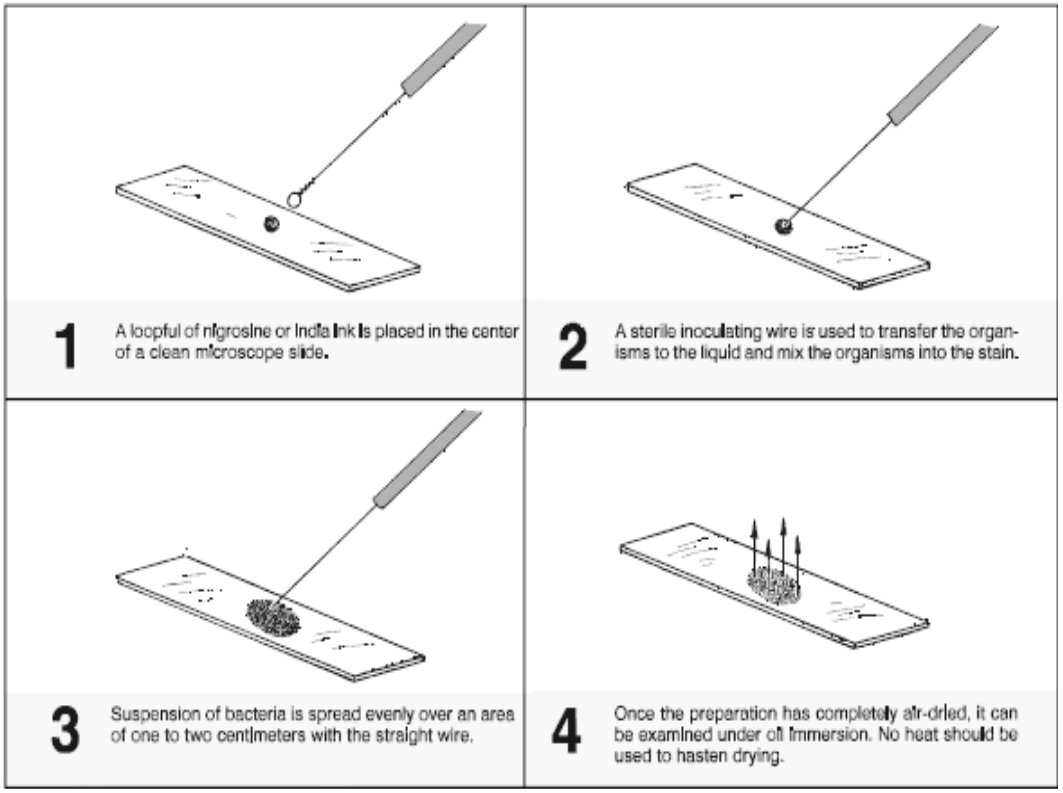
superior to india ink.) Since these two pigments are not really bacterial stains, they do not penetrate the microorganisms. Instead they stain the background, leaving the organisms transparent and visible in a darkened field. Although this technique has limitations, it can be useful for determining cell morphology and size. Since no heat is applied to the slide, there is no shrinkage of the cells, and, consequently, more accurate cell- size determinations result than with some other methods. This method is also useful for studying spirochaetes that don't stain readily with ordinary dyes.



Negative staining technique, using a spreader slide

Negative staining can be done by one of three different methods. The Figure above illustrates the more commonly used method in which the organisms are mixed in a drop of nigrosine and spread over the slide with another slide. The goal is to produce a smear that is thick at one end and feather- thin at the other end. Somewhere between the too thick and too thin areas will be an ideal spot to study the organisms.

The Figure below illustrates a second method, in which organisms are mixed in only a loopful of nigrosine instead of a full drop. In this method the organisms are spread over a smaller area in the center of the slide with an inoculating needle. No spreader slide is used in this method.



A second method for negative staining

The third procedure (Woeste- Demchick's method), which is not illustrated here, involves applying ink to a conventional smear with a black felt marking pen. If this method is used, it should be done on a smear prepared in the manner described in the next exercise. Simply put, the technique involves applying a single coat of felt- pen ink over a smear.

Note in the procedure below that slides may be made from organisms between your teeth or from specific bacterial cultures. Your instructor will indicate which method or methods you should use and demonstrate some basic aseptic techniques. Various options are provided here to ensure success.

Materials

- microscope slides (with polished edges)
- nigrosine solution or india ink
- slant cultures of *S. aureus* and *B. megaterium* (or as instructed)
- inoculating straight wire and loop
- sterile toothpicks
- Bunsen burner
- china marking pencil
- felt marking pen

Procedure

1. Swab down your tabletop with disinfectant in preparation for making slides.

2. Clean two or three microscope slides with alcohol to rid them of all dirt and grease.

3. Place the proper amount of stain on the slide (as in the figures above).

4. Oral Organisms: Remove a small amount of material from between your teeth with a sterile straight toothpick or inoculating needle and mix it into the stain on the slide. Be sure to break up any clumps of organisms with the wire or toothpick. When using a wire, be sure to flame it first to make it sterile.

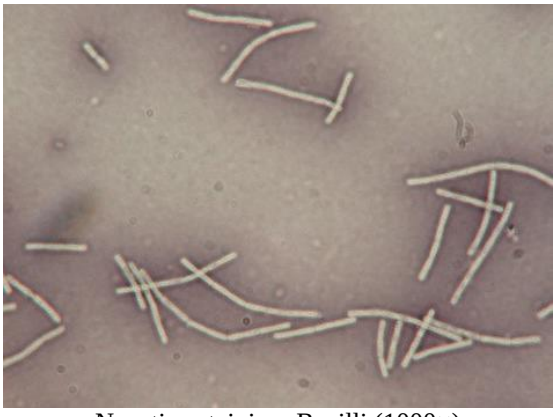
5. From Cultures: With a sterile straight wire, transfer a very small amount of bacteria from the slant to the center of the stain on the slide.

6. Spread the mixture over the slide according to the procedure used in figures above.

7. Allow the slide to air- dry and examine with an oil immersion objective

CAUTION

If you use a toothpick, discard it into a beaker of disinfectant.



Negative staining: Bacilli (1000×)

Notes and Results

(Draw and describe)

SMEAR PREPARATION

While negative staining is a simple enough process to make bacteria more visible with a brightfield microscope, it is of little help when one attempts to observe anatomical microstructures such as flagella, granules, and endospores. Only by applying specific bacteriological stains to organisms can such organelles be seen. However, success at bacterial staining depends first of all on the preparation of a suitable **smear** (thin film) of the organisms. A properly prepared bacterial smear is one that withstands one or more washings during staining without loss of organisms, is not too thick, and does not result in excessive distortion due to cell shrinkage.

The procedure for making such a smear is illustrated
in the following figure.

The first step in preparing a bacteriological smear differs according to the source of the organisms. If the bacteria are growing in a liquid medium (broths, milk, saliva, urine, etc.), one starts by placing one or two loopfuls of the liquid medium directly on the slide. From solid media such as nutrient agar, blood agar, or some part of the body, one starts by placing one or two loopfuls of water on the slide and then uses a straight inoculating wire to disperse the organisms in the water. Bacteria growing on solid media tend to cling to each other and must be dispersed sufficiently by dilution in water; unless this is done, the smear will be too thick. The most difficult concept for students to understand about making slides from solid media is that it takes only a very small amount of material to make a good smear. When your instructor demonstrates this step, pay very careful attention to the amount of material that is placed on the slide. Your instructor will indicate which cultures to use.

From liquid media (Broths, saliva, milk, etc.)

If you are preparing a bacterial smear from liquid media, follow this routine, which is depicted on the left side of the figure.

Materials

- microscope slides
- Bunsen burner
- wire loop
- marking pencil
- slide holder, optional

Procedure

1. Wash a slide with soap alcohol and hot water, removing all dirt and grease. Handle the clean slide by its edges.

2. Write the initials of the organism or organisms on the left- hand side of the slide with a china marking pencil.

3. To provide a target on which to place the organisms, make about 1 cm circle on the bottom side of the slide, centrally located, with a marking pencil. Later on, when you become more skilled, you may wish to omit the use of this "target circle."

4. Shake the culture vigorously and transfer two loopfuls of organisms to the center of the slide over the target circle. Follow the routine for inoculations. Be sure to flame the loop after it has touched the slide.

CAUTION

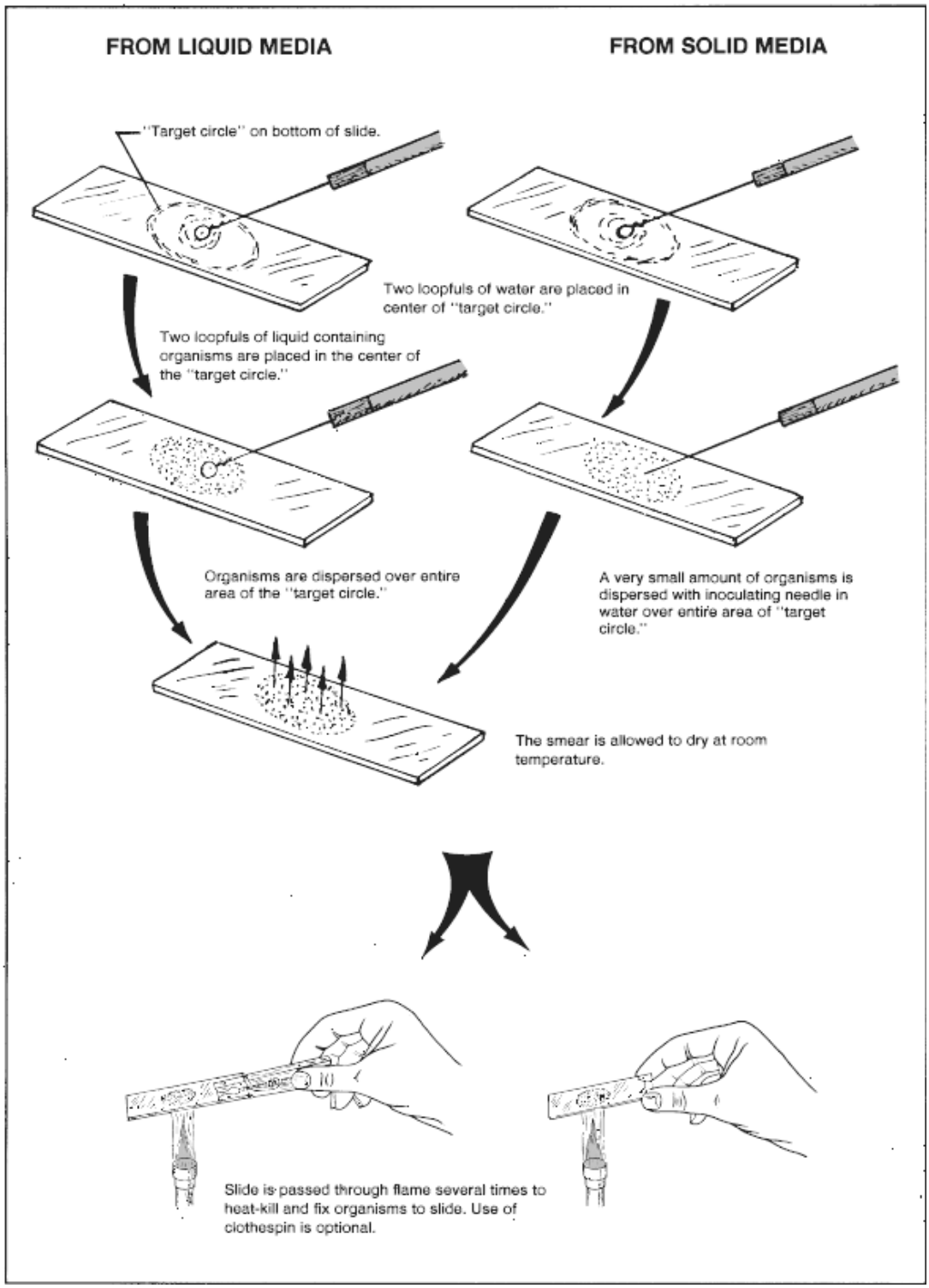
Be sure to cool the loop completely before inserting it into a medium. A loop that is too hot will spatter the medium and move bacteria into the air.

5. Spread the organisms over the area of the target circle.

6. Allow the slide to dry by normal evaporation of the water (at room temperature). Don't apply heat.

7. After the smear has become completely dry, pass the slide over a Bunsen burner flame to heat- kill the organisms and fix them to the slide.

Note that in this step one has the option of using or not using a slide holder.



Procedure for making a bacterial smear

FROM SOLID MEDIA

When preparing a bacterial smear from solid media, such as nutrient agar or a part of the body, follow this routine, which is depicted on the right side of the above figure.

Materials

- microscope slides
- inoculating needle and loop
- china marking pencil
- slide holder, optional
- Bunsen burner

Procedure

1. Wash a slide with soap or alcohol and hot water, removing all dirt and grease. Handle the clean slide by its edges.

2. Write the initials of the organism or organisms on the left- hand side of the slide with a china marking pencil.

3. Mark a "target circle" on the bottom side of the slide with a china marking pencil.

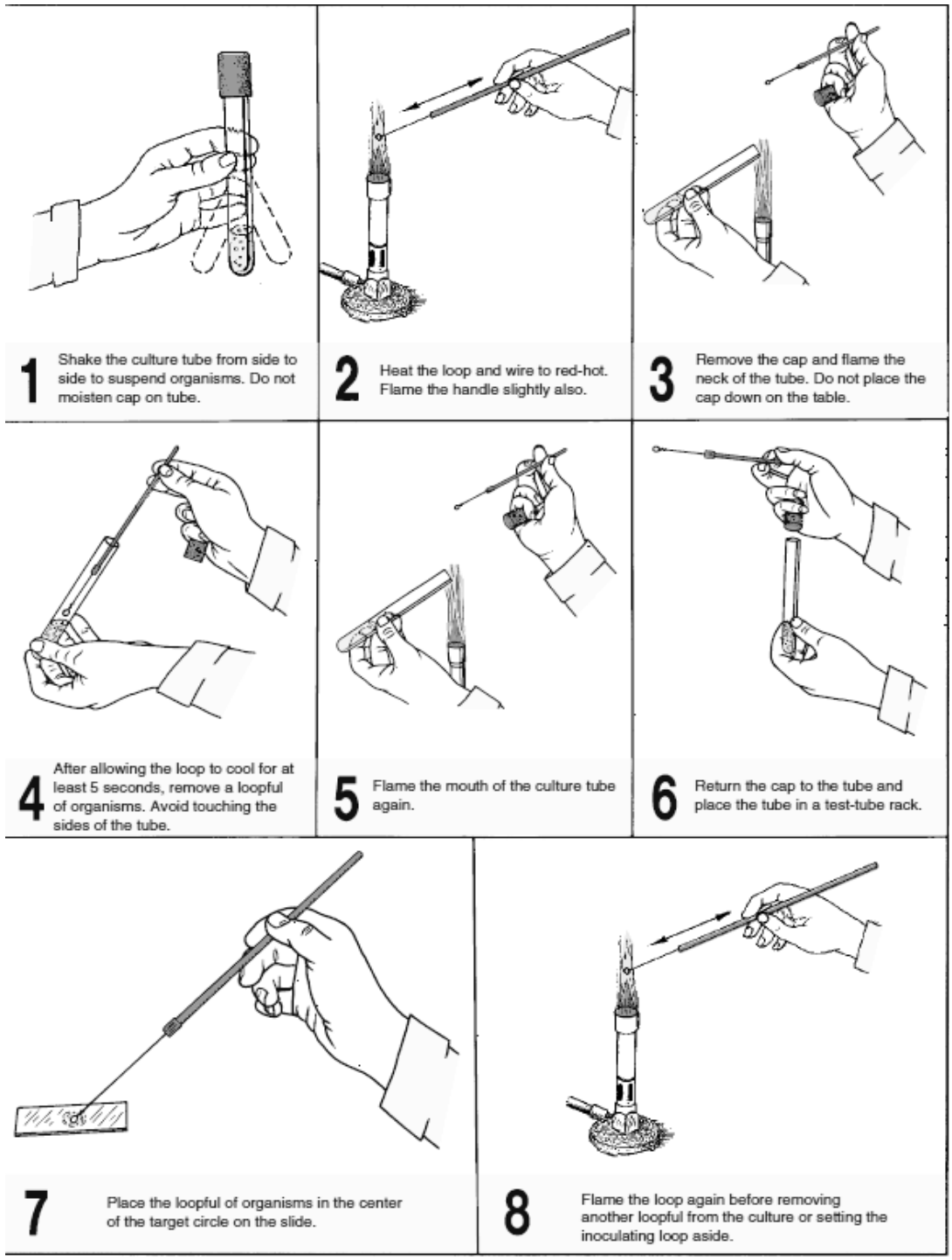
4. Flame an inoculating loop, let it cool, and transfer two loopfuls of water to the center of the target circle.

5. Flame an inoculating needle then let it cool. Pick up a very small amount of the organisms, and mix it into the water on the slide. Disperse the mixture over the area of the target circle. Be certain that the organisms have been well emulsified in the liquid. Be sure to flame the inoculating needle before placing it aside.

6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.

7. Once the smear is completely dry, pass the slide over the flame of a Bunsen burner to heat- kill the organisms and fix them to the slide. Use a slide holder if it is preferred by your instructor. Some workers prefer to

hold the slide with their fingers so that they can monitor the temperature of the slide (to prevent overheating).



Aseptic procedure for organism removal

Notes and Results

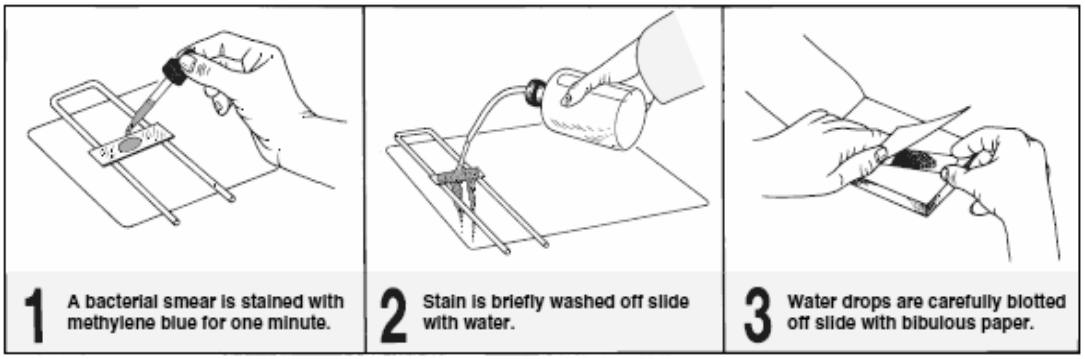
SIMPLE STAINING

The use of a single stain to color a bacterial organism is commonly referred to as **simple staining.** Some of the most commonly used dyes for simple staining are methylene blue, basic fuchsin, and crystal violet. All of these dyes work well on bacteria because they have color- bearing ions (chromophores) that are positively charged (cationic). The fact that bacteria are slightly negatively charged produces a pronounced attraction between these cationic chromophores and the organism. Such dyes are classified as **basic dyes.** Those dyes that have anionic chromophores are called **acidic dyes.** Eosin (sodium eosinate) is such a dye. The anionic chromophore, eosinate, will not stain bacteria because of the electrostatic repelling forces that are involved.

The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

PROCEDURE

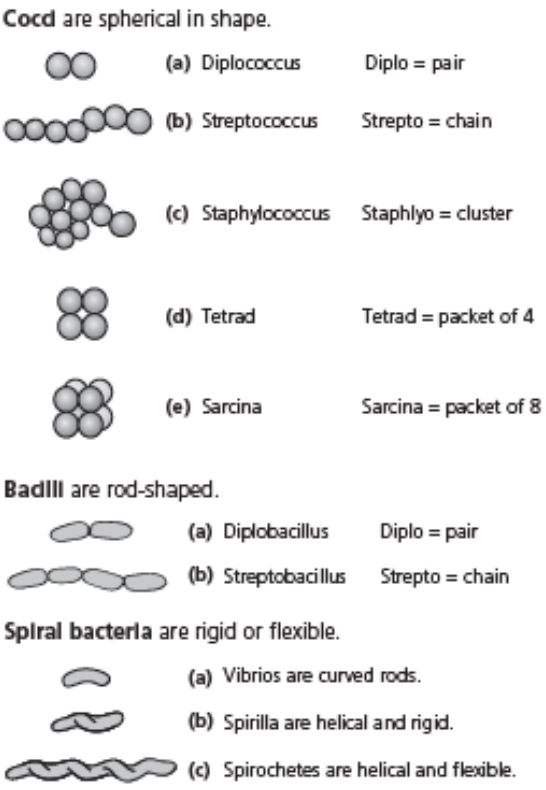
Prepare a slide of *Bacillus subtilis,* using the procedure outlined in the following figure.



Procedure for simple staining

Materials

- slant culture of *B. subtilis*
- methylene blue (Loeffler's)
- wash bottle
- bibulous paper



Bacterial shapes and arrangements

Notes and Results

CAPSULAR STAINING

Some bacterial cells are surrounded by a pronounced gelatinous or slimy layer called a **capsule.** There is considerable evidence to support the view that all bacteria have some amount of slime material surrounding their cells. In most instances, however, the layer is not of sufficient magnitude to be readily discernible. Although some capsules appear to be made of glycoprotein, others contain polypeptides. All appear to be water-soluble.

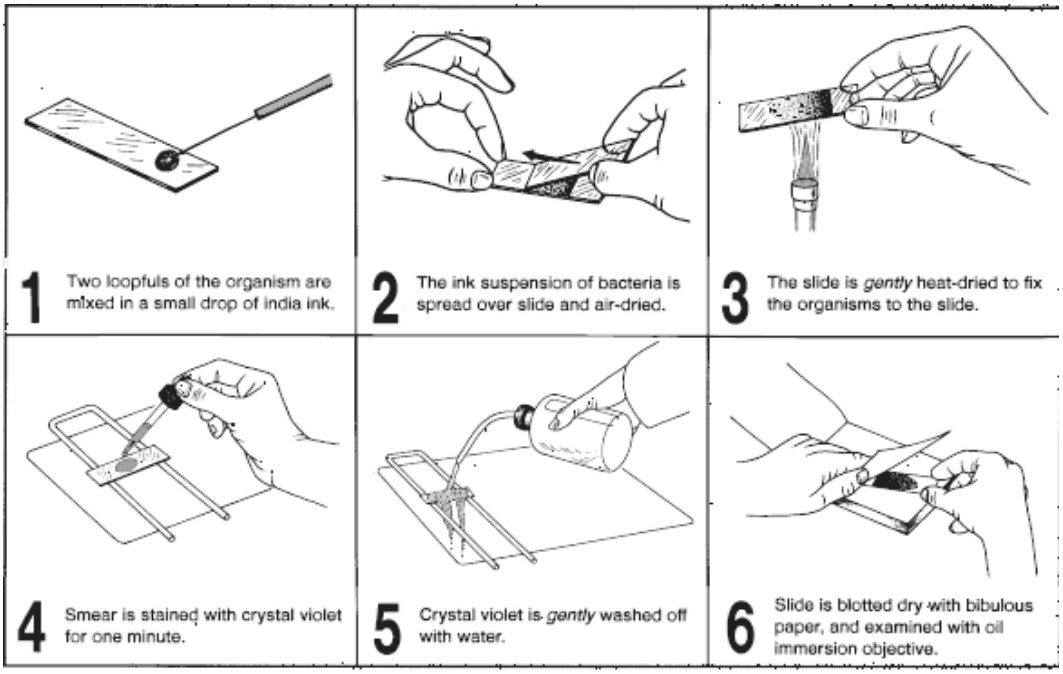
Staining the bacterial capsule cannot be accomplished by ordinary simple staining procedures. The problem with trying to stain capsules is that if you prepare a heat- fixed smear of the organism by ordinary methods, you will destroy the capsule; and, if you do not heat- fix the slide, the organism will slide off the slide during washing. In most of our bacteriological studies our principal concern is simply to demonstrate the presence or absence of a pronounced capsule. This can be easily achieved by combining negative and simple staining techniques, as in the following figure. To learn about this technique prepare a capsule "stained" slide of *Klebsiella pneumoniae* (or as instructed), using the procedure outlined in the figure.

Materials

- 36–48 hour milk culture of the used strain
- india ink
- crystal violet

Observation: Examine the slide under oil immersion and describe your results.

Note that you have always to draw the shape and arrangement of cells in each exercise.



Procedure for demonstration of capsule presence

Your Results

GRAM STAINING

In 1884 the Danish bacteriologist Christian Gram developed a staining technique that separates bacteria into two groups: those that are Gram-positive and those that are Gram- negative. The procedure is based on the ability of microorganisms to retain the purple color of crystal violet during decolorization with alcohol. Gram- negative bacteria are decolorized by the alcohol, losing the purple color of crystal violet. Gram- positive bacteria are not decolorized and remain purple. After decolorization, safranin, a red counterstain, is used to impart a pink color to the decolorized Gram- negative organisms. Note that crystal violet, the **primary stain,** causes both Gram- positive and Gram- negative organisms to become purple after 20 seconds of staining. When Gram's iodine, the **mordant,** is applied to the cells for one minute, the color of Gram-positive and Gram- negative bacteria remains the same: purple. The function of the mordant here is to combine with crystal violet to form a relatively insoluble compound in the Gram- positive bacteria. When the **decolorizing agent,** 95% ethanol, is added to the cells for 10– 20 seconds, the Gram- negative bacteria are leached colorless, but the Gram- positive bacteria remain purple. In the final step a **counterstain,** safranin, adds a pink color to the decolorized Gram- negative bacteria without affecting the color of the purple Gram-positive bacteria.

Of all the staining techniques you will use in the identification of unknown bacteria, Gram staining is, undoubtedly, the most important tool you will use. Although this technique seems quite simple, performing it with a high degree of reliability is a goal that requires some practice and experience. Here are two suggestions that can be helpful: first, don't make your smears too thick, and second, pay particular attention to the comments in step 4 on the next page that pertain to decolorization. When working with unknowns keep in mind that old cultures of Gram- positive bacteria tend

to decolorize more rapidly than young ones, causing them to appear Gram-negative instead of Gram- positive. For reliable results one should use cultures that are approximately 16 hours old. Another point to remember is that some species of *Bacillus* tend to be Gram variable i.e., sometimes positive and sometimes negative.

During this laboratory period you will be provided an opportunity to stain several different kinds of bacteria to see if you can achieve the degree of success that is required. Remember, if you don't master this technique now, you will have difficulty with your unknowns later.

Materials

- slides with heat- fixed smears
- Gram- staining kit and wash bottle
- bibulous paper

Procedure

1. Cover the smear with **crystal violet** and let stand for 20 seconds.

2. Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water (it is possible to use tap water).

3. Cover the smear with **Gram's iodine** solution and let it stand for one minute. (Your instructor may prefer only 30 seconds for this step.)

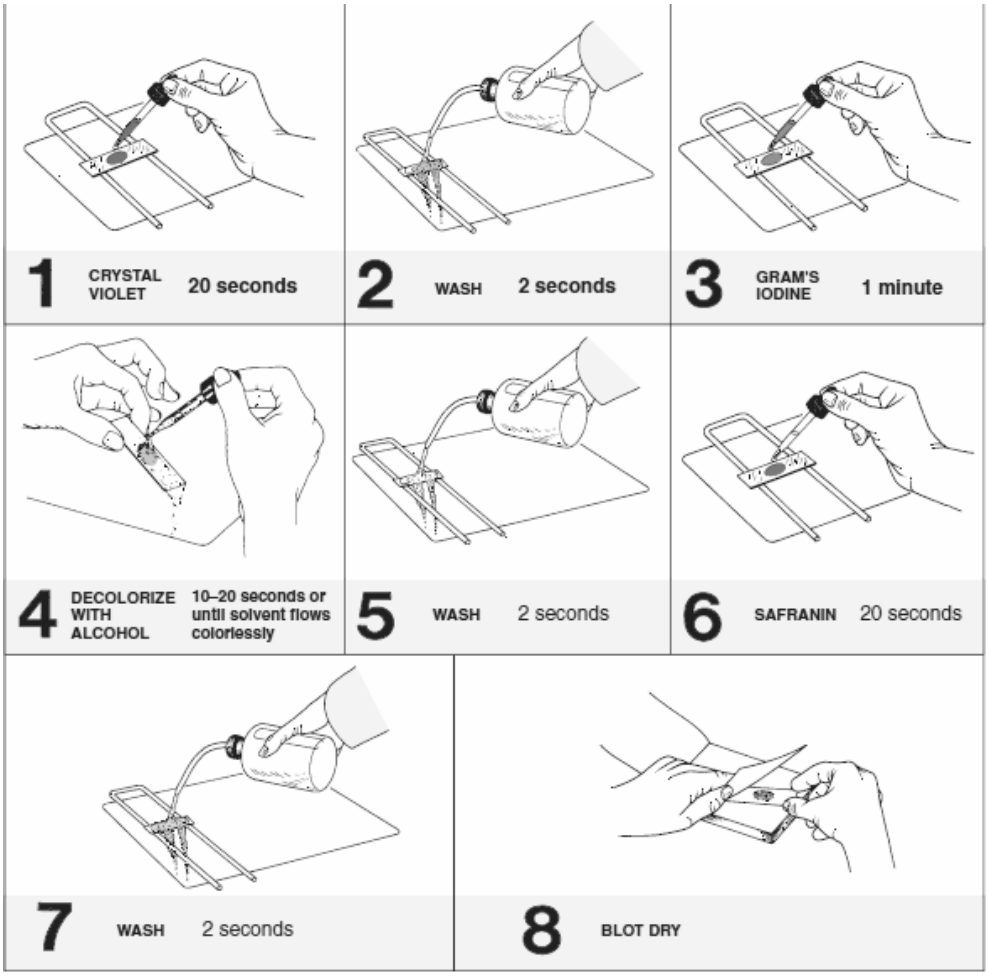
4. Pour off the Gram's iodine and flood the smear with **95% ethyl alcohol** for 10 to 20 seconds. This step is critical. Thick smears will require more time than thin ones. Decolorization has occurred when the solvent flows colorlessly from the slide.

5. Stop action of the alcohol by rinsing the slide with water for a few seconds.

6. Cover the smear with **safranin** for 20 seconds. (Some technicians prefer more time here.)

7. Wash gently (few seconds), blot dry with bibulous paper and air- dry.

8. Examine the slide under oil immersion.



The gram-staining procedure

Notes and Results

SPORE STAINING (TWO METHODS)

The genera *Bacillus* and *Clostridium*, produce extremely heat resistant structures called **endospores.** In addition to being heat- resistant, they are very resistant to many chemicals that destroy non spore forming bacteria.

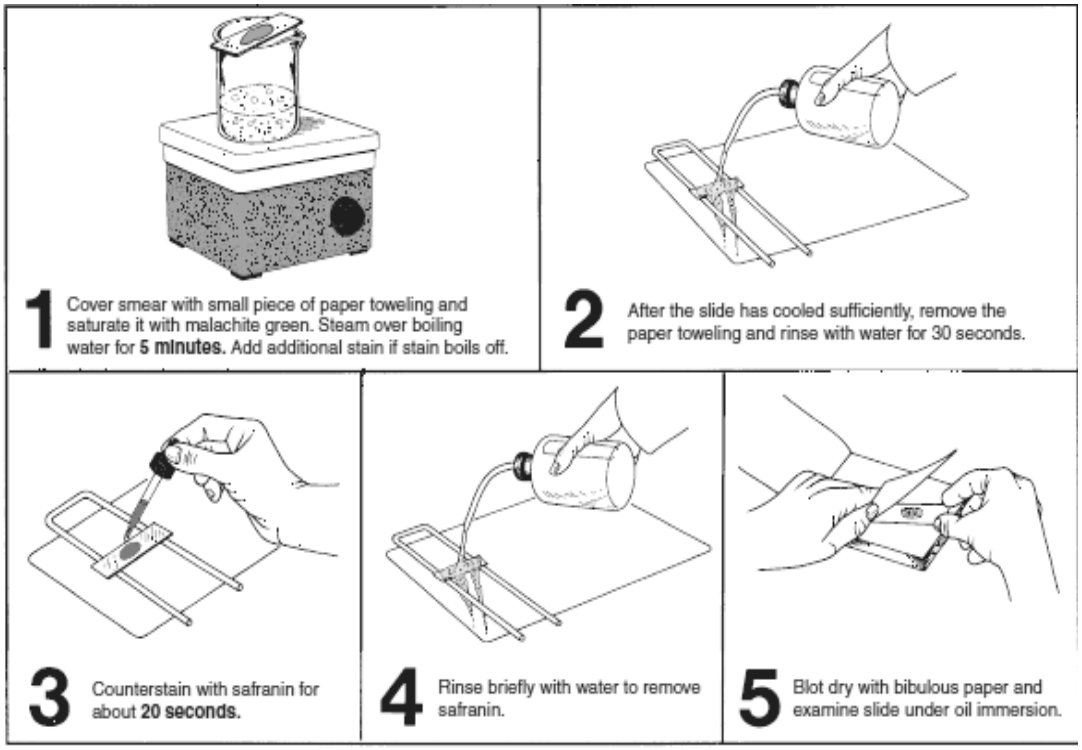
This resistance to heat and chemicals is due primarily to a thick, tough spore coat. It was observed in that Gram staining will not stain endospores. Only if considerable heat is applied to a suitable stain can the stain penetrate the spore coat. Once the stain has entered the spore, however, it is not easily removed with decolorizing agents or water. Several methods are available that employ heat to provide stain penetration. However, since the Schaeffer- Fulton and Dorner methods are the principal ones used by most bacteriologists, both have been included in this exercise. Your instructor will indicate which procedure is preferred in this laboratory.

SCHAEFFER- FULTON METHOD

This method (see the following figure) utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. Utilizing this technique, a properly stained spore- former will have a green endospore contained in a pink sporangium. After preparing a smear of *Bacillus cereus,* follow the steps outlined in the figure to stain the spores.

Materials

- 24–36 hour nutrient agar slant culture of *Bacillus cereus*
- electric hot plate and small beaker (25 ml size)
- spore- staining kit consisting of a bottle each of 5% malachite green and safranin



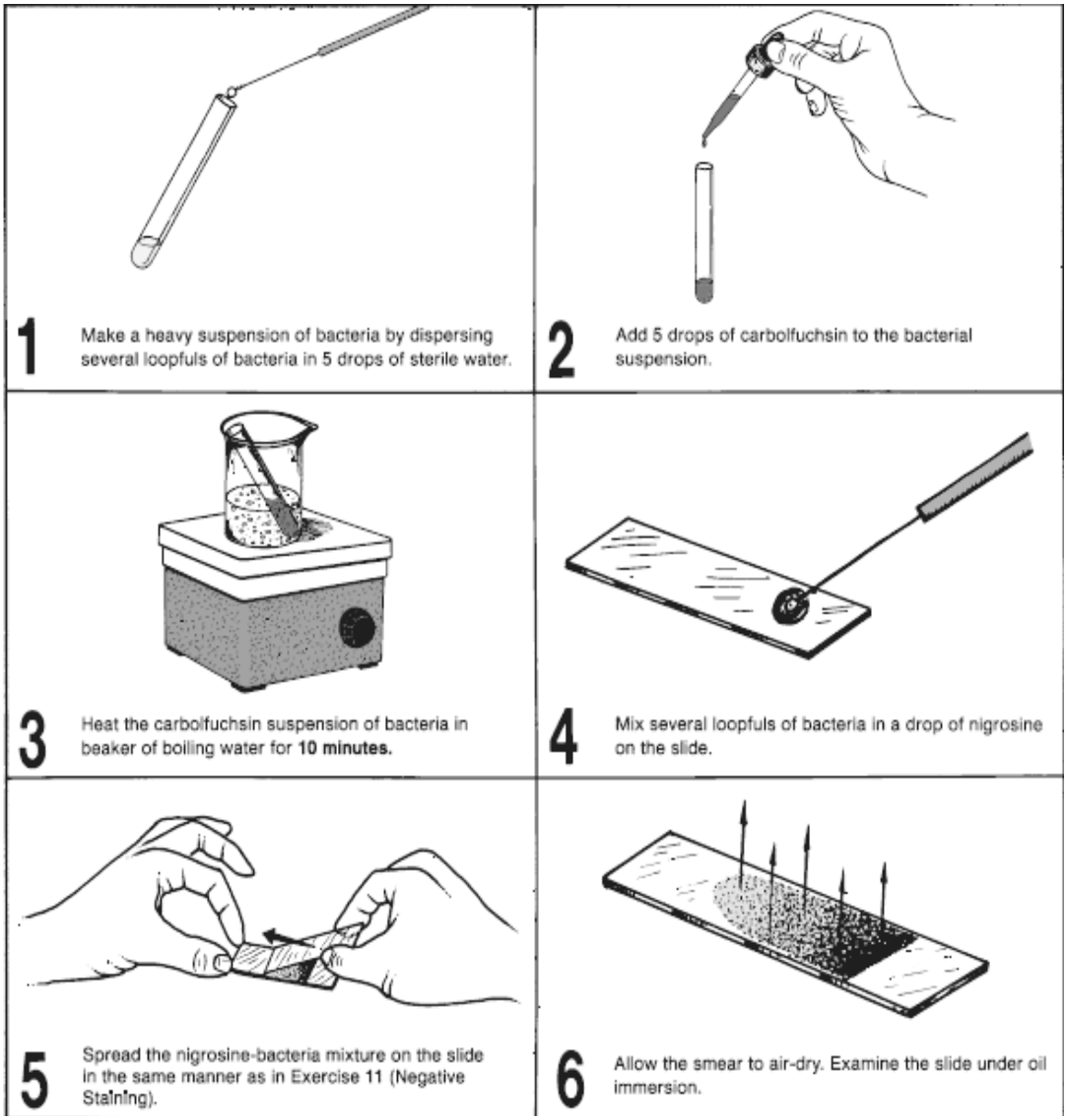
The Schaeffer-Fulton spore stain method

DORNER METHOD

The Dorner method for staining endospores produces a red spore within a colorless sporangium. Nigrosine is used to provide a dark background for contrast. The six steps involved in this technique are shown in the following figure. Although both the sporangium and endospore are stained during boiling in step 3, the sporangium is decolorized by the diffusion of safranin molecules into the nigrosine. Prepare a slide of *Bacillus cereus* that utilizes the Dorner method. Follow the steps in the figure.

Materials

- nigrosine
- electric hot plate and small beaker (25 ml size)
- small test tube
- test tube holder
- 24–36 hour nutrient agar slant culture of *Bacillus cereus*



The Dorner spore stain method

Notes and Results

ACID- FAST STAINING (Ziehl- Neelsen Method)

Most bacteria in the genus *Mycobacterium* contain considerable amounts of wax- like lipoidal material, which affects their staining properties. Unlike most other bacteria, once they are properly stained with carbol fuchsin, they resist decolorization with acid alcohol. Since they are not easily decolorized they are said to be **acid- fast.** This property sets them apart from many other bacteria. This stain is used primarily in the identification of the tuberculosis bacillus, *Mycobacterium tuberculosis,* and the leprosy organism, *Mycobacterium leprae.* After decolorization, methylene blue is added to the organisms to counterstain any material that is not acid- fast; thus, a properly stained slide of a mixture of acid- fast organisms, tissue cells, and non acid- fast bacteria will reveal red acid- fast rods with bluish tissue cells and bacteria. An example of acid- fast staining is shown in the following figure. The two organisms used in this staining exercise are *Mycobacterium smegmatis,* a nonpathogenic acid- fast rod found in soil, and *Staphylococcus aureus,* a non acid- fast coccus.

Materials

- nutrient agar slant culture of *Mycobacterium smegmatis* (48-hour culture) or as instructed.
- nutrient broth culture of *S. aureus*
- electric hot plate and small beaker
- acid- fast staining kit (carbol fuchsin, acid alcohol, and methylene blue)

Smear Preparation

Prepare a mixed culture smear by placing two loopfuls of *S. aureus* on a slide and transferring a small amount of *M. smegmatis* to the broth on the slide with an inoculating needle. Since the *M. smegmatis* bacilli are waxy and tend to cling to each other in clumps, break up the masses of

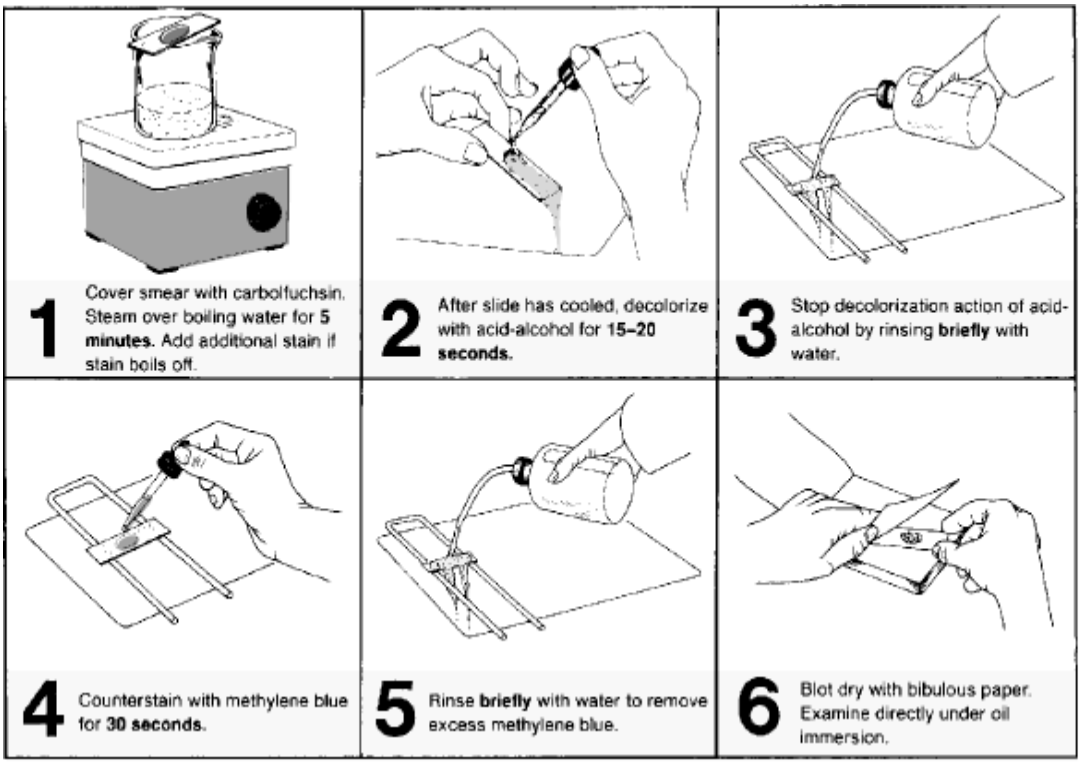
organisms with the inoculating needle. After air- drying the smear, heat-fix it.

Staining

Follow the staining procedure outlined in the figure.

Examination

Examine under oil immersion



Ziehl-Neelsen acid-fast staining procedure

Notes and Results

MOTILITY DETERMINATION

When attempting to identify an unknown bacterium it is usually necessary to determine whether the microorganism is motile. Although one might think that this determination would be easily arrived at, such is not always the case. For the beginner there are many opportunities to err.

FOUR METHODS

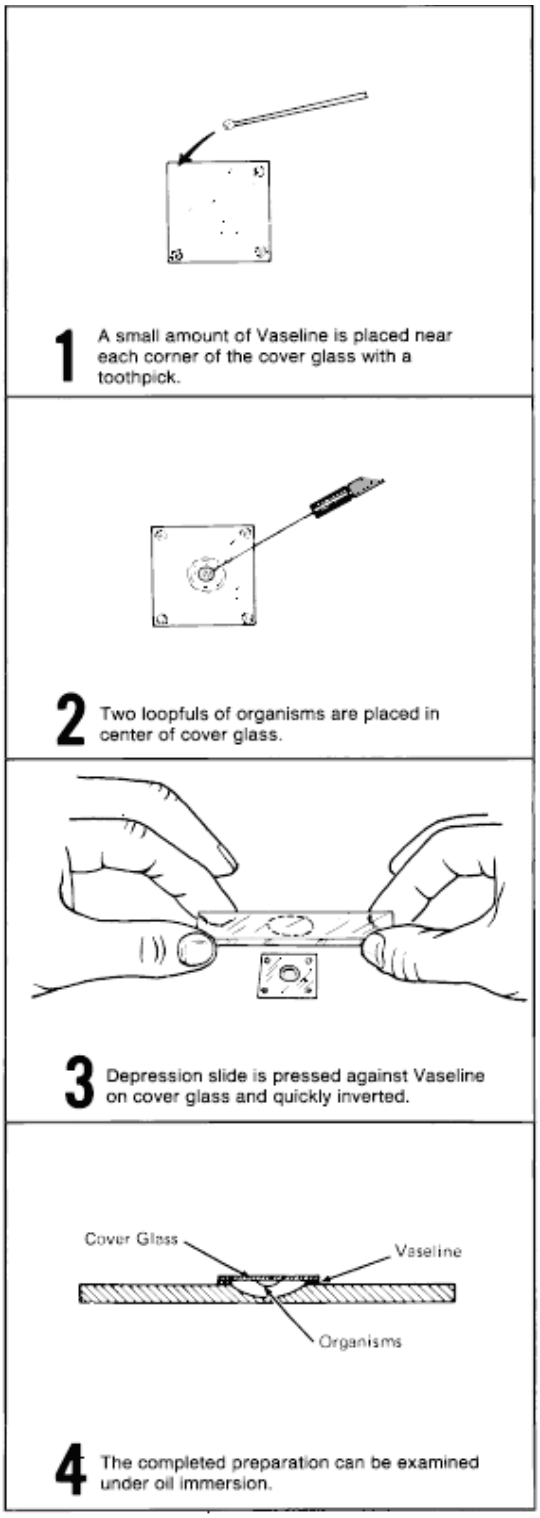
For non pathogens, there are two slide techniques that one might use. For pathogens, one tube and one Petri plate method can be used. Each method has its advantages and limitations. The method you use will depend on which one is most suitable for the situation at hand.

The Wet Mount Slide

When working with nonpathogens, the simplest way to determine motility is to place a few loopfuls of the organism on a clean slide and cover it with a cover glass. In addition to being able to determine the presence or absence of motility, this method is useful in determining cellular shape (rod, coccus, or spiral) and arrangement (irregular clusters, packets, pairs, or long chains). A wet mount is especially useful if **phase optics** are used. Unlike stained slides that are heat- fixed for staining, there is no distortion of cells on a wet mount. One problem for beginners is the difficulty of being able to see the organisms on the slide. Since bacteria are generally colorless and very transparent, the novice has to learn how to bring them into focus.

The Hanging Drop Slide

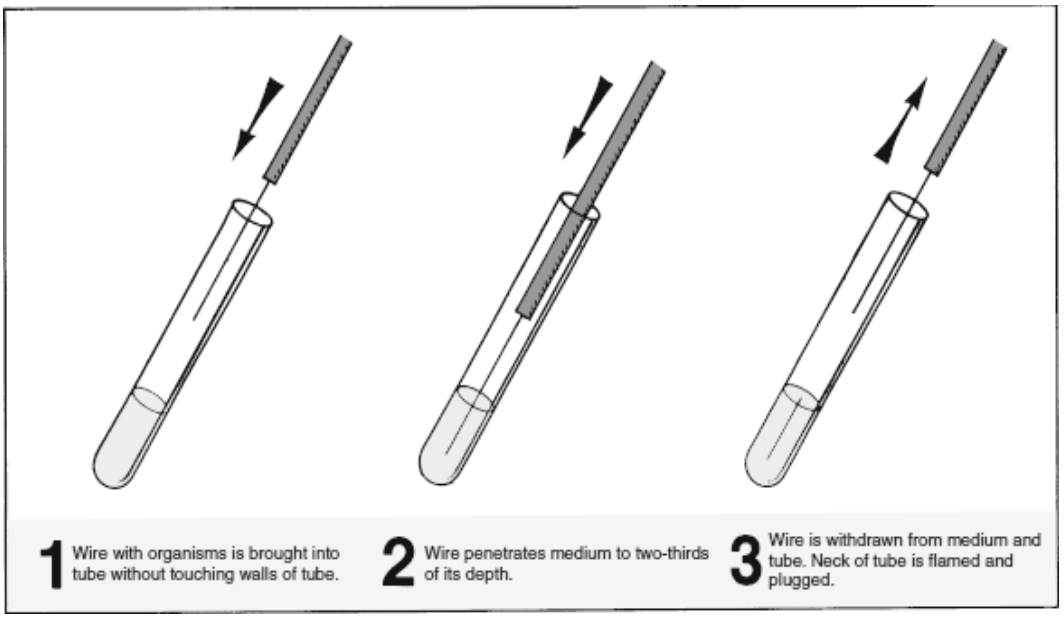
If it is necessary to study viable organisms on a microscope slide for a longer period of time than is possible with a wet mount, one can resort to a hanging drop slide. As shown in the following figure, organisms are observed in a drop that is suspended under a cover glass in a concave depression slide. Since the drop lies within an enclosed glass chamber, drying out occurs very slowly.



The hanging drop slide

Tube Method

When working with pathogenic microorganisms such as the typhoid bacillus, it is too dangerous to attempt to determine motility with slide techniques. A much safer method is to culture the organisms in a special medium that can demonstrate the presence of motility. The procedure is to inoculate a tube of semisolid medium that can demonstrate the presence of motility. The used medium has a very soft consistency that allows motile bacteria to migrate readily through it causing cloudiness. The following Figure illustrates the inoculation procedure.



Stab technique for motility test

Soft Agar Plate Method

Although the tube method is the generally accepted procedure for determining motility of pathogens, it is often very difficult for beginners to interpret. Richard Roller at the University of Iowa suggests that incubating a Petri plate of soft agar that has been stab inoculated with a motile organism will show up motility more clearly than an inoculated tube.

FIRST PERIOD

Tube Method

Inoculate tubes of semisolid medium with *M. luteus* and *E. coli* according to the following instructions:

1. Label the tubes of semisolid medium with the names of the organisms. Place your initials on the tubes, also.

2. Flame and cool the inoculating needle, and insert it into the culture after flaming the neck of the tube.

3. Remove the cap from the tube of medium, flame the neck, and stab it 2⁄3 of the way down to the bottom, as shown in the figure. Flame the neck of the tube again before returning the cap to the tube.

4. Repeat steps 2 and 3 for the other culture.

5. Incubate the tubes at room temperature for 24 to 48 hours.

Plate Method

Mark the bottom of a plate of soft agar with two one- half inch circles about one inch apart. Label one circle ML and the other EC. These circles will be targets for your culture stabs. Put your initials on the plate also. Using proper aseptic techniques, stab the medium in the center of the ML circle with *M. luteus* and the center of the other circle with *E. coli.* Incubate the plate for 24 to 48 hours at room temperature.

SECOND PERIOD

Assemble the following materials:

- culture tubes of motility medium that have been incubated
- inoculated Petri plate that has been incubated
- Compare the two tubes that were inoculated with *M. luteus* and *E. coli.* Look for cloudiness as evidence of motility.
- Does the plate method provide any better differentiation of results than the tube method?

Notes and Results

PURE CULTURE TECHNIQUES

When we try to study the bacterial flora of the body, soil, water, food, or any other part of our environment, we soon discover that bacteria exist in mixed populations. It is only in very rare situations that they occur as a single species. To be able to study the cultural, morphological, and physiological characteristics of an individual species, it is essential, first of all, that the organism be separated from the other species that are normally found in its habitat; in other words, we must have a **pure culture** of the microorganism.

Several different methods of getting a pure culture from a mixed culture are available to us. The two most frequently used methods involve making a streak plate or a pour plate. Both plate techniques involve thinning the organisms so that the individual species can be selected from the others. In this exercise you will have an opportunity to use both methods in an attempt to separate three distinct species from a tube that contains a mixture. The principal difference between the three organisms will be their colors: *Serratia marcescens* is red, *Micrococcus luteus* is yellow, and *Escherichia coli* is white.

STREAK PLATE METHOD

For economy of materials and time, this method is best. It requires a certain amount of skill, however, which is forthcoming with experience. A properly executed streak plate will give as good an isolation as is desired for most work. The following Figure illustrates how colonies of a mixed culture should be spread out on a properly made streak plate. The important thing is to produce good spacing between colonies.

Materials

- electric hot plate
- Bunsen burner and beaker of water
- wire loop, thermometer, and china marking pencil

- 1 nutrient agar pour and 1 sterile Petri plate
- 1 mixed culture of *Serratia marcescens, Escherichia coli,* and *Micrococcus luteus*

If your streak plate reveals well- isolated colonies you will have a plate suitable for subculturing.

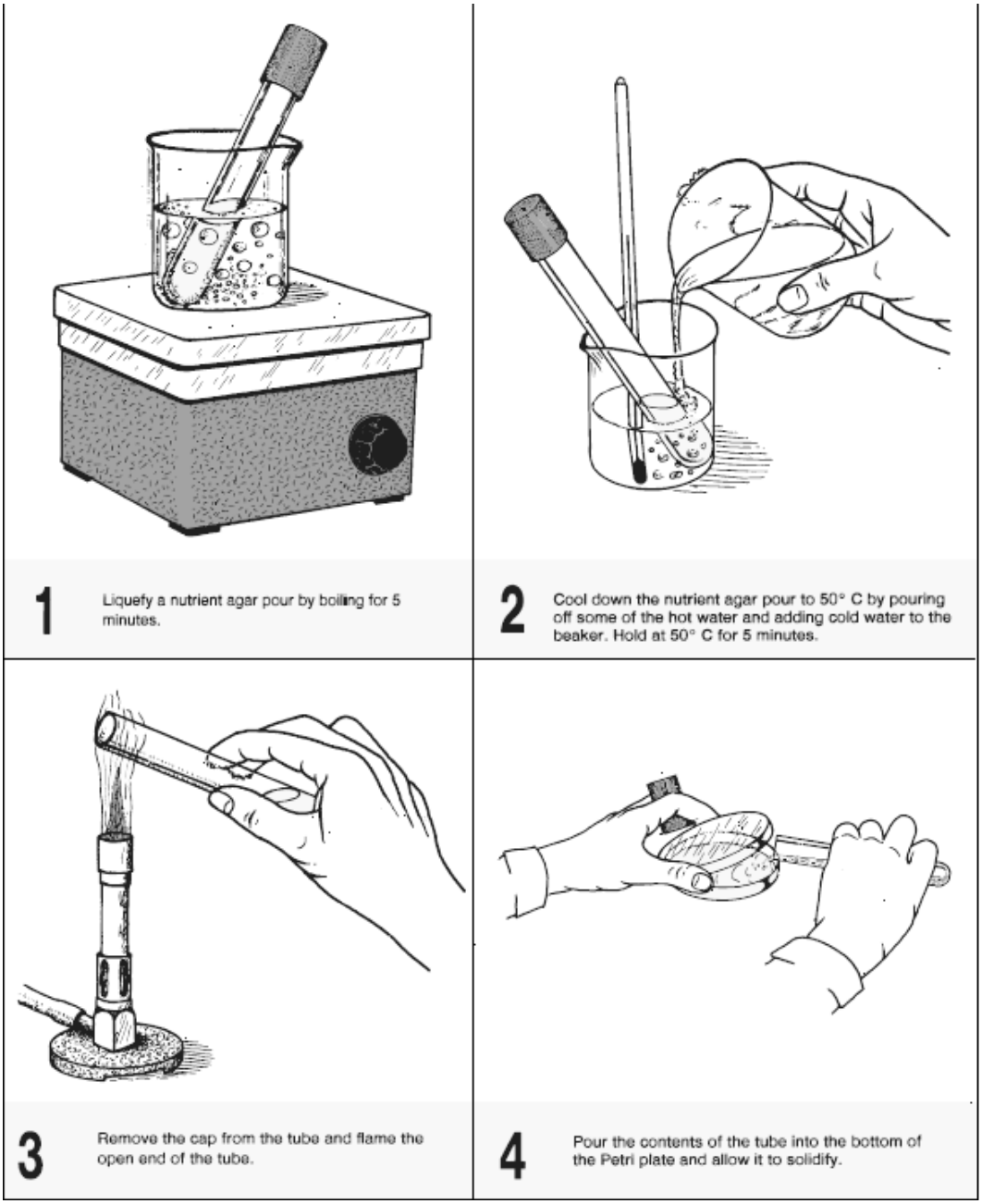
Procedure

1. Prepare your tabletop by disinfecting its surface with the disinfectant that is available in the laboratory. Use a sponge to scrub it clean.

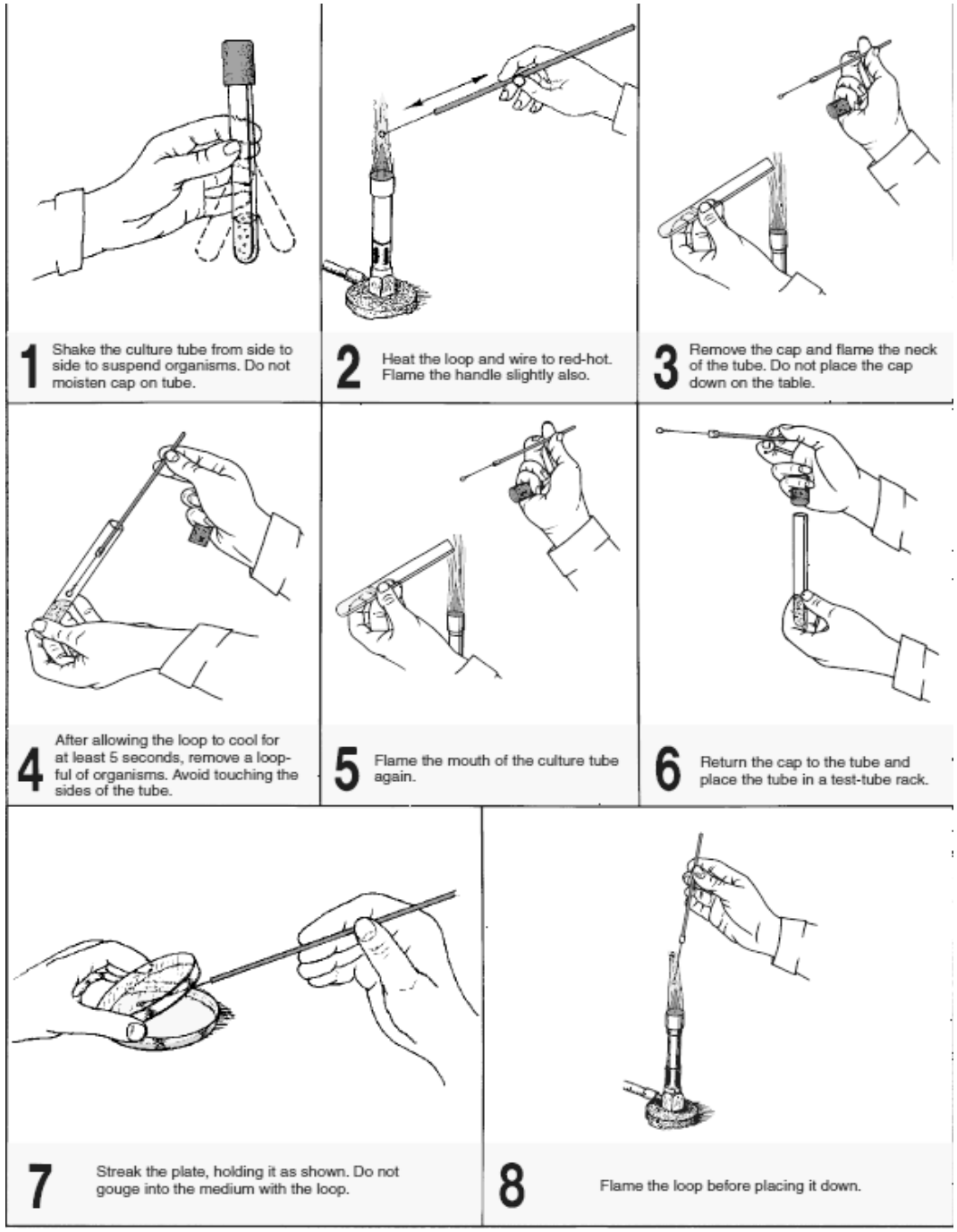
2. Label the bottom surface of a sterile Petri plate with your name and date. Use a china marking pencil.

3. Liquefy a tube of nutrient agar, cool to 50° C, and pour the medium into the bottom of the plate, following the procedure illustrated in the following figure. Be sure to flame the neck of the tube prior to pouring to destroy any bacteria around the end of the tube. After pouring the medium into the plate, gently rotate the plate so that it becomes evenly distributed, but do not splash any medium up over the sides. Agar- agar, the solidifying agent in this medium becomes liquid when boiled and resolidifies at around 42° C. Failure to cool it prior to pouring into the plate will result in condensation of moisture on the cover. Any moisture on the cover is undesirable because if it drops down on the colonies, the

organisms of one colony can spread to other colonies, defeating the entire isolation technique.



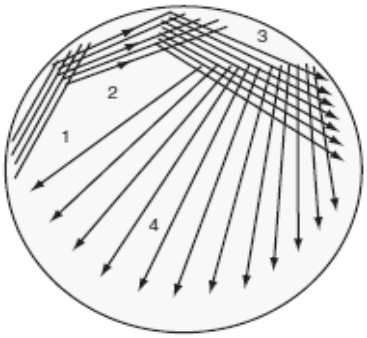
Procedure for pouring an agar plate for streaking



Routine for inoculating a Petri plate

4. Streak the plate by one of the methods shown in the following figure. Your instructor will indicate which technique you should use.

Caution: Be sure to follow the routine in the figure for getting the organism out of culture.



QUADRANT STREAK

(Method A)

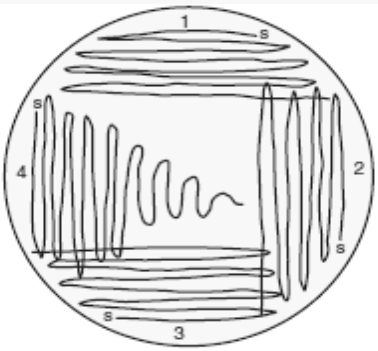
1. Streak one loopful of organisms over Area 1 near edge of the plate. Apply the loop lightly. Don't gouge into the medium.

2. Flame the loop, cool 5 seconds, and make 5 of 6 streaks from Area 1 through Area 2. Momentarily touching the loop to a sterile area of the medium before streaking insures a cool loop.

3. Flame the loop again, cool it, and make 6 or 7 streaks from Area 2 through Area 3.

4. Flame the loop again and make as many streaks as possible from Area 3 into Area 4, using up the remainder of the plate surface.

5. Flame the loop before putting it aside.



QUADRANT STREAK

(Method B)

1. Streak one loopful of organisms back and forth over Area 1, starting at point designated by "s". Apply loop lightly. Don't gouge into the medium.

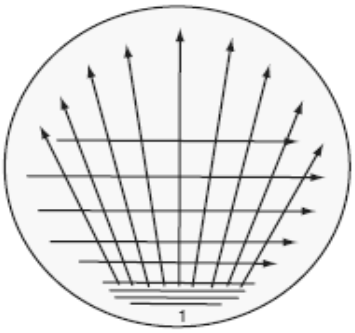
2. Flame the loop, cool 5 seconds and touch the medium in sterile area momentarily to insure coolness.

3. Rotate the dish 90 degrees while keeping the dish closed. Streak Area 2 with several back and forth strokes, hitting the original streak a few times.

4. Flame the loop again. Rotate the dish and streak Area 3 several times, hitting last area several times.

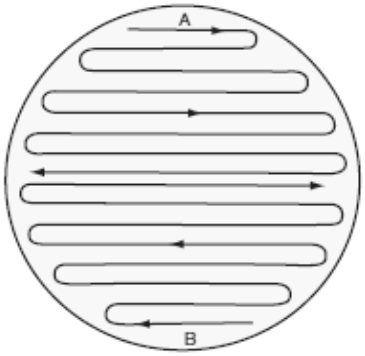
5. Flame the loop, cool it, and rotate the dish 90 degrees again. Streak Area 4, contacting Area 3 several times and drag out the culture as illustrated.

6. Flame the loop before putting it aside.



RADIANT STREAK

1. Spread a loopful of organisms in small area near the edge of the plate in Area 1. Don't gouge medium.
2. Flame the loop and allow it to cool for 5 seconds. Touching a sterile area of the medium will insure coolness.
3. **From the edge** of Area 1 make 7 or 8 straight streaks to the opposite side of the plate.
4. Flame the loop again, cool it sufficiently, and cross streak over the last streaks, **starting near Area 1.**
5. Flame the loop again before putting it aside.



CONTINUOUS STREAK

1. Starting at the edge of the plate (Area A) with a loopful of organisms, spread the organisms in a single continuous movement to the center of the plate. Use light pressure and avoid gouging the medium.
2. Rotate the plate 180 degrees so that the uninoculated portion of the plate is away from you.
3. Without flaming loop, and using the same face of the loop, continue streaking the other half of the plate by starting at Area B and working toward the center.
4. Flame your loop before putting it aside.

5. Incubate the plate in an inverted position at 25° C for 24– 48 hours. By incubating plates upside down, the problem of moisture on the cover is minimized.

POUR PLATE METHOD (Loop Dilution)

This method of separating one species of bacteria from another consists of diluting out one loopful of organisms with three tubes of liquefied nutrient

agar in such a manner that one of the plates poured will have an optimum number of organisms to provide good isolation. The following Figure illustrates the general procedure.

One advantage of this method is that it requires somewhat less skill than that required for a good streak plate; a disadvantage, however, is that it requires more media, tubes, and plates. Proceed as follows to make three dilution pour plates, using the same mixed culture for streak plate.

Materials

- mixed culture of bacteria
- 3 nutrient agar pours
- 3 sterile Petri plates
- electric hot plate
- beaker of water
- thermometer
- inoculating loop and china marking pencil

Procedure

1. Label the three nutrient agar pours **I, II,** and **III** with a marking pencil and place them in a beaker of water on an electric hot plate to be liquefied. To save time, start with hot tap water if it is available.

2. While the tubes of media are being heated, label the bottoms of the three Petri plates **I, II,** and **III.**

3. Cool down the tubes of media to 50° C, using the same method that was used for the streak plate.

4. Following the routine in the figure, inoculate tube I with one loopful of organisms from the mixed culture. Note the sequence and manner of handling the tubes in the figure.

5. Inoculate tube II with one loopful from tube I after thoroughly mixing the organisms in tube I by shaking the tube from side to side or by rolling

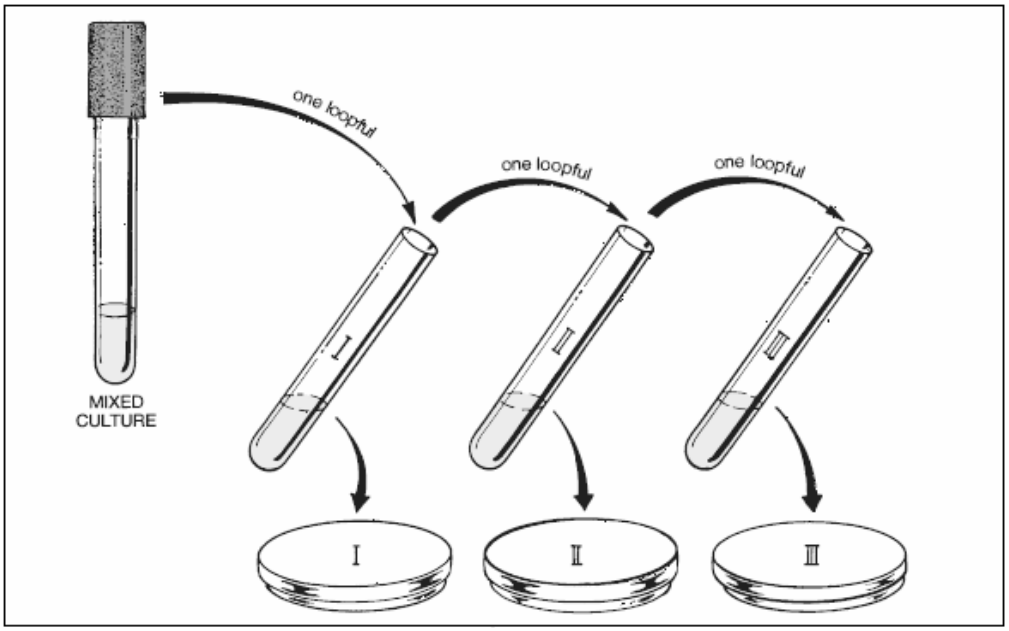
the tube vigorously between the palms of both hands. **Do not splash any of the medium up onto the tube closure.** Return tube I to the water bath.

6. Agitate tube II to completely disperse the organisms and inoculate tube III with one loopful from tube II. Return tube II to the water bath.

7. Agitate tube III, flame its neck, and pour its contents into plate III.

8. Flame the necks of tubes I and II and pour their contents into their respective plates.

9. After the medium has completely solidified, incubate the inverted plates at 25° C for 24–48 hours.



Three steps in the loop dilution technique for separating out organisms

EVALUATION OF THE TWO METHODS

Examine all four Petri plates after 24 to 48 hours of incubation. Look for colonies that are well isolated from the others. Note how crowded the colonies appear on plate I as compared with plates II and III. Plate I will be unusable. Either plate II or III will have the most favorable isolation of colonies. Can you pick out three well- isolated colonies that are white, yellow, and red? Draw the appearance of your streak plate and pour plates.

Notes and Results

BACTERIAL POPULATION COUNTS

Many bacteriological studies require that we be able to determine the number of organisms that are present in a given unit of volume. Several different methods are available to us for such population counts. The method one uses is determined by the purpose of the study. To get by with a minimum of equipment, it is possible to do a population count by diluting out the organisms and counting the organisms in a number of microscopic fields on a slide. Direct examination of milk samples with this technique can be performed very quickly, and the results obtained are quite reliable. A technique similar to this can be performed on a Petrof-Hauser counting chamber.

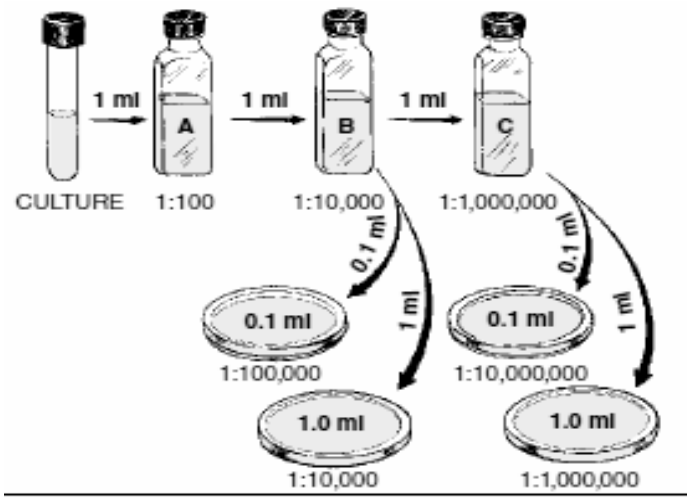
Bacterial counts of gas- forming bacteria can be made by inoculating a series of tubes of lactose broth and using statistical probability tables to estimate bacterial numbers. This method, used to estimate numbers of coliform bacteria in water samples, is easy to use, works well in water testing, but is limited to water, milk, and food testing.

In this exercise we will use **quantitative plating** (Standard Plate Count, or SPC) and **turbidity measurements** to determine the number of bacteria in a culture sample. Although the two methods are somewhat parallel in the results they yield, there are distinct differences. For one thing, the SPC reveals information only as related to viable organisms; that is, colonies that are seen on the plates after incubation represent only living organisms, not dead ones. Turbidimetry results, on the other hand, reflect the presence of all organisms in a culture, dead and living.

Quantitative plating method (Standard Plate Count)

In determining the number of organisms present in water, milk, and food, the **standard plate count** (SPC) is universally used. It is relatively easy to perform and gives excellent results. We can also use this basic technique to calculate the number of organisms in a bacterial culture. It is in this

respect that this assignment is set up. The procedure consists of diluting the organisms with a series of sterile water blanks as illustrated in the following figure. Generally, only three bottles are needed, but more could be used if necessary. By using the dilution procedure indicated here, a final dilution of 1:1,000,000 occurs in blank C. From blanks B and C, measured amounts of the diluted organisms are transferred into empty Petri plates. Nutrient agar, cooled to 50° C, is then poured into each plate. After the nutrient agar has solidified, the plates are incubated for 24 to 48 hours and examined. A plate that has between 30 and 300 colonies is selected for counting. From the count it is a simple matter to calculate the number of organisms per milliliter of the original culture. It should be pointed out that greater accuracy can be achieved by pouring two plates for each dilution and averaging the counts. Duplicate plating, however, has been avoided for obvious economic reasons.



Quantitative plating procedure

Notes and Results

Diluting and Plating Procedure

Proceed as follows to dilute out a culture of *E. coli* and pour four plates.

Materials

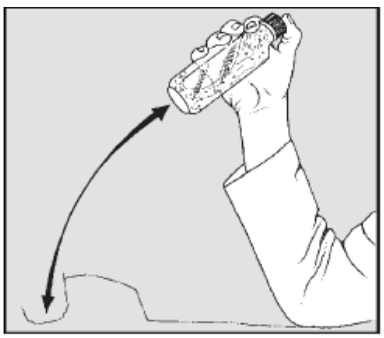
- 1 bottle (40 ml) broth culture of *E. coli*
- 1 bottle (80 ml) nutrient agar
- 4 Petri plates
- 1 pipettes 1 ml
- 3 sterile 99 ml water blanks
- cannister for discarded pipettes

procedure

1. Liquefy a bottle of nutrient agar. While it is being heated, label three 99 ml sterile water blanks **A, B,** and **C.** Also, label the four Petri plates **1:10,000, 1:100,000, 1:1,000,000,** and **1:10,000,000.** In addition, indicate with labels the amount to be pipetted into each plate (**0.1 ml** or **1.0 ml**).

2. Shake the culture of *E. coli* and transfer 1 ml of the organisms to blank A, using a sterile 1 ml pipette. After using the pipette, place it in the discard cannister.

3. Shake blank A 25 times in an arc of 1 foot for 7 seconds with your elbow on the table. Forceful shaking not only brings about good distribution, but it also breaks up clumps of bacteria.



Standard procedure for shaking water blanks requires elbow to remain fixed on table

4. With a different 1 ml pipette, transfer 1 ml from blank A to blank B.

5. Shake water blank B 25 times in same manner.

6. With another sterile pipette, transfer 0.1 ml from blank B to the 1:100,000 plate and 1.0 ml to the 1:10,000 plate. With the same pipette, transfer 1.0 ml to blank C.

7. Shake blank C 25 times.

8. With another sterile pipette, transfer from blank C 0.1 ml to the 1:10,000,000 plate and 1.0 ml to the 1:1,000,000 plate.

9. After the bottle of nutrient agar has boiled for 8 minutes, cool it down in a water bath at 50° C for **at least 10 minutes.**

10. Pour one- fourth of the nutrient agar (20 ml) into each of 4 plates. Rotate the plates **gently** to get adequate mixing of medium and organisms. **This step is critical!** Too little action will result in poor dispersion and too much action may slop inoculated medium over the edge.

11. After the medium has cooled completely, incubate at 35° C for 48 hours, inverted.

Counting and Calculations

Materials

- 4 culture plates
- colony counter
- mechanical hand counter
- felt pen (optional)

Procedure

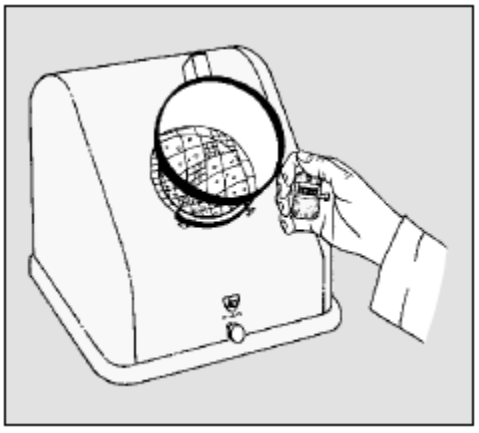
1. Lay out the plates on the table in order of dilution and compare them. Select the plates that have no fewer than 30 nor more than 300 colonies for your count. Plates with less than 30 or more than 300 colonies are statistically unreliable.

2. Place the plate on the colony counter with the lid removed. Start counting at the top of the plate, using the grid lines to prevent counting the same colony twice. Use a mechanical hand counter. Count every colony,

regardless of how small or insignificant and record your counts. **Alternative Counting Method:** Another way to do the count is to remove the lid and place the plate upside down on the colony counter. Instead of using the grid to keep track, use a felt pen to mark off each colony as you do the count.

3. Calculate the number of bacteria per ml of undiluted culture using the data recorded above. Multiply the number of colonies counted by the dilution factor (the reciprocal of the dilution).

Example: If you counted 220 colonies on the plate that received 1.0 ml of the 1:1,000,000 dilution: 220 x 1,000,000 (or 2.2 x 10^8) bacteria per ml. If 220 colonies were counted on the plate that received 0.1 ml of the 1:1,000,000 dilution, then the above results would be multiplied by 10 to convert from number of bacteria per 0.1 ml to number of bacteria per 1.0 ml (2,200,000,000, or 2.2 x 10^9). If the number of bacteria per ml was calculated to be 227,000,000, it should be recorded as 230,000,000, or 2.3 x 10^8.



Colony counts are made on a colony counter, using a mechanical hand tally

Notes and Results

ISOLATION OF ANAEROBIC PHOTOTROPHIC BACTERIA (the Winogradsky Column method)

The culture of photosynthetic bacteria requires special culture methods to promote their growth. These prokaryotes contain photopigments, such as chlorophyll and carotenoids, which convert solar energy into cellular constituents. There are two groups of phototrophic bacteria: (1) the aerobic phototrophic **cyanobacteria,** and (2) the anaerobic phototrophic bacteria, which include the **purple** and **green bacteria.** It is this latter group that will be studied in this exercise. The cyanobacteria contain chlorophyll *a,* carotenoids, and phycobilisomes. The nonchlorophyll pigments in this group are accessory pigments for capturing light. They resemble higher plants in that they split water for a source of reducing power and evolve oxygen in the process. The anaerobic phototrophic bacteria, on the other hand, differ in that they contain bacteriochlorophyll, which is chemically distinct from chlorophyll. Instead of utilizing water as a source of reducing power, the purple and green bacteria use sulfide or organic acids for the reduction of carbon dioxide. The purple bacteria that utilize organic acids instead of sulfide are essentially photoheterotrophic since they derive their carbon from organic acids rather than carbon dioxide. These bacteria are ubiquitous in the sediment of ditches, ponds, and lakes: i.e., mostly everywhere that freshwater lies relatively stagnant for long periods of time and subject to sunlight. In this environment, fermentation processes produce the sulfides and organic acids that are essential to their existence.

Characterization

According to *Bergey's Manual* (Section 18, Vol. 3), there are approximately 30 genera of anaerobic phototrophic bacteria. The purple bacteria belong to the family *Chromatiaceae.* The green ones are in the

family *Chlorobiaceae.* The morphological, cultural, and physiological differences between the purple and green sulfur bacteria are as follows:

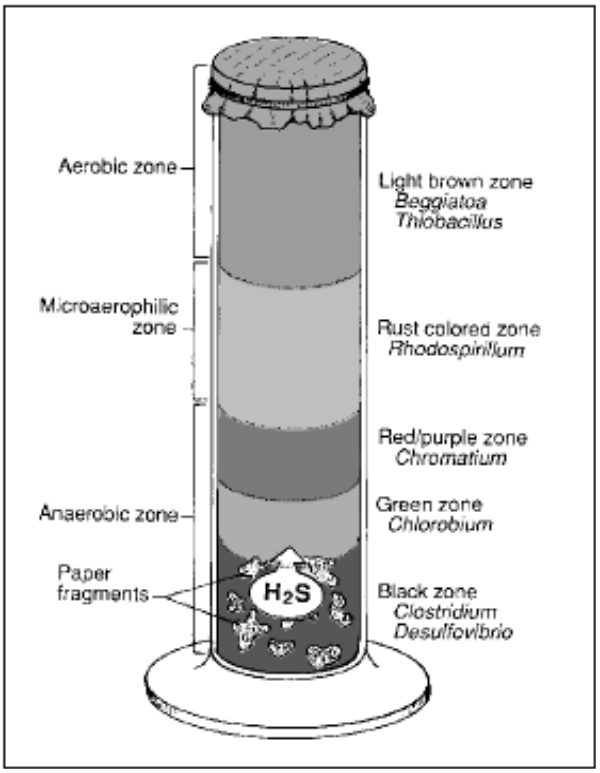
Purple Sulfur Bacteria Members of this group are all Gram negative, straight or slightly curved rods that are motile with polar flagellation. Colors of the various genera vary considerably—from orange- brown to brown, brownish- red to pink, and purple- red to purple- violet. Color variability is due to the blend of bacteriochlorophyll with the type of carotenoid present. All species contain elemental-sulfur internally in the form of globules. Some species are able to fix nitrogen. Sulfides are required as electron donors; bicarbonate, acetate, and pyrovate are also required. They cannot utilize thiosulfate, sugars, alcohols, amino acids, or benzoates.

Green Sulfur Bacteria All of these bacteria are Gram negative, spherical to straight, or curved rods. Arrangement of cells may be in chains like streptococci. Some are motile by gliding, others are nonmotile. Color may be grass- green or brown. Sulfur byproduct is excreted, not retained in cells. Some are able to utilize thiosulfate. Many are mixotrophic in that they can photoassimilate simple organic compounds in the presence of sulfide and bicarbonate.

WINOGRADSKY'S COLUMN

To create a small ecosystem that is suitable for the growth of these bacteria, one can set up a **Winogradsky column** as illustrated in the following figure. Sergii Winogradsky, a Russian microbiologist, developed this culture technique to study the bacteria that are involved in the sulfur cycle. From his studies he defined the chemoautotrophic bacteria. This setup consists of a large test tube or graduated cylinder that is packed with pond sediments, sulfate, carbonate, and some source of cellulose (shredded paper or cellulose powder). It is incubated for a period of time (up to 8 weeks) while being exposed to incandescent light. Note

that different layers of microorganisms develop, much in the same manner that is found in nature.



Winogradsky's Column

Observe that in the bottom of the column the cellulose is degraded to fermentation products by *Clostridium.* The fermentation products and sulfate are then acted upon by other bacteria (*Desulfovibrio*) to produce hydrogen sulfide, which diffuses upward toward the oxygenated zone, creating a stable hydrogen sulfide gradient. Note, also, that the *Chlorobium* species produce an olive- green zone deep in the column. A red to purple zone is produced by *Chromatium* a little farther up. Ascending the column farther where the oxygen gradient increases, other phototrophic bacteria such as *Rhodospirillum, Beggiatoa,* and *Thiobacillus* will flourish. Once the column has matured, one can make subcultures from the different layers, using an enrichment medium. The subcultures

can be used for making slides to study the morphological characteristics of the various types of organisms. Proceed as follows:

FIRST PERIOD

You will set up your Winogradsky column in a 100 ml glass graduate. It will be filled with mud, sulfate, water, phosphate, carbonate, and a source of fermentable cellulose. The cellulose, in this case, will be in the form of a shredded paper slurry. The column will be covered completely at first with aluminum foil to prevent the overgrowth of amoeba and then later uncovered and illuminated with incandescent light to promote the growth of phototrophic bacteria. The column will be examined at 2- week intervals to look for the development of different- colored layers. Once distinct colored layers develop, subcultures will be made to tubes of enrichment medium with a pipette. The subcultures will be incubated at room temperature with exposure to incandescent light and examined periodically for color changes.

Materials

- graduated cylinder (100 ml size)
- cellulose source (cellulose powder, newspaper, or filter paper)
- calcium sulfate, calcium carbonate, dipotassium phosphate
- mud from various sources (freshly collected)
- water from ponds (freshly collected)
- beaker (100 ml size)
- glass stirring rod
- aluminum foil
- rubber bands
- incandescent lamp (60–75 watt)

Procedure

1. Using cellulose powder or some form of paper, prepare a thick slurry with water in a beaker. If you are using paper, tear the paper up into small

pieces and macerate it in a small volume of water with a glass rod. If you are using cellulose powder, start with 1– 2 g of powder in a small amount of water. The slurry should be thick but not a paste.

2. Fill the cylinder with the slurry until it is one- third full.

3. To 200 g of mud, add 1.64 g of calcium sulfate and 1.3 g each of calcium carbonate and dipotassium phosphate. Keep a record of the source of the mud you are using.

4. Add some "self water" (pond water collected with the mud) to the mud and chemical mixture and mix the ingredients well.

5. Pour the mud mixture into the cylinder on top of the cellulose slurry.

6. With a glass rod, gently mix and pack the contents of the cylinder. As packing occurs, you may find that you need to add more "self water" to bring the level up to two- thirds or three- fourths of the graduate. Make sure all trapped air bubbles are released.

7. Top off the cylinder by adding pond water until 90% full.

8. Cap the cylinder with foil, using a rubber band to secure the cover.

9. Record the initial appearance of the cylinder.

10. Wrap the sides of the cylinder completely with aluminum foil to exclude light.

11. Incubate the cylinder at room temperature for one and a half to two weeks.

TWO WEEKS LATER

Remove the aluminum foil from the sides of the cylinder. Note the color of the mud, particularly in the bottom. Its black appearance will indicate sulfur respiration with the formation of sulfides by *Desulfovibrio* and other related bacteria. Record the color differences of different layers and the overall appearance of the entire cylinder. Place a lamp with a 75 watt bulb within a few inches of the cylinder and continue to incubate the cylinder at room temperature.

SUBSEQUENT EXAMINATIONS

Examine the cylinder periodically at each laboratory period, looking for the color changes that might occur. The presence of green, purple, red, or brown areas on the surface of the mud should indicate the presence of blooms of anaerobic phototrophic bacterial growth. Record your results.

SUBCULTURING

After 6 to 8 weeks, make several subcultures from your Winogradsky column.

Notes and Results

ENVIRONMENTAL FACTORS AND CONTROL OF MICROBIAL GROWTH

Microbial control by chemical and physical means involves the use of antiseptics, disinfectants, antibiotics, ultraviolet light, and many other agents. Some factors will be tested as examples of the environmental factors that include temperature, oxygen, pH, heavy metals and dyes.

OSMOTIC PRESSURE AND BACTERIAL GROWTH

Growth of bacteria can be profoundly affected by the amount of water entering or leaving the cell. When the medium surrounding an organism is **hypotonic** (low solute content), a resultant higher osmotic pressure occurs in the cell. Except for some marine forms, this situation is not harmful to most bacteria. The cell wall structure of most bacteria is so strong and rigid that even slight cellular swelling is generally inapparent.

In the reverse situation, however, when bacteria are placed in a **hypertonic** solution (high solute content), their growth may be considerably inhibited. The degree of inhibition will depend on the type of solute and the nature of the organism. In media of growth- inhibiting osmotic pressure, the cytoplasm becomes dehydrated and shrinks away from the cell wall. Such **plasmolyzed** cells are often simply inhibited in the absence of sufficient cellular water and return to normal when placed in an **isotonic** solution. In other instances, the organisms are irreversibly affected due to permanent inactivation of enzyme systems.

Organisms that thrive in hypertonic solutions are designated as halophiles or osmophiles. If they require minimum concentrations of salt (NaCl and other cations and anions) they are called **halophiles.** Obligate halophiles require a minimum of 13% sodium chloride. **Osmophiles,** on the other hand, require high concentrations of an organic solute, such as sugar.

In this exercise we will test the degree of inhibition of organisms that results with media containing different concentrations of sodium chloride.

To accomplish this, you will streak three different organisms on four plates of media. The specific organisms used differ in their tolerance of salt concentrations. The salt concentrations will be 0.5, 5, 10, and 15%. After incubation for 48 hours and several more days, comparisons will be made of growth differences to determine their degrees of salt tolerances.

Materials

- 1 Petri plate of nutrient agar (0.5% NaCl)
- 1 Petri plate of nutrient agar (5% NaCl)
- 1 Petri plate of nutrient agar (10% NaCl)
- 1 Petri plate of milk salt agar (15% NaCl)
- cultures of *Escherichia coli* (nutrient broth), *Staphylococcus aureus* (nutrient broth) and *Halobacterium salinarium* (slant culture) or as instructed.

Procedure

1. Mark the bottoms of the four Petri plates.

2. Streak each organism in a straight line on the agar, using a wire loop.

3. Incubate all the plates for 48 hours at room temperature with exposure to light (the pigmentation of *H. salinarium* requires light to develop). Record your results.

4. Continue the incubation of the milk salt agar plate for several more days in the same manner, and record your results again.

Notes and Results

EFFECT OF TEMPERATURE

Materials

- 24- to 48-hour tryptic soy broth cultures of *Escherichia coli* (ATCC 11229), *Bacillus stearothermophilus* (ATCC 7953), *Bacillus globisporus* (ATCC 23301), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 25923), and spore suspension of *Bacillus subtilis* (ATCC 6051). (To produce endospores, grow *B. subtilis* for 48 hours at 35°C on endospore agar, nutrient agar plus 0.002% $MnCl2 \cdot 4H2O$. Resuspend the paste in at least 7 ml of sterile diluent.)
- 12 tryptic soy agar slants
- Bunsen burner
- inoculating loop
- 15 tryptic soy broth tubes (9.9 ml per tube)
- test tube rack
- 18 sterile 1-ml pipettes with pipettor
- 3 sterile test tubes
- refrigerator set at 4°C
- incubators or water baths set at 4°, 23° to 25° (room temperature), 60°, 85°, and 100°C (The instructor or students are not limited to these temperatures. Modifications can be instituted based on incubators or water baths available.)
- wax pencil
- sterile water

Principles

Microbial growth is directly dependent on how temperature affects cellular enzymes. With increasing temperatures, enzyme activity increases until the three dimensional configuration of these molecules is lost because of denaturation of their protein structure. As the temperature is

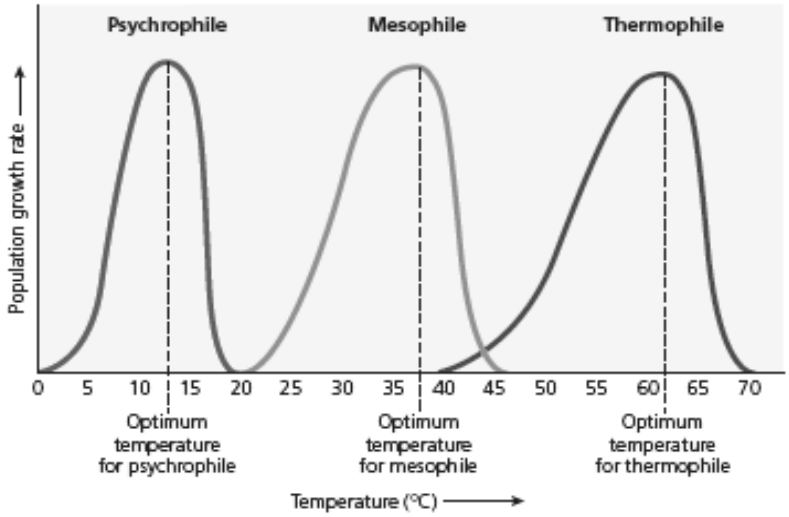
lowered toward the freezing point, enzyme inactivation occurs and cellular metabolism gradually diminishes. At 0°C, biochemical reactions cease in most cells.

Bacteria, as a group of living organisms, are capable of growth within an overall temperature range of minus 5°C to 80°C. Each species, however, requires a narrower range that is determined by the heat sensitivity of its enzyme systems. Specific temperature ranges consist of the following **cardinal (**significant**) temperature points** (see the Figure below):

1. Minimum growth temperature: The lowest temperature at which growth will occur. Below this temperature, enzyme activity is inhibited and the cells are metabolically inactive so that growth is negligible or absent.

2. Maximum growth temperature: The highest temperature at which growth will occur. Above this temperature, most cell enzymes are destroyed and the organism dies.

3. Optimum growth temperature: The temperature at which the rate of reproduction is most rapid; however, it is not necessarily optimum or ideal for all enzymatic activities of the cell.



The effect of temperature on the growth of microorganisms

All bacteria can be classified into one of three major groups, depending on their temperature requirements:

1. Psychrophiles: Bacterial species that will grow within a temperature range of −5°C to 20°C. The distinguishing characteristic of all psychrophiles is that they will grow between 0° and 5°C.

2. Mesophiles: Bacterial species that will grow within a temperature range of 20°C to 45°C. The distinguishing characteristics of all mesophiles are their ability to grow at human body temperature (37°C) and their inability to grow at temperatures above 45°C. Included among the mesophiles are two distinct groups:

a. Mesophiles with optimum growth temperature between 20°C and 30°C are plant saprophytes.

b. Mesophiles with optimum growth temperature between 35°C to 40°C are organisms that prefer to grow in the bodies of warmblooded hosts.

3. Thermophiles: Bacterial species that will grow at 35°C and above. Two groups of thermophiles exist:

a. Facultative thermophiles: Organisms that will grow at 37°C, with an optimum growth temperature of 45°C to 60°C.

b. Obligate thermophiles: Organisms that will grow only at temperatures above 50°C, with optimum growth temperatures above 60°C.

The ideal temperature for specific enzymatic activities may not coincide with the optimum growth temperature for a given organism. To understand this concept, you will investigate pigment production and carbohydrate fermentation by selected organisms at a variety of incubation temperatures.

1. The production of an endogenous red or magenta pigment by *Serratia marcescens* is determined by the presence of an orange to deep red coloration on the surface of the colonial growth.

2. Carbohydrate fermentation by *Saccharomyces cerevisiae* is indicated by the presence of gas, one of the end products of this fermentative process.

Detection of this accumulated gas may be noted as an air pocket, of varying size, in an inverted inner vial (Durham tube) within the culture tube.

Procedure

First Period

1. Work in groups of three to four students. Each group of students will be assigned one temperature to study: 4°, 23° to 25°, 60°, 85°, or 100°C.

2. Label each of the tryptic soy agar slants with the name of the test bacterium to be inoculated (*E. coli, B. stearothermophilus,* and *B. globisporus*), your name, and date.

3. Using aseptic technique, streak the surface of each slant with the appropriate bacterium. Incubate the slants for 24 to 48 hours at the temperature assigned to your group.

4. Take three sterile test tubes and label one *S. aureus,* the second *B. subtilis spores,* and the third *P. aeruginosa.* Add your name and date.

5. With a sterile pipette, aseptically add 1 ml of bacterial culture or spore suspension to the respective tubes.

6. Subject your tubes to the temperature you are studying for 15 minutes (i.e., either place them in the refrigerator, let them stand at room temperature, or put them in one of the water baths).

7. After 15 minutes, let the samples cool or warm up to room temperature. For each bacterial sample, make a dilution series as follows:

Pipette 0.1 ml of the incubated sample into the 9.9 ml tryptic soy broth (10^{-2} dilution). Mix the tube thoroughly. With a fresh pipette, transfer 0.1 ml of this 10^{-2} dilution into 9.9 ml of broth (10^{-4} dilution) and mix. In the same way, prepare a 10^{-6}, 10^{-8}, and 10^{-10} dilution.

8. Incubate all dilutions at 35°C for 24 to 48 hours.

Second Period

1. At the end of incubation, observe the slants for the presence of growth. Record your observations and those of your classmates; use a + for the

presence of growth and a – for the absence of growth in Part 1 of the exercise.

2. Observe your dilution series to see which tubes have bacterial growth as indicated by turbidity. The logic of this procedure is that reproduction will have occurred in each tube that received at least one living bacterium. The greater the number of bacteria present in the particular sample, the more such a sample can be diluted and still contain bacteria in the aliquot transferred. Thus, if bacteria **A** are less susceptible to heat than bacteria **B,** bacteria **A** will require more dilutions in order to obtain a sterile sample as indicated by no growth.

3. From your results and those of your classmates, indicate the last dilution in which growth occurred.

Notes and Results

EFFECT OF PH

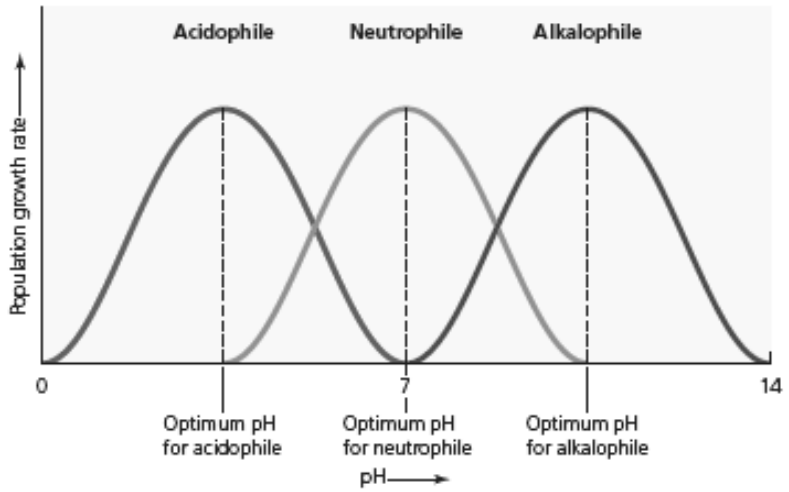
Materials

- saline suspensions of 24- hour tryptic soy broth cultures of *Alcaligenes faecalis* (ATCC 8750), *Escherichia coli* (ATCC 11229), and a Sabouraud dextrose agar slant (48 hours at 30°C) of *Saccharomyces cerevisiae* (ATCC 2366, a yeast). Add 3 ml of sterile saline to each *S. cerevisiae* slant and resuspend the yeast with a sterile inoculating loop. Transfer the suspension to a sterile culture tube. Adjust the suspensions to an absorbance of 0.05 at a wavelength of 550 to 600 nm by adding either more saline or culture.
- pH meter or pH paper
- 4 tryptic soy broth tubes, pH 3.0
- 4 tryptic soy broth tubes, pH 5.0
- 4 tryptic soy broth tubes, pH 7.0
- 4 tryptic soy broth tubes, pH 9.0
  (the pH of the above tubes is adjusted with either 1 N sodium hydroxide or 1 N hydrochloric acid)
- Bunsen burner
- sterile 1-ml pipettes with pipettor
- spectrophotometer
- cuvettes
- wax pencil
- test-tube rack

Principles

It is not surprising that **pH** (acidity; log 1/ $[H^+]$ dramatically affects bacterial growth. The pH affects the activity of enzymes—especially those that are involved in biosynthesis and growth. Each microbial species possesses a definite pH **growth range** and a distinct pH **growth**

optimum. Acidophiles have a growth optimum between pH 0.0 and 5.5; **neutrophiles** between 5.5 and 8.0; and **alkalophiles** 8.5 to 11.5. In general, different microbial groups have characteristic pH optima. The majority of bacteria and protozoa are neutrophiles. Most molds and yeasts occupy slightly acidic environments in the pH range of 4 to 6; algae also seem to favor acidity Many bacteria produce metabolic acids that may lower the pH and inhibit their growth. To prevent this, **buffers** that produce a pH equilibrium are added to culture media to neutralize these acids. For example, the peptones in complex media act as buffers. Phosphate salts are often added as buffers in chemically defined media.



The effect of ph on the growth of microorganisms

Procedure

First Period

1. Label each of the tryptic soy broth tubes with the pH of the medium, your name, date, and the microorganism to be inoculated.

2. Using a sterile pipette, add 0.1 ml of the *E. coli* saline culture to the tube that has a pH of 3.0. Do the same for the tubes that have pH values of 5.0, 7.0, and 9.0.

3. Repeat the above for *A. faecalis* and *S. cerevisiae.*

4. Incubate the *E. coli* and *A. faecalis* cultures for 24 to 48 hours at 35°C, and the *S. cerevisiae* culture for 48 to 72 hours at room temperature.

Second Period

1. By using the spectrophotometer, set the wavelength at 550 to 600 nm. Calibrate the spectrophotometer, using a tryptic soy broth blank of each pH for each respective set of cultures.

2. Fill each cuvette 2/ 3 full of the respective pH culture and read the absorbance. Blank the spectrophotometer with tryptic soy broth.

3. If no spectrophotometer is available, record your visual results as – (no growth), +, ++, +++, and ++++ (for increasing growth, respectively).

4. Record your.

Notes and Results

EFFECTS OF DISINFECTANTS

Materials

- 20- hour tryptic soy broth cultures of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 10145)
- 2 sterile screw cap test tubes
- 1 sterile 5 ml pipette with pipettor
- 12 sterile 1 ml pipettes
- 48 tryptic soy broth tubes (10 ml per tube)
- sterile water in Erlenmeyer flask
- 12 sterile tubes for making dilutions
- commercial disinfectants such as 3% hydrogen peroxide, 70% isopropyl alcohol, bleach, or any cleaner or students can bring in their own to test. If commercial disinfectants are used, note the use dilution and active ingredients. Dilute with normal tap water. The tap water need not be sterilized for commercial disinfectants.
- phenol (carbolic acid)
- wax pencil
- 35°C incubator
- test-tube rack
- Bunsen burner
- inoculating loop

Principles

Many factors influence the effectiveness of chemical disinfectants and antiseptics. The **microbicidal** (to kill) or **microbiostatic** (to inhibit) **efficiency** of a chemical is often determined with respect to its ability to deter microbial growth. The first part of this exercise will examine this effect of several chemicals. More specifically, the microbicidal efficiency of a chemical is often determined with respect to phenol and is known as the **phenol coefficient (PC).**

The phenol coefficient is calculated by dividing the highest dilution of the antimicrobial of interest, which kills all organisms after incubation for 10 minutes but not after 5 minutes, by the highest dilution of phenol that has the same characteristics. Chemicals that have a phenol coefficient greater than 1 are more effective than phenol, and those that have a phenol coefficient less than 1 are less effective than phenol. However, this comparison should only be used for phenol- like compounds that do not exert bacteriostatic effects and are not neutralized by the subculture media used. The second part of this experiment will enable you to calculate a phenol coefficient for a select chemical.

Procedure

First Period

A- Growth Inhibition

1. Each group of students should select one of the disinfectants and, if necessary, dilute it according to the specifications on the label (the **use dilution**).

2. Place 5 ml of disinfectant into two sterile tubes. Add 0.05 ml of *P. aeruginosa* to one tube and 0.05 ml of *S. aureus* to the other.

3. Using the wax pencil, label the tubes with your name and those of the respective bacteria. Mix each of the tubes in order to obtain a homogeneous suspension.

4. At intervals of 1, 2, 5, 10, and 15 minutes, transfer 0.1 ml of the mixture containing the bacteria and disinfectant to separate tubes oftryptic soy broth. Do this for both bacteria. Also inoculate two tubes of broth with 0.1 ml of both bacteria and mark these "controls."

5. Incubate all tubes for 48 hours at 35°C.

B- Phenol Coefficient

1. Dilute phenol in sterile distilled water 1/ 80, 1/ 90,

and 1/ 100; dilute the used cleaner 1/ 400, 1/ 450, and 1/ 500 so that the final volume in each tube is 5 ml.

2. Label 18 tryptic soy broth tubes with the name and dilution of disinfectant, the time interval of the subculture (e.g., 5 minutes, phenol 1/ 80), and your name. Each dilution should be tested after 5, 10, and 15 minute incubations.

3. Place in order in a test tube rack, one test tube of each of the different cleaner and phenol dilutions for each time interval.

4. Add 0.5 ml of *S. aureus* to each tube of disinfectant and note the time. Mix each of the tubes in order to obtain a homogeneous suspension and allow the disinfectant to come into contact with the bacteria.

5. Using aseptic technique, at intervals of 5, 10, and 15 minutes, transfer one loopful from each disinfectant tube into the appropriately labeled tryptic soy broth tube.

6. Incubate all tubes for 48 hours at 35°C.

7. The experiment can be repeated with *P. aeruginosa.*

Second Period

A- Growth Inhibition

1. Shake and observe each of the tubes for growth. Record the presence of growth as + and the absence of growth as –. Tabulate your results as well as the results of the class.

B- Phenol Coefficient

1. Shake and observe all tryptic soy broth cultures for the presence (+) or absence (–) of growth.

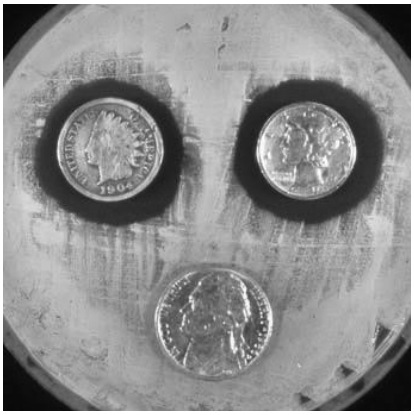
2. Record your observations.

Notes and Results

EFFECT OF HEAVY METALS

Principle

Metals such as mercury, silver, lead and copper are called **heavy metals** because of their large atomic weights and complex electron configurations. Salts of heavy metals catch onto sulfhydryl groups (–SH) on certain amino acids, thereby interfering with proteins and killing microbial cells (**see the following Figure**). Because many of the proteins involved are enzymes, cellular metabolism is disrupted, and the microorganism dies. However, heavy metals are not sporicidal.



The early-20th-century cent and dime were composed mainly of copper and silver, respectively. The clear zones around these two coins are "zones of inhibition" where the bacteria are unable to survive because these metals are present. The metal found in the nickel has little negative effect on the bacterial species in this culture.

In the following experiment, we will study the effect of both iron and copper on both Gram +ve and Gram –ve bacteria.

Materials

- Liquid cultures of *E. coli* and *B. cereus* or *subtilis*
- Two plates of sterilized nutrient agar
- Different metal coins (old and new preferably of different metals) and two nails (4- 6 cm long)
- Marker pen
- Triangular spreader
- Ethanol

- 2 sterile 1 ml pipettes
- Metal forceps
- Bunsen burner

Procedure

1- Sterilize the spreader by alcoholic flaming.

2- By using the sterile spreader, spread 0.1 ml of each cultures on one plates using a sterile pipette.

3- Hold the metal coin by forceps and sterilize by alcoholic flaming. Wait for seconds to cool and put in one plate and repeat for other coins. Repeat the procedure for metal nails in the second plate.

4- Incubate the plates inverted at 30°C until next day.

5- Observe and draw the growth and clear zones around the metal objects and record your results and notes.

Results

EFFECT OF DYES

Principle

The inhibitory effects of dyes on bacteria were first studied by Churchman and Kline in 1912, who tested gentian violet (crystal violet) and brilliant Green on bacterial cultures. Early works in this area were centered on the elucidation of the chemotherapeutic potential of dyes.

The incorporation of dyes into culture media for the purposes of isolation and differentiation of bacteria was described by Endo in 1904, who used basic fuchsin decolorized with sodium sulfite for the isolation of coliform organisms. Modifications of Endo's medium were developed in subsequent years, and a number of other dyes came into use. These dye-containing media found their greatest application in the field of sanitary microbiology where they were used for the detection of fecal coliforms from milk and water samples and for the isolation and detection of typhoid and other intestinal pathogens from fecal specimens. Dyes used extensively for their inhibitory and differential properties (especially selective for Gram negative organisms) are basic fuchsin, crystal violet, eosine Y, methylene blue, and Brilliant Green. Acridine orange, ethyl violet, aniline blue, and trypan blue were also used for selectivity for streptococci. Since these dyes are useful in differentiating between certain bacteria or groups of bacteria on the basis of inhibition, it is possible that many other dyes could be as effective or more effective in a similar manner.

Materials

- Solid or liquid cultures of *E. coli* and *B. cereus* or *subtilis*
- Inoculating loop
- Crystal violet solution (prepared for Gram staining)
- 4 test tubes containing 9 ml distilled water, sterilized
- 5 sterile Petri plates

- 5 sterile 1 ml pipettes
- Water bath adjusted to 45°C
- Sterile 10 ml measuring cylinder
- Marker pen

Procedure

1- Make a series of dilutions from the crystal violet solution using the tubes of distilled water by adding one ml in the first tube. Shake and transfer one ml to the second tube. Repeat this for the rest of the tubes until you have dilutions as follows: original stain, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.

2- Pour 9 ml of nutrient agar in each plate.

3- Immediately add one ml of each dye dilutions to one of the plates and carefully shake in a "number 8 shape" until homogenous.

4- Now you have 5 agar plates with different dye dilutions as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}.

5- After solidification, divide the plates from the reverse side into two halves by the marker.

6- Inoculate *E. coli* in one half of each plate and the other with *Bacillus* in the middle of the used space (but not reaching the end). Try to make equal lines of inoculations in all plates.

7- Incubate all the plates at 30°C until next day.

8- Observe and draw the bacterial growth results for each plate and record your results.

Your Results

DETERMINATION OF BACTERIAL GROWTH CURVE (Two-Hour Method)

Materials

- 6-hour starter culture of *E. coli* (or as instructed)
- 500 ml flask containing approximately 300 ml of nutrient broth
- and 10-ml pipettes with pipettor
- water bath or incubator at 37°C

Principles

The four phases **(lag, logarithmic, stationary,** and **death or decline)** of growth of a bacterial population can be determined by measuring the **turbidity** of the population in a broth culture. Turbidity is not a direct measure of bacterial numbers but an indirect measure of biomass, which can be correlated with cell density during the log growth phase. Since about 10^7 bacterial cells per milliliter must be present to detect turbidity with the unaided eye, a spectrophotometer can be used to achieve increased sensitivity and obtain quantitative data. The construction of a complete **bacterial growth curve** (increase and decrease in cell numbers versus time) requires that aliquots of a shake- flask culture be measured for population size at intervals over an extended period. Because this may take many hours, such a procedure does not lend itself to a regular laboratory session. *E coli* is a rapid growth bacterium (a generation time of less than 20 minutes) makes this bacterium useful for determining a complete bacterial growth curve. The growth cycle, from lag through log and stationary phases, can be measured in approximately 2- 3 hours.

Procedure (Two-Hour Method)

1. Zero the spectrophotometer at 550 to 600 nm with the medium.

2. Place the flask containing the nutrient broth medium in the 37°C water bath or incubator for 15 minutes.

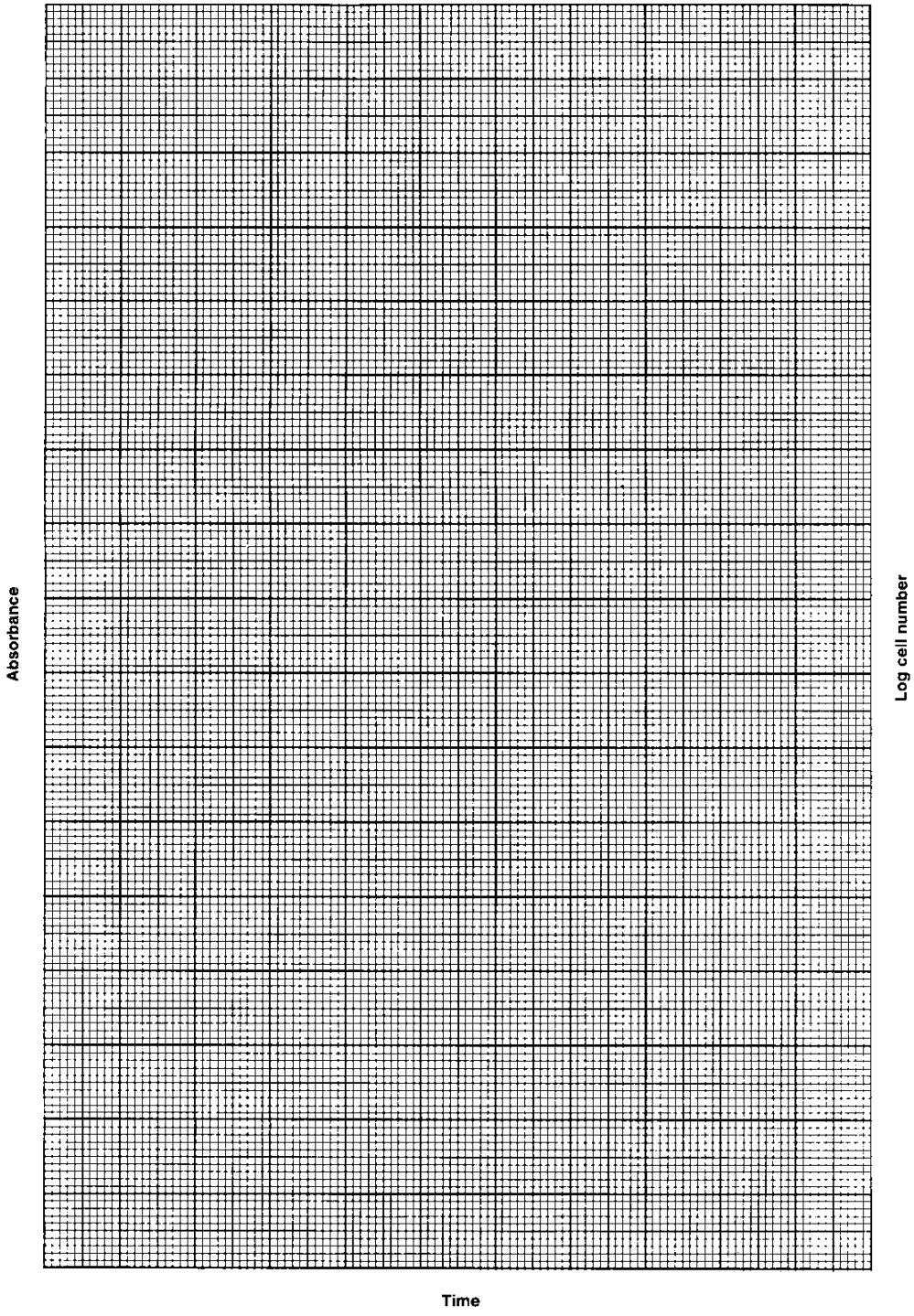
3. While slowly agitating the flask in the water bath, inoculate it with 10 ml of 6 hour *E. coli* culture.

4. Read and record the absorbance *(A)* of this initial culture (0 time) and every 20 minutes thereafter for about 2 hours. Be sure to suspend the bacteria thoroughly each time before taking a sample.

5. Construct a growth curve by plotting *A* against time on semilog graph paper.

Results

Graph paper



MICROBIOLOGY OF MILK AND FOOD PRODUCTS

Milk and food provide excellent growth media for bacteria when suitable temperatures exist. This is in direct contrast to natural waters, which lack the essential nutrients for pathogens. The introduction of a few pathogens into food or milk products becomes a much more serious problem because of the ability of these substances to support tremendous increases in bacterial numbers. Many milk- borne epidemics of human diseases have been spread by contamination of milk by soiled hands of dairy workers, unsanitary utensils, flies, and polluted water supplies. The same thing can be said for improper handling of foods in the home, restaurants, hospitals, and other institutions. Bacteriological testing of milk and food may also be performed in this same manner like water testing, using similar media and procedures to detect the presence of coliforms. However, most testing by public health authorities is quantitative. Although the presence of small numbers of bacteria in these substances does not necessarily mean that pathogens are lacking, low counts do reflect better care in handling of food and milk than is true when high counts are present.

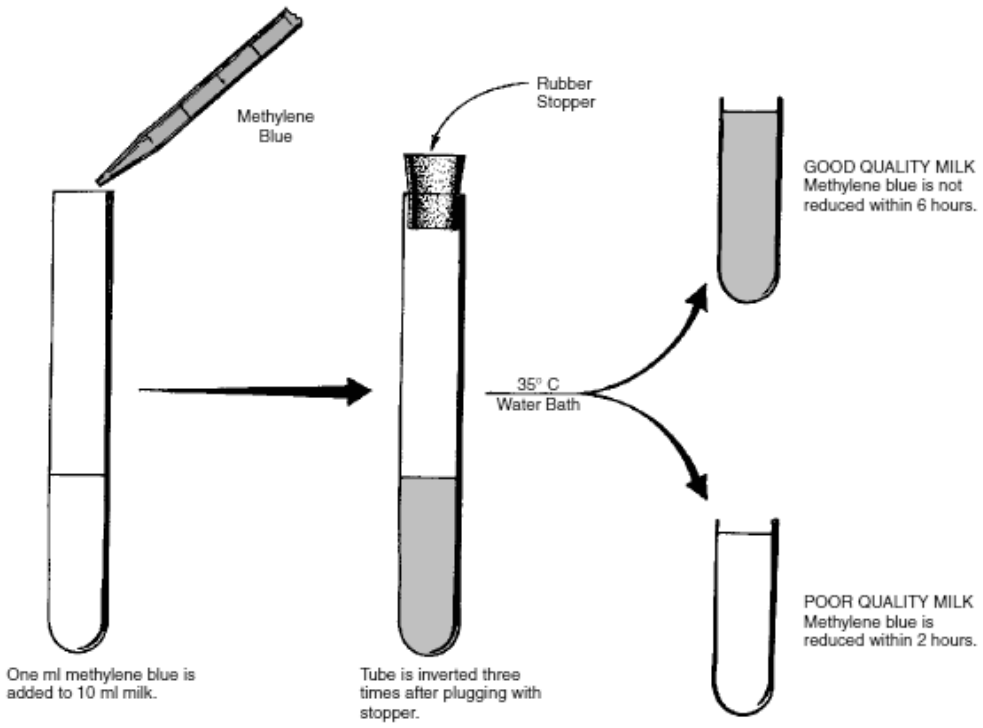
Standardized testing procedures for milk products are outlined by the American Public Health Association in Standard Methods for the Examination of Dairy Products. Since bacterial counts in foods are performed with some of the techniques you have learned in previous exercises, you will have an opportunity to apply some of those skills here.

Reductase Test

Milk that contains large numbers of actively growing bacteria will have a lowered oxidation- reduction potential due to the exhaustion of dissolved oxygen by microorganisms. The fact that methylene blue loses its color (becomes reduced) in such an environment is the basis for the **reductase test.** In this test, 1 ml of methylene blue (1:25,000) is added to 10 ml of milk. The tube is sealed with a rubber stopper and slowly inverted three

times to mix. It is placed in a water bath at 35° C and examined at intervals up to 6 hours (or up to the end of class). The time it takes for the methylene blue to become colorless is the **methylene blue reduction time** (MBRT). The shorter the MBRT, the lower the quality of milk. An MBRT of 6 hours is very good. Milk with an MBRT of 30 minutes is of very poor quality. The validity of this test is based on the assumption that all bacteria in milk lower the oxidation reduction potential at 35° C. Large numbers of psychrophiles, thermophiles, and thermodurics, which do not grow at this temperature, would not produce a positive test. Raw milk, however, will contain primarily *Streptococcus lactis* and *Escherichia coli*, which are strong reducers; thus, this test is suitable for screening raw milk at receiving stations. Its principal value is that less technical training of personnel is required for its performance.

In this exercise, samples of low- and high-quality raw milk will be tested.



Procedure for testing raw milk with reductase test

Materials:

- 2 sterile test tubes with rubber stoppers
- raw milk samples of low- and high-quality (samples A and B)
- water bath set at 35° C
- methylene blue (1:25,000)
- 10 ml pipettes
- 1 ml pipettes
- gummed labels

1. Attach gummed labels with your name and type of milk to two test tubes (a good- quality as well as a poor-quality milk).

2. Using separate 10 ml pipettes for each type of milk, transfer 10 ml to each test tube. To the milk in the tubes add 1 ml of methylene blue with a 1 ml pipette. Insert rubber stoppers and gently invert three times to mix. Record your name and the time on the labels and place the tubes in the water bath, which is set at 35° C.

3. After 5 minutes incubation, remove the tubes from the bath and invert once to mix. This is the last time they should be mixed.

4. Carefully remove the tubes from the water bath 30 minutes later and every half hour until the end of the laboratory period. When at least four-fifths of the tube has turned white, the end point of reduction has taken place. Record this time on the Laboratory Report. The classification of milk quality is as follows:

Class 1: Excellent, not decolorized in 8 hours.

Class 2: Good, decolorized in less than 8 hours, but not less than 6 hours.

Class 3: Fair, decolorized in less than 6 hours, but not less than 2 hours.

Class 4: Poor, decolorized in less than 2 hours.

Notes and Results

Microbiology of Yogurt Production

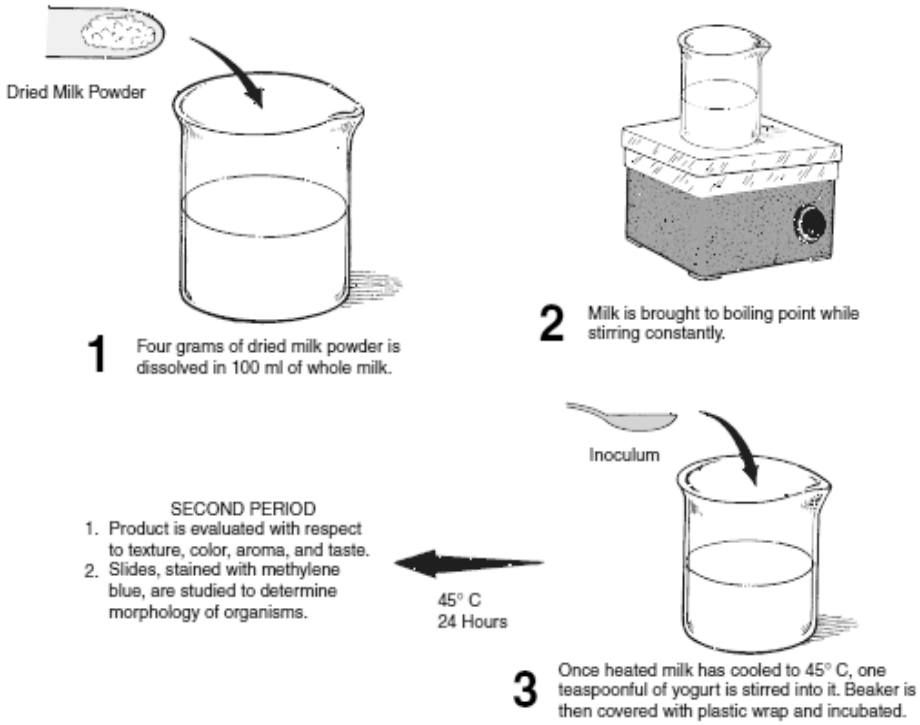
For centuries, people throughout the world have been producing fermented milk products using yeasts and lactic acid bacteria. The **yogurt** of eastern central Europe, the **kefir** of the Cossacks, the **koumiss** of central Asia, and the **leben** of Egypt are just a few examples. In all of these fermented milks, lactobacilli act together with some other microorganisms to curdle and thicken milk, producing a distinctive flavor desired by the producer.

Kefir of the Cossacks is made by charging milk with small cauliflower-like grains that contain *Streptococcus lactis, Saccharomyces delbrueckii,* and *Lactobacillus brevis.* As the grains swell in the milk they release the growing microorganisms to ferment the milk. The usual method for producing yogurt in large scale production is to add pure cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to pasteurized milk.

In this exercise you will produce a batch of yogurt from milk by using an inoculum from commercial yogurt. Gram- stained slides will be made from the finished product to determine the types of organisms that control the reaction. If proper safety measures are followed, the sample can be tasted. Two slightly different ways of performing this experiment are provided here. Your instructor will indicate which method will be followed.

METHOD A (First Period)

The following Figure illustrates the procedure for this method. Note that 4 g of powdered milk are added to 100 ml of whole milk. This mixture is then heated to boiling and cooled to 45°C. After cooling, the milk is inoculated with yogurt and incubated at 45° C for 24 hours.



Yogurt production by Method A

Materials:

- dried powdered milk
- whole milk
- commercial yogurt (with viable organisms)
- small beaker, graduate, teaspoon, stirring rod
- plastic wrap
- filter paper (for weighing)

Procedure

1. On a piece of filter paper weigh 4 grams of dried powdered milk.

2. To a beaker of 100 ml of whole milk add the powdered milk and stir thoroughly with sterile glass rod to dissolve.

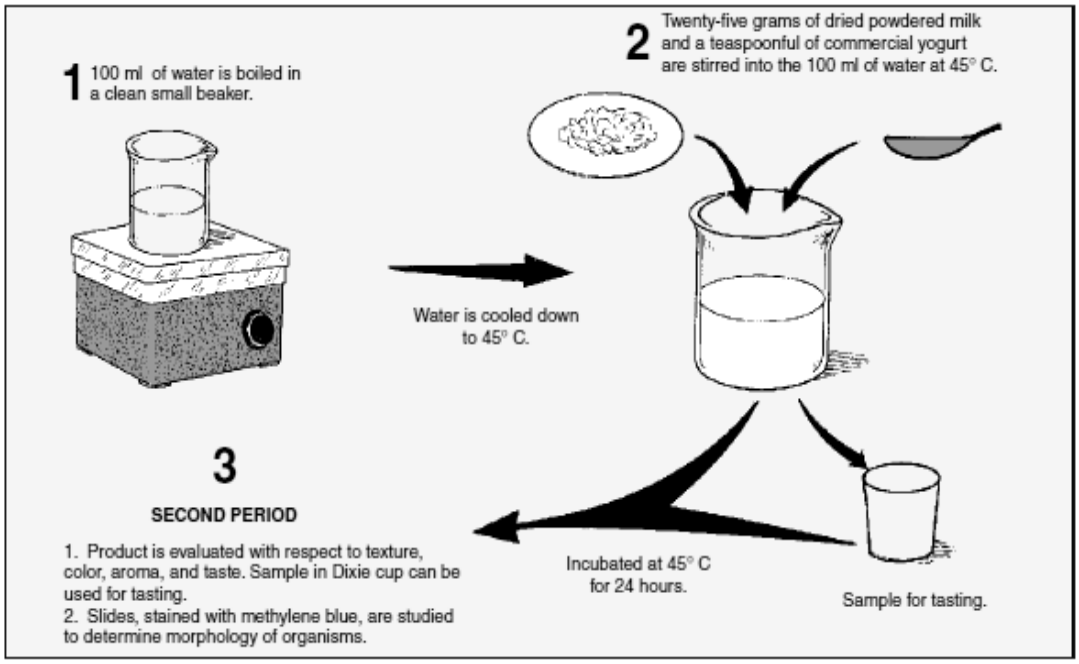
3. Heat to boiling, while stirring constantly.

4. Cool to 45° C and inoculate with 1 teaspoon of the commercial yogurt. Stir. Be sure to check the label to make certain that product contains a live culture. Cover with plastic wrap.

5. Incubate at 45° C for 24 hours.

METHOD B (First Period)

The following Figure illustrates a slightly different method of culturing yogurt, which, due to its simplicity, may be preferred. Note that no whole milk is used and provisions are made for producing a sample for tasting.



Yogurt production by Method B

Materials:

- small beaker, graduate, teaspoon, stirring rod
- dried powdered milk
- commercial yogurt (with viable organisms)
- plastic wrap
- filter paper for weighing
- paper cup (50 or 100 ml size) and cover
- electric hot plate or Bunsen burner and tripod

Procedure

1. On a piece of filter paper weigh 25 grams of dried powdered milk.

2. Heat 100 ml of water in a beaker to boiling and cool to 45° C.

3. Add the 25 grams of powdered milk and 1 teaspoon of yogurt to the beaker of water. Mix the ingredients with a sterile glass rod.

4. Pour some of the mixture into a sterile cup and cover loosely. Cover the remainder in the beaker with plastic wrap.

5. Incubate at 45° C for 24 hours.

SECOND PERIOD (Both Methods)

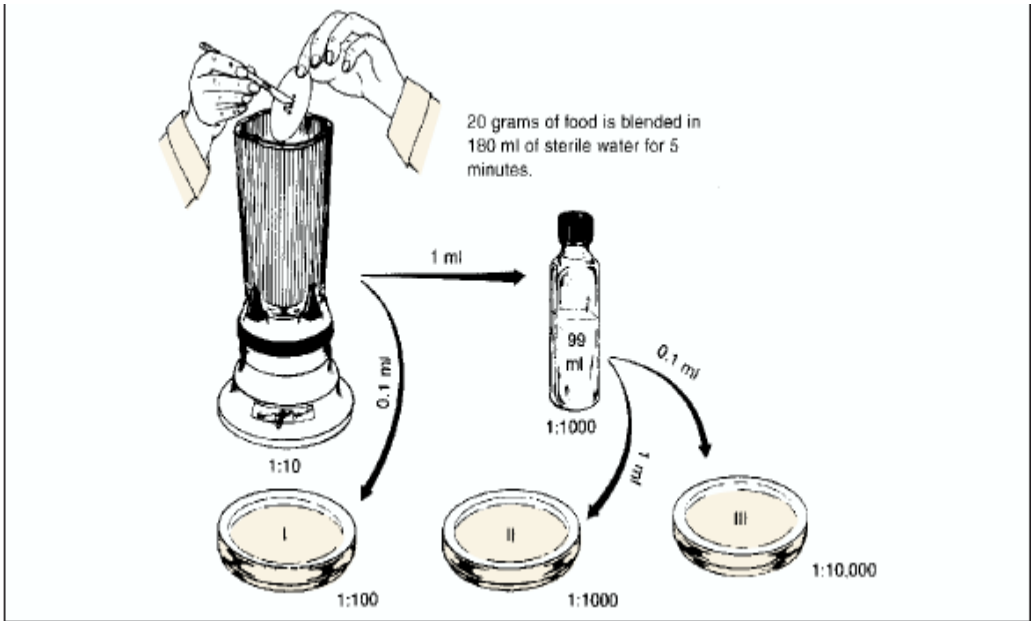
1. Examine the product and record the color, aroma, texture, and, if desired, the taste.

2. Make slide preparations of the yogurt culture. Fix and stain with methylene blue. Examine under oil immersion and record your results.

Notes and Results

Bacterial Count of a Food Product

The standard plate count, as well as the multiple tube test, can be used on foods much in the same manner that they are used on milk and water to determine total counts and the presence of coliforms. To get the organisms in suspension, however, a food blender is necessary. This will not be a coliform count. The instructor will indicate the specific kinds of foods to be tested and make individual assignments. The following Figure illustrates the general procedure.



Dilution procedure for bacterial counts of food

Materials

- 3 Petri plates
- 1 bottle (45 ml) of Plate Count agar or Standard Methods agar
- 1 99 ml sterile water blank
- 2 1 ml dilution pipettes
- food blender
- sterile blender jars (one for each type of food)
- sterile weighing paper

- 180 ml sterile water blanks (one for each type of food)
- samples of ground meat, dried fruit, and frozen vegetables, thawed for 2 hours

Procedure

1. Using aseptic techniques, weigh out on sterile weighing paper 20 grams of food to be tested.

2. Add the food and 180 ml of sterile water to a sterile mechanical blender jar. Blend the mixture for 5 minutes. This suspension will provide a 1:10 dilution. (Alternatively add 10g of food to 190 ml of water).

3. With a 1 ml dilution pipette dispense from the blender 0.1 ml to plate I and 1.0 ml to the water blank. See the figure.

4. Shake the water blank 25 times in an arc for 7 seconds with your elbow on the table as done in the Exercise of "Bacterial Population Counts.

5. Using a fresh pipette, dispense 0.1 ml to plate III and 1.0 ml to plate II.

6. Pour agar (50° C) into the three plates and incubate them at 35° C for 24 hours.

7. Count the colonies on the best plate and record the results.

Notes and Results

BIOCHEMICAL ACTIVITIES OF MICROORGANISMS

Microorganisms must be separated and identified for a wide variety of reasons, including:

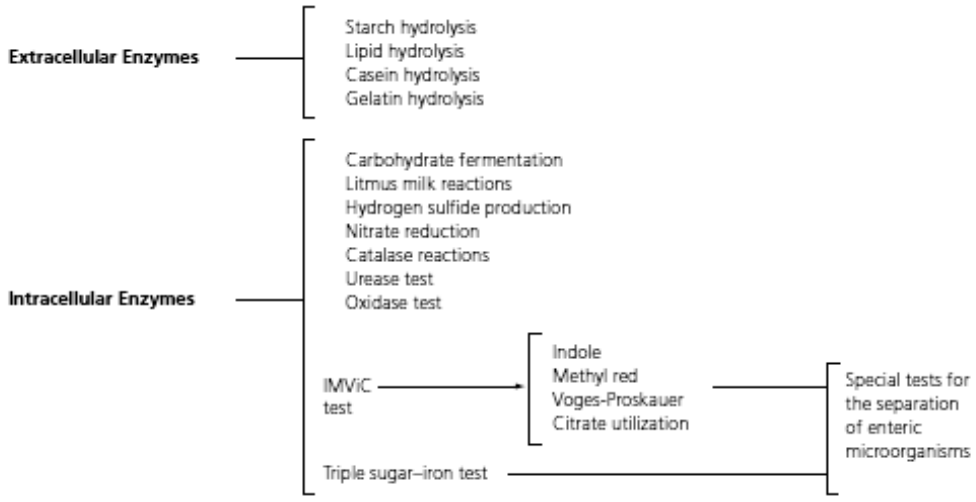
1. Determination of pathogens responsible for infectious diseases.

2. Selection and isolation of strains of fermentative microorganisms necessary for the industrial production of alcohols, solvents, vitamins, organic acids, antibiotics, and industrial enzymes.

3. Isolation and development of suitable microbial strains necessary for the manufacture and the enhancement of quality and flavor in certain food materials, including yogurt, cheeses, and other milk products.

4. Comparison of biochemical activities for taxonomic purposes.

To accomplish these tasks, the microbiologist utilized the fact that microorganisms all have their own identifying biochemical characteristics. These so- called biochemical fingerprints are the properties controlled by the cells' enzymatic activity, and they are responsible for bioenergetics, biosynthesis, and biodegradation. The sum of all these chemical reactions is defined as **cellular metabolism**, and the biochemical transformations that occur both outside and inside the cell are governed by biological catalysts called **enzymes**.



Biochemical activities of microorganisms

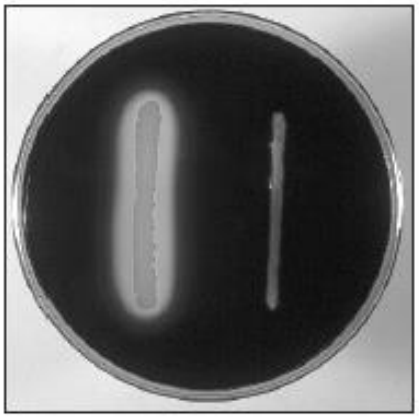
Extracellular enzymatic Activities of Microorganisms

Because of their large sizes, high molecular- weight nutrients such as polysaccharides, lipids, and proteins are not capable of permeating the cell membrane. These macromolecules must first be hydrolyzed by specific extracellular enzymes into their respective basic building blocks. These low molecular- weight substances can then be transported into the cells and used for the synthesis of protoplasmic requirements and energy production. The following procedures are designed to investigate the exoenzymatic activities of different microorganisms.

Starch Hydrolysis

Starch is a high molecular- weight, branching polymer composed of **glucose** molecules linked together by **glycosidic bonds**. The degradation of this macromolecule first requires the presence of the extracellular enzyme **amylase** for its hydrolysis into shorter polysaccharides, namely **dextrins**, and ultimately into **maltose** molecules. The final hydrolysis of this disaccharide, which is catalyzed by **maltase**, yields low molecular-weight, soluble **glucose** molecules that can be transported into the cell and used for energy production through the process of glycolysis.

In this experimental procedure, starch agar is used to demonstrate the hydrolytic activities of these exoenzymes. The medium is composed of nutrient agar supplemented with starch, which serves as the polysaccharide substrate. The detection of the hydrolytic activity following the growth period is made by performing the starch test to determine the presence or absence of starch in the medium. Starch in the presence of iodine will impart a blue- black color to the medium, indicating the absence of starch- splitting enzymes and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the growth of the organism. This is a positive result. Positive and negative results are shown in the following figure.



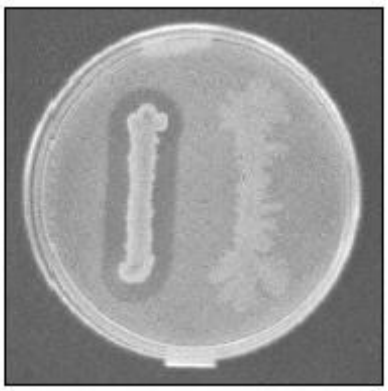
Starch agar plate. Starch hydrolysis on left; no starch hydrolysis on right.

Lipid Hydrolysis

Lipids are high molecular-weight compounds possessing large amounts of energy. The degradation of lipids such as **triglycerides** is accomplished by extracellular hydrolyzing enzymes, called **lipases** (esterases), that cleave the **ester bonds** in this molecule by the addition of water to form the building blocks **glycerol** (an alcohol) and **fatty acids**. The following figure shows this reaction. Once assimilated into the cell, these basic components can be further metabolized through aerobic respiration to produce cellular energy, adenosine triphosphate (ATP). The components may also enter other metabolic pathways for the synthesis of other cellular protoplasmic requirements.

In this experimental procedure, tributyrin agar is used to demonstrate the hydrolytic activities of the exoenzyme lipase. The medium is composed of nutrient agar supplemented with the triglyceride tributyrin as the lipid substrate. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium that is necessary for observing exoenzymatic activity. Following inoculation and incubation of the agar plate cultures, organisms excreting lipase will show a zone of **lipolysis**, which is demonstrated by a clear area surrounding the bacterial growth. This loss of opacity is the result of the hydrolytic reaction yielding soluble

glycerol and fatty acids and represents a positive reaction for lipid hydrolysis. In the absence of lipolytic enzymes, the medium retains its opacity. This is a negative reaction. Positive and negative results are shown in the following figure.



Tributyrin agar plate. Lipid hydrolysis on left; no lipid hydrolysis on right.

Casein Hydrolysis

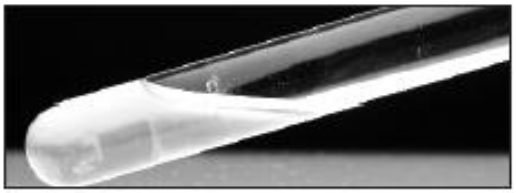
Casein, the major milk protein, is a macromolecule composed of **amino acid** subunits linked together by **peptide bonds** (CO—NH). Before their assimilation into the cell, proteins must undergo step-by-step degradation into **peptones**, **polypeptides**, **dipeptides**, and ultimately into their building blocks, **amino acids**. This process is called peptonization, or **proteolysis**, and it is mediated by extracellular enzymes called **proteases**. The function of these proteases is to cleave the peptide bond CO–NH by introducing water into the molecule. The reaction then liberates the amino acids. The low molecular-weight soluble amino acids can now be transported through the cell membrane into the intracellular amino acid pool for use in the synthesis of structural and functional cellular proteins.

In this experimental procedure, milk agar is used to demonstrate the hydrolytic activity of these exoenzymes. The medium is composed of nutrient agar supplemented with milk that contains the protein substrate casein. Similar to other proteins, milk protein is a colloidal suspension that gives the medium its color and opacity because it deflects light rays rather

than transmitting them. Following inoculation and incubation of the agar plate cultures, organisms secreting proteases will exhibit a zone of proteolysis, which is demonstrated by a clear area surrounding the bacterial growth. This loss of opacity is the result of a hydrolytic reaction yielding soluble, noncolloidal amino acids, and it represents a positive reaction. In the absence of protease activity, the medium surrounding the growth of the organism remains opaque, which is a negative reaction.

Gelatin Hydrolysis

Although the value of gelatin as a nutritional source is questionable (it is an incomplete protein, lacking the essential amino acid tryptophan), its value in identifying bacterial species is well established. Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Below temperatures of 25°C, gelatin will maintain its gel properties and exist as a solid; at temperatures above 25°C, gelatin is liquid. The following Figure shows gelatin hydrolysis.



(a) Positive for gelatin liquefaction

(b) Negative for gelatin liquefaction

Nutrient gelatin hydrolysis

Liquefaction is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called **gelatinase**, which acts to hydrolyze this protein to **amino acids**. Once this degradation occurs, even very low temperatures of 4°C will not restore the gel characteristic.

In this experimental procedure, you will use nutrient gelatin deep tubes to demonstrate the hydrolytic activity of gelatinase. The medium consists of nutrient broth supplemented with 12% gelatin. This high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Following inoculation and incubation for 48 hours, the cultures are placed in a refrigerator at 4°C for 30 minutes. Cultures that remain liquefied produce gelatinase and demonstrate rapid gelatin hydrolysis. Re- incubate all solidified cultures for an additional 5 days. Refrigerate for 30 minutes and observe for liquefaction. Cultures that remain liquefied are indicative of *slow* gelatin hydrolysis.

Materials for the above experiments

Cultures

24- to 48-hour trypticase soy broth cultures of *Escherichia coli, Bacillus cereus, Pseudomonas aeruginos ,* and *Staphylococcus aureus.*

Media and Reagent

- Two plates each of starch agar, tributyrin agar, and milk agar, and three nutrient gelatin deep tubes per designated student group.
- Gram's iodine solution.

Equipment

Microincinerator or Bunsen burner, inoculating loop and needle, glassware marking pencil, test tube rack, and refrigerator.

Controls

Test	Positive Control	Negative Control
Starch Hydrolysis	*B. cereus*	*E. coli*
Lipis Hydrolysis	*S. aureus*	*E. coli*
Casein Hydrolysis	*B. cereus*	*E. coli*
Gelatin Hydrolysis	*B. cereus*	*E. coli*

Procedure for all experiments

Lab One (Inoculation and Incubation)

1. Prepare the starch agar, tributyrin agar, and milk agar plates for inoculation. Using two plates per medium, divide the bottom of each Petri dish into two sections. Label the sections as *E. coli*, *B. cereus*, *P. aeruginosa*, and *S. aureus*, respectively.

2. Using aseptic technique, make a single-line streak inoculation of each test organism on the agar surface of its appropriately labeled section on the agar plates.

3. Using aseptic technique, inoculate each experimental organism in its appropriately labeled gelatin deep tube by means of a stab inoculation.

4. Incubate all plates in an inverted position for 24 to 48 hours at 37°C. Incubate the gelatin deep tube cultures for 48 hours. Re- incubate all negative cultures for an additional 5 days.

Lab Two

Starch Hydrolysis

1. Flood the starch agar plate cultures with Gram's iodine solution, allow the iodine to remain in contact with the medium for 30 seconds, and pour off the excess.

2. Examine the cultures for the presence or absence of a blue- black color surrounding the growth of each test organism. Record your results.

3. Based on your observations, determine and record the organisms that were capable of hydrolyzing the starch.

Lipid Hydrolysis

1. Examine the tributyrin agar plate cultures for the presence or absence of a clear area, or zone of lipolysis, surrounding the growth of each of the organisms. Record your results.

2. Based on your observations, determine and record which organisms were capable of hydrolyzing the lipid.

Casein Hydrolysis

1. Examine the milk agar plate cultures for the presence or absence of a clear area, or zone of proteolysis, surrounding the growth of each of the bacterial test organisms. Record your results.

2. Based on your observations, determine and record which of the organisms were capable of hydrolyzing the milk protein casein.

Gelatin Hydrolysis

1. Place all gelatin deep tube cultures into a refrigerator at 4°C for 30 minutes.

2. Examine all the cultures to determine whether the medium is solid or liquid. Record your results.

3. Based on your observations following the 2-day and 7-day incubation periods, determine and record: (a) which organisms were capable of hydrolyzing gelatin and (b) the rate of hydrolysis.

Notes and Results

DIFFERENTIATION OF ENTERIC BACILLI

The IMViC Tests

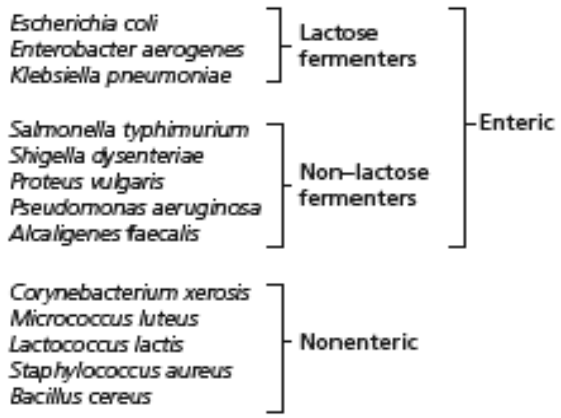
Identification of enteric bacilli is of prime importance in controlling intestinal infections by preventing contamination of food and water supplies. The groups of bacteria that can be found in the intestinal tract of humans and lower mammals are classified as members of the family **Enterobacteriaceae**. They are short, Gram negative, non spore- forming bacilli. Included in this family are:

1. Pathogens, such as members of the genera *Salmonella* and *Shigella.*

2. Occasional pathogens, such as members of the genera *Proteus* and *Klebsiella.*

3. Normal intestinal flora, such as members of the genera *Escherichia* and *Enterobacter*, which are saprophytic inhabitants of the intestinal tract.

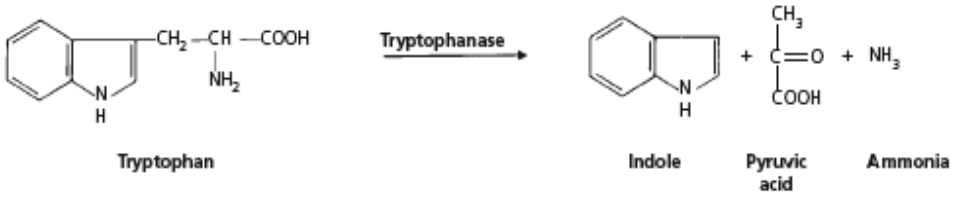
Differentiation of the principal groups of Enterobacteriaceae can be accomplished on the basis of their biochemical properties and enzymatic reactions in the presence of specific substrates. The **IMViC** series of tests (**indole**, **methyl red**, **Voges- Proskauer**, and **citrate utilization**) can be used. The biochemical reactions that occur during the IMViC tests are shown below. The enteric organisms are subdivided as lactose fermenters and non–lactose fermenters.



Principle

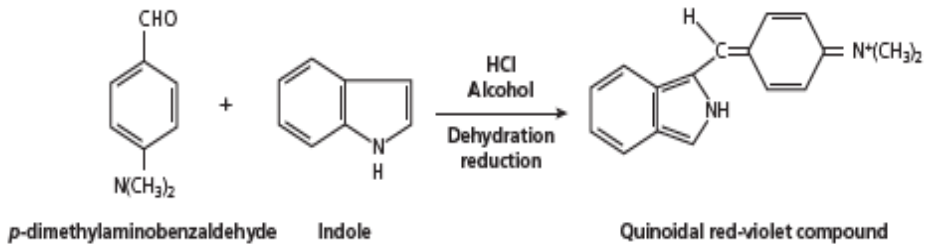
A) Tryptophanase test

Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme **tryptophanase**. The chemistry of this reaction is illustrated in the following Figure. This ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical marker.



Enzymatic degradation of tryptophan

In this experiment, SIM agar, which contains the substrate tryptophan, is used. The presence of indole is detectable by adding Kovac's reagent, which produces a cherry red reagent layer. This color is produced by the reagent, which is composed of *p*-dimethyl aminobenzaldehyde, butanol, and hydrochloric acid. Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms a complex with the *p*-dimethyl aminobenzaldehyde, yielding the cherry red color. The chemistry of this reaction is illustrated in the following Figure. Cultures producing a red reagent layer following addition of Kovac's reagent are indole positive; an example of this is *E. coli*. The absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole negative reaction.



Indole reaction with Kovac's reagent

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *P. vulgaris*, and *E. aerogenes* for the short version.

media

4 SIM agar deep tubes per designated student group:

reagent

Kovac's reagent.

Equipment

Microincinerator or Bunsen burner, inoculating needle, test tube rack, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled deep tube by means of a stab inoculation. The last tube will serve as a control.

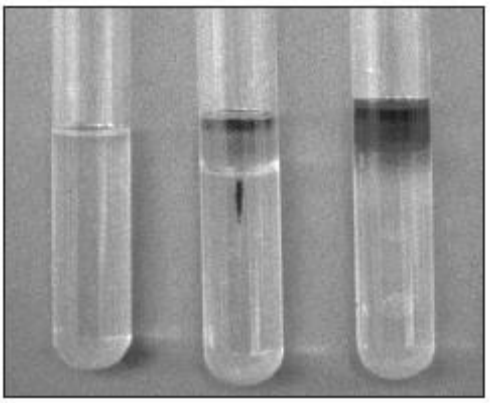
2. Incubate tubes for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.

2. Examine the color of the reagent layer in each culture (refer to the following Figure). Record your results in a chart.

3. Based on your observations, determine and record whether or not each organism was capable of hydrolyzing the tryptophan.



Indole production test.
(a) Uninoculated, (b) negative, and (c) positive.

B) Methyl Red Test

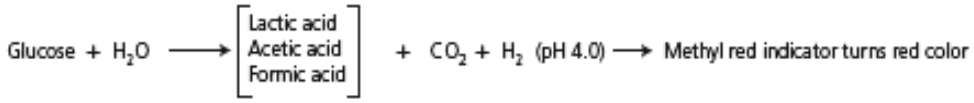
Principle

The hexose monosaccharide **glucose** is the major substrate utilized by all enteric organisms for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acid end products. Although most enteric microorganisms ferment glucose with the production of organic acids, this test is of value in the separation of *E. coli* and *E. aerogenes.*

Both of these organisms initially produce organic acid end products during the early incubation period. The low acidic pH (4) is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period, *E. aerogenes* enzymatically converts these acids to nonacidic end products, such as 2,3-butanediol and acetoin (acetylmethyl carbinol), resulting in an elevated pH of approximately 6. The glucose fermentation reaction generated by *E. coli* is illustrated

in the following Figure. As shown, at a pH of 4.4 or lower, the methyl red indicator in the pH range of 4 will turn red, which is indicative of a positive test. At a pH of 6.2 or higher, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicator turns yellow

and is a negative test. Production and detection of the nonacidic end products from glucose fermentation by *E. aerogenes* is amplified in Part C of this exercise, the Voges- Proskauer test, which is performed simultaneously with the methyl red test.



Glucose fermentation reaction with methyl red pH reagent

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes*, and *K. pneumoniae.* In Lab Two, aliquots of these experimental cultures must be set aside for the Voges- Proskauer test.

media

4 MR- VP broth per designated student group.

reagent

Methyl red indicator.

Equipment

Microincinerator or Bunsen burner, inoculating loop, test tubes, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube of medium by means of a loop inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

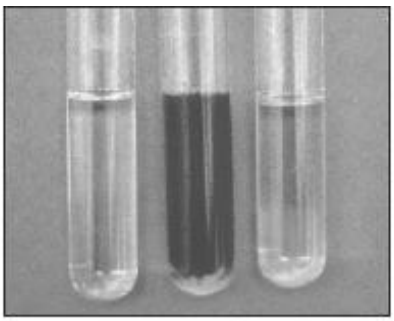
Procedure Lab Two

1. Transfer approximately one-third of each culture into an empty test tube and set these tubes aside for the Voges- Proskauer test.

2. Add five drops of the methyl red indicator to the remaining aliquot of each culture.

3. Examine the color of all cultures (refer to the following Figure). Record the results in a chart.

4. Based on your observations, determine and record whether or not each organism was capable of fermenting glucose with the production and maintenance of a high concentration of acid.



Methyl red test.
(a) Uninoculated, (b) positive, and (c) negative.

C) Voges- Proskauer Test

Principle

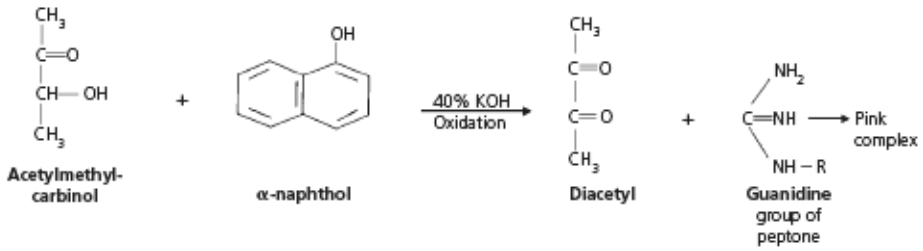
The Voges- Proskauer test determines the capability of some organisms to produce nonacidic or neutral end products, such as acetylmethylcarbinol, from the organic acids that result from glucose metabolism. This glucose fermentation, which is characteristic of *E. aerogenes*, is illustrated in the following Figure.

$$\text{Glucose} + O_2 \longrightarrow \underset{\text{acid}}{\text{Acetic}} \longrightarrow \left[\begin{array}{l}\text{2,3-butanediol} \\ \text{acetylmethylcarbinol}\end{array}\right] + CO_2 + H_2\ (\text{pH } 6.0)$$

Glucose fermentation by E. aerogenes

The reagent used in this test, Barritt's reagent, consists of a mixture of alcoholic anaphthol and 40% potassium hydroxide solution. Detection of acetylmethyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α- naphthol catalyst and a guanidine group that is present in the peptone of the MR-

VP medium. As a result, a pink complex is formed, imparting a rose color to the medium. The chemistry of this reaction is illustrated
in the following Figure.



Acetylmethylcarbinol reaction with Barritt's reagent

Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetylmethyl carbinol and represents a positive result. The absence of rose coloration is a negative result.

Cultures

24- to 48-hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes*, and *K. pneumoniae*. Note: Aliquots of these experimental cultures must be set aside from the methyl red test.

reagent

Barritt's reagents A and B.

Equipment

Microincinerator or Bunsen burner, inoculating loop, and glassware marking pencil.

Procedure Lab One

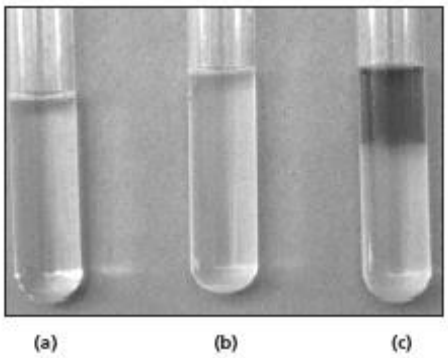
Refer to the methyl red test in Part B of this exercise.

Procedure Lab Two

1. To the aliquots of each broth culture separated during the methyl red test, add 10 drops of Barritt's reagent A and shake the cultures. Immediately add 10 drops of Barritt's reagent B and shake. Re shake the cultures every 3 to 4 minutes.

2. Examine the color of the cultures 15 minutes after the addition of Barritt's reagent. Record your results.

3. Based on your observations, determine and record whether or not each organism was capable of fermenting glucose with ultimate production of acetylmethyl carbinol.



Voges- Proskauer test
(a) Uninoculated, (b) negative, and (c) positive

Notes and Results

CITRATE UTILIZATION TEST

Principle

In the absence of fermentable glucose or lactose, some microorganisms are capable of using **citrate** as a carbon source for their energy. This ability depends on the presence of a **citrate permease** that facilitates the transport of citrate in the cell. Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme **citrase**, which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide. During this reaction, the medium becomes alkaline—the carbon dioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep Prussian blue. Following incubation, citrate- positive cultures are identified by the presence of growth on the surface of the slant, which is accompanied by blue coloration, as seen with *E. aerogenes*. Citrate-negative cultures will show no growth, and the medium will remain green.

Cultures

24- to 48- hour Trypticase soy broth cultures of *E. coli*, *E. aerogenes,* and *K. pneumoniae*.

media

4 Simmons citrate agar slants per designated student group.

Equipment

Microincinerator or Bunsen burner, inoculating needle, test tube rack, and glassware marking pencil.

Procedure Lab One

1. Using aseptic technique, inoculate each organism into its appropriately labeled tube by means of streak inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Examine all agar slant cultures for the presence or absence of growth and coloration of the medium. Record your results.

2. Based on your observations, determine and record whether or not each organism was capable of using citrate as its sole source of carbon.

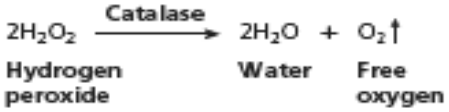
Note that negative tubes, showing no growth on slant surface, will have green colour and positive tubes, showing growth on slant surface will have blue colour.

Notes and Results

CATALASE TEST

Principle

During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing **catalase** rapidly degrade hydrogen peroxide as illustrated:



Aerobic organisms that lack catalase can degrade especially toxic superoxides using the enzyme **superoxide dismutase**; the end product of a superoxide dismutase is H_2O_2, but this is less toxic to the bacterial cells than are the superoxides. The inability of strict anaerobes to synthesize catalase, peroxidase, or superoxide dismutase may explain why oxygen is poisonous to these microorganisms. In the absence of these enzymes, the toxic concentration of H_2O_2 cannot be degraded when these organisms are cultivated in the presence of oxygen.

Catalase production can be determined by adding the substrate H_2O_2 to an appropriately incubated culture. If catalase is present, the chemical reaction mentioned is indicated by bubbles of free oxygen gas O_2. This is a positive catalase test; the absence of bubble formation is a negative catalase test. The following Figure shows the results of the catalase test using (a) the tube method, (b) the plate method, and (c) slide method.

Cultures

24- to 48-hour broth cultures of *Staphylococcus aureus* , *Micrococcus luteus*, and *Lactococcus lactis* for the short version.

media

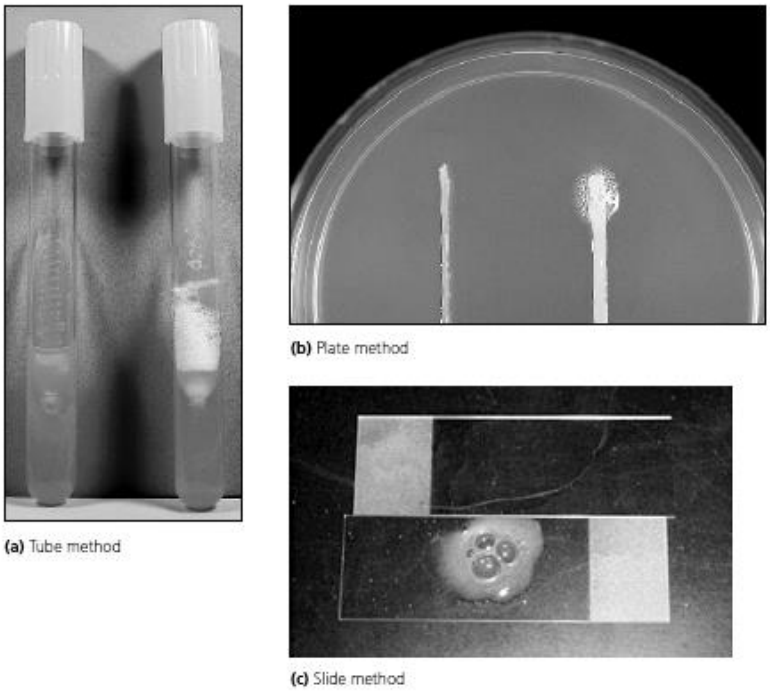
4 Trypticase soy agar slants per designated student group: for the short version.

reagent

3% hydrogen peroxide.

Equipment

Tube method: Microincinerator or Bunsen burner, inoculating loop, test tube rack, and glassware marking pencil.



Catalase test. Negative results are shown on the left and positive results on the right in the (a) tube method and (b) plate method. Negative results are shown on the top and positive results on the bottom in the (c) slide method.

Slide method: Microincinerator or Bunsen burner, inoculating loop, glassware marking pencil, 4 glass microscope slides, Petri dish and cover.

Procedure Lab One

(Tube method)

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube by means of a streak inoculation. The last tube will serve as a control.

2. Incubate all cultures for 24 to 48 hours at 37°C.

Procedure Lab Two

1. Allow three or four drops of the 3% hydrogen peroxide to flow over the entire surface of each slant culture.

2. Examine each culture for the presence or absence of bubbling or foaming. Record your results.

3. Based on your observations, determine and record whether or not each organism was capable of catalase activity.

Slide method

1. Label slides with the names of the organisms.

2. Using a sterile loop, collect a small sample of the first organism from the culture tube and transfer it to the appropriately labeled slide.

3. Place the slide in the Petri dish.

4. Place one drop of 3% hydrogen peroxide on the sample. Do not mix. Place the cover on the Petri dish to contain any aerosols.

5. Observe for immediate presence of bubble formation. Record your results.

6. Repeat Steps 2 through 5 for the remaining test organisms.

Notes and Results

MICROBIOLOGY OF SOIL

Soil is often thought of as an inert substance by the average person. However, contrary to this belief, it serves as a repository for many life forms, including a huge and diverse microbial population. The beneficial activities of these soil inhabitants far outweigh their detrimental effects. Life on this planet could not be sustained in the absence of microorganisms that inhabit the soil. This flora is essential for degradation of organic matter deposited in the soil, such as dead plant and animal tissues and animal wastes. Hydrolysis of these macromolecules by microbial enzymes supplies and replenishes the soil with basic elemental nutrients. By means of enzymatic transformations, plants assimilate these nutrients into organic compounds essential for their growth and reproduction. In turn, these plants serve as a source of nutrition for animals and man. Thus, many soil microorganisms play a vital role in a number of elemental cycles, such as the nitrogen cycle, the carbon cycle, and the sulfur cycle.

Nitrogen Cycle

The nitrogen cycle is concerned with the enzymatic conversion of complex nitrogenous compounds in the soil and atmosphere into nitrogen compounds that plants are able to use for the synthesis of essential macromolecules, including nucleic acids, amino acids, and proteins. The four distinct phases in this cycle are as follows:

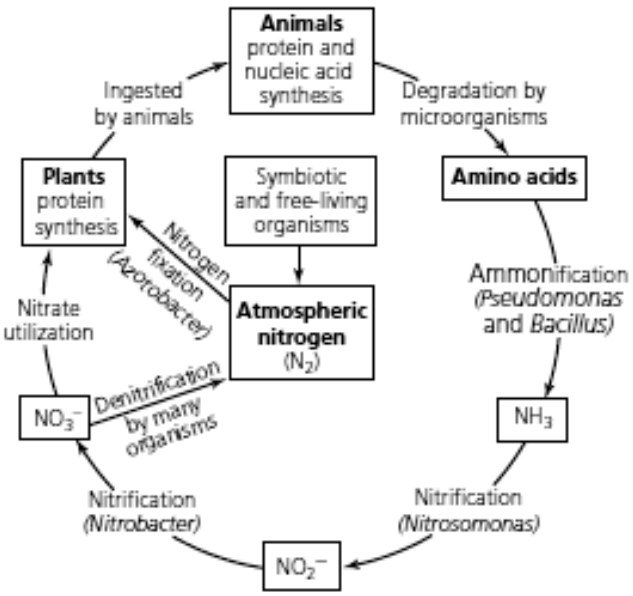
1. Ammonification: Soil microorganisms sequentially degrade nitrogenous organic compounds derived from dead plants and animals deposited in the soil. The degraded nitrogenous organic compounds are converted to inorganic nitrogen compounds and then to ammonia.

2. Nitrification: In this two- step process, (1) ammonia is oxidized to nitrite ions $NO2^{-2}$ by an aerobic species of *Nitrosomonas*, and then (2) nitrites are converted to nitrate ions $NO3^{-2}$ by another aerobic species,

Nitrobacter. Nitrates are released into the soil and are assimilated as a nutritional source by plants.

3. Denitrification: Nitrates $NO3^{-2}$ that are not used by plants are reduced to gaseous nitrogen (N_2) and are liberated back into the atmosphere by certain groups of microorganisms.

4. Nitrogen fixation: This vital process involves the chemical combination of gaseous nitrogen (N_2) with other elements to form fixed nitrogen (nitrogen- containing compounds), which are useful for plant growth.



The nitrogen cycle

The two types of microorganisms involved in this process are free- living and symbiotic. Free- living microorganisms include *Azotobacter, Pseudomonas, Clostridium,* and *Bacillus,* as well as some species of yeast. Symbiotic microorganisms, such as *Rhizobium*, grow in tumor- like nodules in the roots of leguminous plants, and use nutrients in the plant sap to fix gaseous nitrogen as ammonia for its subsequent assimilation into plant proteins. Animals then consume the leguminous plants and convert

plant protein to animal protein, completing the process. The nitrogen cycle is shown above.

Carbon Cycle

Carbon dioxide is the major carbon source for the synthesis of organic compounds. The carbon cycle is basically represented by two steps:

1. Oxidation of organic compounds to carbon dioxide with the production of energy and heat by heterotrophs.

2. Fixation of carbon dioxide into organic compounds by green plants and some bacteria, the autotrophic soil flora.

Sulfur Cycle

Elemental sulfur and proteins cannot be utilized by plants for growth. They must first undergo enzymatic conversions into inorganic sulfur-containing compounds. The basic steps in the sulfur cycle are:

1. Degradation of proteins into hydrogen sulfide (H_2S) by many heterotrophic microorganisms.

2. Oxidation of H_2S to sulfur (S) by a number of bacterial genera, such as *Beggiatoa.*

3. Oxidation of sulfur to utilizable sulfate SO_4 by several chemoautotrophic genera, such as *Thiobacillus.*

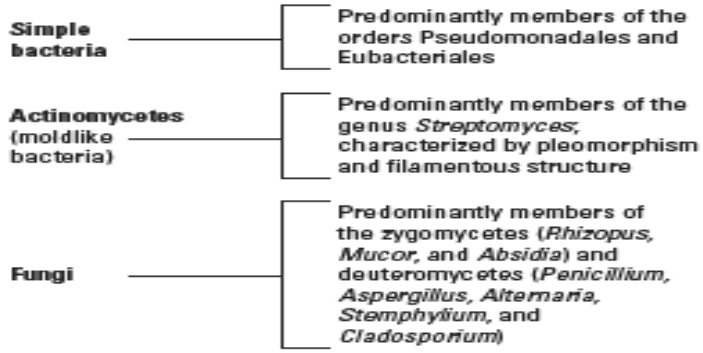
Some soil microorganisms also play a role in the enzymatic transformation of other elements, such as phosphorus, iron, potassium, zinc, manganese, and selenium. These biochemical changes make the minerals available to plants in a soluble form.

ENUMERATION OF SOIL MICROBES

Principle

Soil contains very diverse of microorganisms, including bacteria, fungi, protozoa, algae, and viruses. The most prevalent are bacteria, including the mold- like actinomycetes, and fungi. It is essential to bear in mind that the soil environment differs from one location to another and from one period

of time to another. Therefore, factors, including moisture, pH, temperature, gaseous oxygen content, and organic and inorganic composition of soil are crucial in determining the specific microbial flora of a particular sample.



Just as the soil differs, microbiological methods used to analyze soil also vary. A single technique cannot be used to count all the different types of microorganisms present in a given soil sample because no one laboratory cultivation procedure can provide all the physical and nutritional requirements necessary for the growth of a greatly diverse microbial population. In this experiment, only the relative numbers of bacteria, are determined. The method used is the serial dilution– agar plate procedure.

Materials

Soil

1 g sample of fine soil in a flask containing 99 ml of sterile water; flask labeled 1:100 dilution (10^{-2}).

Medium

four nutrient agar deep tubes, and two 99 ml flasks

of sterile water.

Equipment

Bunsen burner, 12 Petri dishes, colony counter, mechanical hand counter, sterile 1-ml pipettes, mechanical pipetting device, L- shaped bent glass

rod, turntable (optional), 95% alcohol in a 500 ml beaker, and glassware marking pencil.

Procedure Lab One

1. Liquefy the nutrient agar deep tubes in an autoclave or by boiling. Cool the molten agar tubes and maintain in a water bath at 45°C.

2. Using a glassware marking pencil, label the Petri dishes of nutrient agar as follows: 10−4, 10−5, 10−6, and 10−7 (to
be used for enumeration of bacteria).

3. With a glassware marking pencil, label the soil sample flask as Flask 1, and label the 99 ml sterile water Flasks 2 and 3.

4. Vigorously shake the provided soil sample dilution of 1:100 (10^{-2}) approximately 30 times, with your elbow resting on the table.

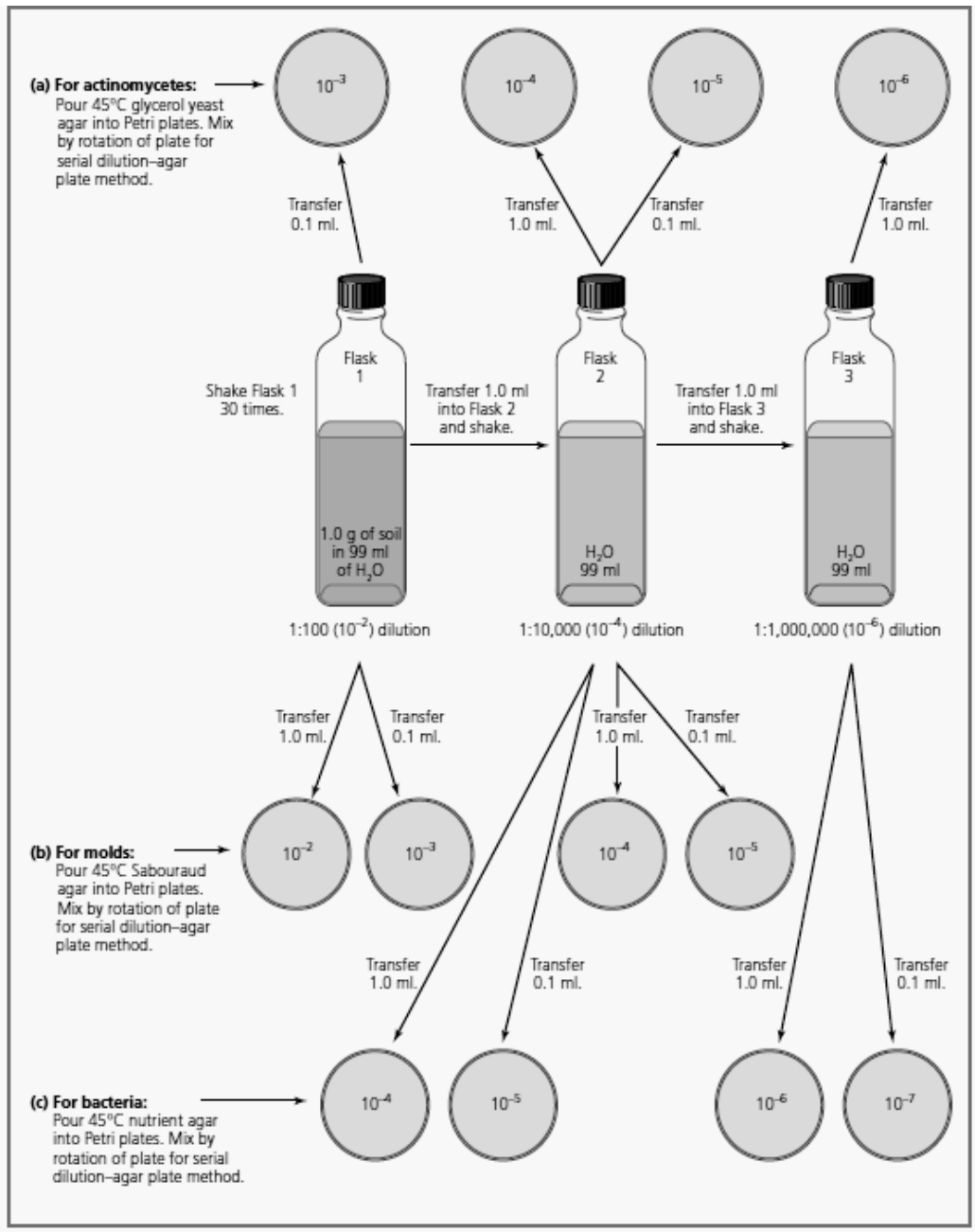
5. With a sterile 1 ml pipette, transfer 1 ml of the provided soil sample dilution to Flask 2 and shake vigorously as before. The final dilution is 1:10,000 (10^{-4}).

6. Using another sterile 1 ml pipette, transfer 1 ml of Dilution 2 to Flask 3 and shake vigorously as before. The final dilution is 1:1,000,000 (10^{-6}).

7. Using sterile 1 ml pipettes and aseptic technique, transfer 1 ml of dilution 2 into plate to effect a 10^{-4} dilution. Transfer 0.1 ml of Dilution 2 into plate to effect a 10^{-5} dilution. Transfer 1 ml of Dilution 3 into plate to effect a 10^{-6} dilution. Transfer 0.1 ml of Dilution 3 into plate to effect a 10^{-7} dilution.

8. Check the temperature of the molten agar medium to be sure that the temperature is 45°C. Remove the tubes from the water bath and wipe the outside surface dry with a paper towel. Using the pour plate technique, pour the liquefied agar into the plates as shown in the following Figure and rotate gently to ensure uniform distribution of the cells in the medium.

9. Incubate the plates in an inverted position at 25°C. Perform colony counts on nutrient agar plate cultures in 2 to 3 days.



Procedure for enumeration of soil microorganisms

Procedure Lab Two

1. Using an electronic colony counter or a colony counter and a mechanical hand counter, observe all the colonies on each nutrient agar plate 2 to 3 days after incubation begins. Plates with more than 300 colonies cannot be counted and should be designated as **too numerous to**

count (TNTC); plates with fewer than 30 colonies should be designated as **too few to count (TFTC)**. Count only plates with between 30 and 300 colonies.

2. Determine the number of organisms per milliliter of original culture on all plates other than those designated as TFTC or TNTC by multiplying the number of colonies counted by the dilution factor.

3. Record your observations and calculated cell counts per gram of sample in the Lab Report chart.

Organism	Dilution	Number of Colonies	Organisms per Gram of Soil
Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		
Actinomycetes	10^{-3}		
	10^{-4}		
	10^{-5}		
	10^{-6}		
Molds	10^{-2}		
	10^{-3}		
	10^{-4}		
	10^{-5}		

Results and observations

Nitrogen- Fixing Bacteria

Among the most beneficial microorganisms of the soil are those that are able to convert gaseous nitrogen of the air to "fixed forms" of nitrogen that can be utilized by other bacteria and plants. Without these nitrogen-fixers, life on this planet would probably disappear within a relatively short period of time. The utilization of free nitrogen gas by fixation can be accomplished by organisms that are able to produce the essential enzyme **nitrogenase**. This enzyme, in the presence of traces of molybdenum, enables the organisms to combine atmospheric nitrogen with other elements to form organic compounds in living cells. In organic combinations nitrogen is more reduced than when it is free. From these organic compounds, upon their decomposition, the nitrogen is liberated in a fixed form, available to plants either directly or through further microbial action.

The most important nitrogen-fixers belong to two families: **Azotobacteraceae** and **Rhizobiaceae.** Other organisms of less importance that have this ability are a few strains of *Klebsiella,* some species of *Clostridium,* the cyanobacteria, and photosynthetic bacteria.

In this exercise we will concern ourselves with two activities: the isolation of *Azotobacter* from garden soil and the demonstration of *Rhizobium* in root nodules of legumes.

AZOTOBACTERACEAE

Bergey's Manual of Systematic Bacteriology, volume 1, section 4, lists two genera of bacteria in family Azotobacteraceae that fix nitrogen as free-living organisms under aerobic conditions: *Azotobacter* and *Azomonas.* The basic difference between these two genera is that *Azotobacter* produces drought resistant cysts and *Azomonas* does not. Aside from the presence or absence of cysts, these two genera are very

similar. Both are large gram- negative motile rods that may be ovoid or coccoidal in shape (pleomorphic). Catalase is produced by both genera.

There are six species of *Azotobacter* and three species of *Azomonas.*

The following Figure illustrates the overall procedure for isolating Azotobacteraceae from garden soil. Note that a small amount of rich garden soil is added to a bottle of nitrogen- free medium that contains glucose as a carbon source. The bottle of medium is incubated in a horizontal position for 4 to 7 days at 30° C.

After incubation, a wet mount slide is made from surface growth to see if typical azotobacterlike organisms are present. If organisms are present, an agar plate of the same medium, less iron, is used to streak out for isolated colonies. After another 4 to 7 days incubation, colonies on the plate are studied and more slides are made in an attempt to identify the isolates.

The N_2- free medium used here contains glucose for a carbon source and is completely lacking in nitrogen. It is selective in that only organisms that can use nitrogen from the air and use the carbon in glucose will grow on it. All species of *Azotobacter* and *Azomonas* are able to grow on it. The metallic ion molybdenum is included to activate the enzyme nitrogenase, which is involved in this process.

FIRST PERIOD (ENRICHMENT)

Proceed as follows to inoculate a bottle of the nitrogen- free glucose medium with a sample of garden soil.

Materials:

- 1 bottle (50 ml) N_2-free glucose medium (Thompson-Skerman)
- rich garden soil (neutral or alkaline)
- spatula

1. With a small spatula, put about 1 gm of soil into the bottle of medium. Cap the bottle and shake it sufficiently to mix the soil and medium.

2. Loosen the cap slightly and incubate the bottle at 30° C for 4 to 7 days. Since the organisms are strict aerobes, it is best to incubate the bottle horizontally to provide maximum surface exposure to air.

SECOND PERIOD (PLATING OUT)

During this period a slide will be made to make certain that organisms have grown on the medium. If the culture has been successful, a streak plate will be made on nitrogen- free, iron- free agar. Proceed as follows:

Materials:

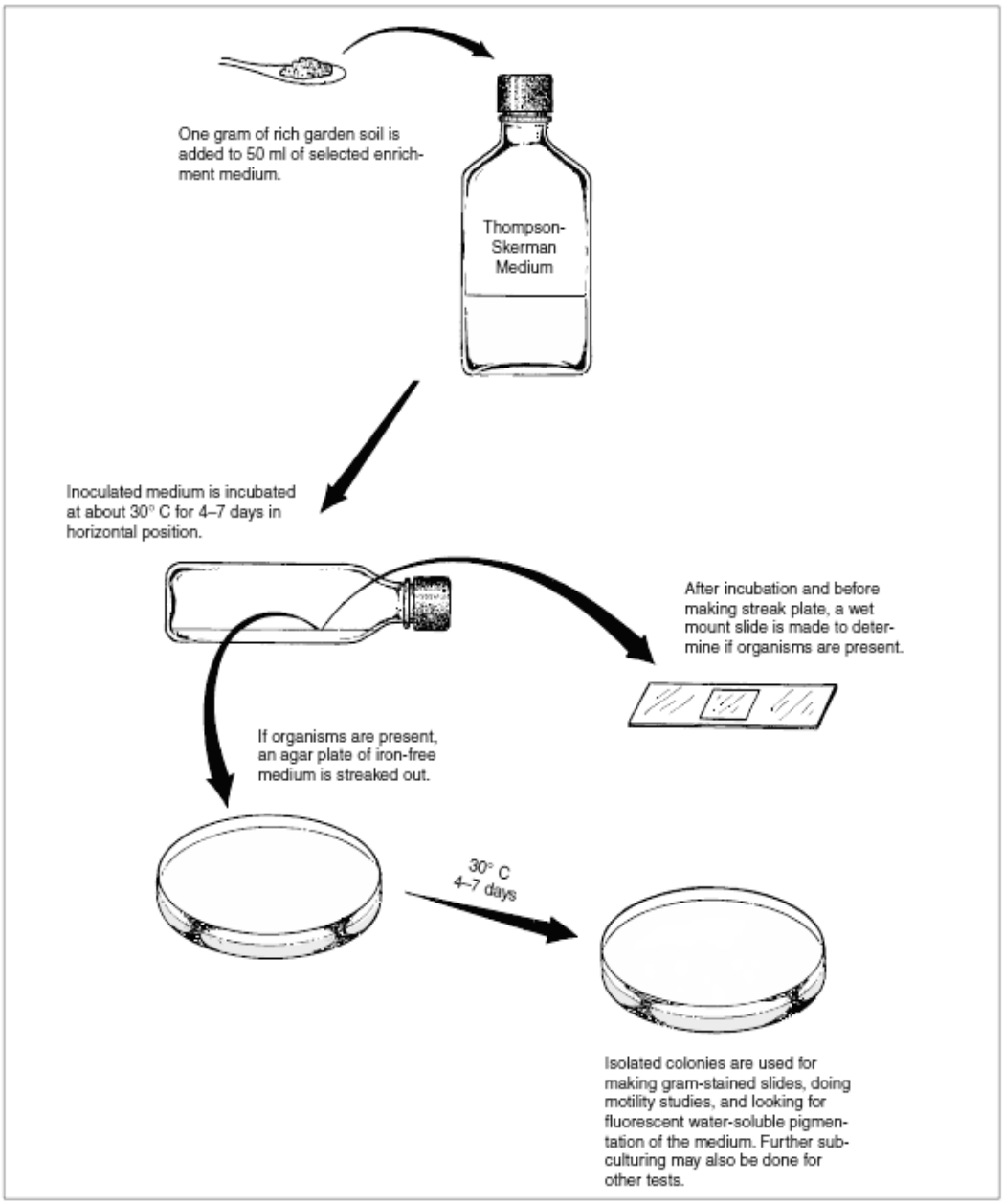
- microscope slides and cover glasses
- microscope with phase-contrast optics
- 1 agar plate of nitrogen- free, iron- free glucose medium

1. After 4 to 7 days incubation, carefully move the bottle of medium to your desktop without agitating the culture.
2. Make a wet mount slide with a few loopfuls from the surface of the medium and examine under oil immersion, preferably with phase- contrast optics. Look for large ovoid to rod- shaped organisms, singly and in pairs.
3. If azotobacter- like organisms are seen, note whether or not they are motile and if cysts are present. Cysts look much like endospores in that they are refractile. Since cysts often take 2 weeks to form, they may not be seen.
4. If the presence of azotobacter- like organisms is confirmed, streak an agar plate of nitrogen- free, iron- free medium, using a good isolation streak pattern. Ferrous sulfate has been left out of this medium to facilitate the detection of water- soluble pigments.
5. Incubate the plate at 30° C for 4 or 5 days. A longer period of incubation is desirable for cyst formation.

Alternatively, a more simple procedure can be as follows:

The N- free medium is inoculated in one or two plates by spreading some soil o the surface. After incubation for one to two days mucous colonies

on the surface of solid medium is indicative of N- fixing organisms. Take some of the mucous growth on a clean slide , stain with Gram staining and observe under light microscope by using oil immersion lens then proceed with step (3) above.



Enrichment and isolation procedure for *Azotobacter* and *Azomonas*

Microbiology of Water

The microorganisms of natural waters are extremely diverse. The numbers and types of bacteria present will depend on the amounts of organic matter present, the presence of toxic substances, the water's saline content, and environmental factors such as pH, temperature, and aeration. The largest numbers of heterotrophic forms will exist on the bottoms and banks of rivers and lakes where organic matter predominates. Open water in the center of large bodies of water, free of floating debris, will have small numbers of bacteria. Many species of autotrophic types are present, however, that require only the dissolved inorganic salts and minerals that are present.

The threat to human welfare by contamination of water supplies with sewage is a prime concern of everyone. The enteric diseases such as cholera, typhoid fever, and bacillary dysentery often result in epidemics when water supplies are not properly protected or treated. Thus, our prime concern in this unit is the sanitary phase of water microbiology.

Bacteriological Examination of Water:

Qualitative Tests

Principle

The three basic tests to detect coliform bacteria in water are presumptive, confirmed, and completed. The tests are performed sequentially on each sample under analysis. They detect the presence of coliform bacteria (indicators of fecal contamination), the Gram -ve, non–spore- forming bacilli that ferment lactose with the production of acid and gas that is detectable following a 24-hour incubation period at 37°C.

The Presumptive test

The **presumptive test** is specific for detection of coliform bacteria. Measured aliquots of the water to be tested are added to a lactose fermentation broth containing an inverted gas vial. Because these bacteria

are capable of using lactose as a carbon source (the other enteric organisms are not), their detection is facilitated by the use of this medium. In this experiment, you will use lactosefermentation broth containing an inverted Durhamtube for gas collection.

Tubes of this lactose medium are inoculated with 10-ml, 1-ml, and 0.1-ml aliquots of the water sample. The series consists of at least three groups, each composed of five tubes of the specified medium. The tubes in each group are then inoculated with the designated volume of the water sample, as described under "Procedure: Lab One."

The greater the number of tubes per group, the greater the sensitivity of the test. Development of gas in any of the tubes is **presumptive** evidence of the presence of coliform bacteria in the sample. The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the **most probable number (MPN) test**. The MPN is estimated by determining the number of tubes in each group that show gas following the incubation period (**MPN Tables are shown later**).

The Confirmed test

The presence of a positive or doubtful presumptive test immediately suggests that the water sample is nonpotable. Confirmation of these results is necessary because positive presumptive tests may be the result of organisms of noncoliform origin that are not recognized as indicators of fecal pollution. The **confirmed test** requires that selective and differential media (e.g., eosin–methylene blue (EMB) or Endo agar) be streaked from a positive lactose broth tube obtained from the presumptive test. The nature of the differential and selective media was discussed before. Eosin–methylene blue contains the dye methylene blue, which inhibits the growth of Gram +ve organisms. In the presence of an acid environment, EMB forms a complex that precipitates out onto the coliform colonies,

producing dark centers and a green metallic sheen. The reaction is characteristic for *Escherichia coli*, the major indicator of fecal pollution. Endo agar is a nutrient medium containing the dye fuchsin, which is present in the decolorized state. In the presence of acid produced by the coliform bacteria, fuchsin forms a dark pink complex that turns the *E. coli* colonies and the surrounding medium pink.

The Completed test

The **completed test** is the final analysis of the water sample. It is used to examine the coliform colonies that appeared on the EMB or Endo agar plates used in the confirmed test. An isolated colony is picked up from the confirmatory test plate and inoculated into a tube of lactose broth and streaked on a nutrient agar slant to perform a Gram stain. Following inoculation and incubation, tubes showing acid and gas in the lactose broth and presence of Gram -ve bacilli on microscopic examination are further confirmation of the presence of *E. coli,* and they are indicative of a positive completed test.

Media

Lab One (per student group): 15 double strength lactose fermentation broths (LB2X) and 30 single strength lactose fermentation broths (LB1X).
Lab Two (three each per student group): eosin–methylene blue agar plates or Endo agar plates.
Lab Three (three each per student group): nutrient agar slants and lactose fermentation broths.

Reagents

Lab Three: Crystal violet, Gram's iodine, 95% ethyl alcohol, and safranin.

Equipment

Lab One: Bunsen burner, 45 test tubes, test tube rack, sterile 10-ml pipettes, sterile 1-ml pipettes, sterile 0.1-ml pipettes, mechanical pipetting device, and glassware marking pencil.

Lab Two: Bunsen burner, glassware marking pencil, and inoculating loop.

Lab Three: Bunsen burner, staining tray, inoculating loop, lens paper, bibulous paper, microscope, and glassware marking pencil.

Procedure Lab One

Presumptive test

Exercise care in handling sewage waste water sample because enteric pathogens may be present**.**

1. Set up three separate series consisting of three groups, a total of 15 tubes per series, in a test tube rack; for each tube, label the water source and volume of sample inoculated as illustrated below.

Series 1: Sewage water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml
Series 2: Pond water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml
Series 3: Tap water	5 tubes of LB2X-10 ml 5 tubes of LB1X-1 ml 5 tubes of LB1X-0.1 ml

2. Mix sewage plant water sample by shaking thoroughly.

3. Flame bottle and then, using a 10-ml pipette, transfer 10-ml aliquots of water sample to the five tubes labeled LB2X-10 ml.

4. Flame bottle and then, using a 1-ml pipette, transfer 1-ml aliquots of water sample to the five tubes labeled LB1X-1 ml.

5. Flame bottle and then, using a 0.1-ml pipette, transfer 0.1-ml aliquots of water sample to the five tubes labeled LB1X-0.1 ml.

6. Repeat Steps 2 through 5 for the tap and pond water samples.

7. Incubate all tubes for 48 hours at 37°C.

Procedure Lab Two

Presumptive test

1. Examine the tubes from your presumptive test after 24 and 48 hours of incubation. Your results are positive if the Durham tube fills 10% or more

with gas in 24 hours, doubtful if gas develops in the tube after 48 hours, and negative if there is no gas in the tube after 48 hours. Record your results in the Lab Report.

2. Determine the MPN using the following Table, and record your results in the Lab Report.

Confirmed test

1. Label the covers of the three EMB plates or the three Endo agar plates with the source of the water sample (sewage, pond, and tap).

2. Using a positive 24-hour lactose broth culture from the sewage water series from the presumptive test, streak the surface of one EMB or one Endo agar plate, as described in Experiment 3, to obtain discrete colonies.

3. Repeat Step 2 using the positive lactose broth cultures from the pond and tap water series from the presumptive test to inoculate the remaining plates.

4. Incubate all plate cultures in an inverted position for 24 hours at 37°C.

Procedure Lab Three

Confirmed test

1. Examine all the plates from your confirmed test for the presence or absence of *E. coli* colonies (refer to the description of the confirmed test in the experiment introduction). Record your results in the Lab Report.

2. Based on your results, determine whether each of the samples is potable or nonpotable.

The presence of *E. coli* is a positive confirmed test, indicating that the water is nonpotable. The absence of *E. coli* is a negative test, indicating that the water is not contaminated with fecal wastes and is therefore potable. Record your results in the Lab Report.

Completed test

1. Label each tube of nutrient agar slants and lactose fermentation broths with the source of its water sample.

2. Inoculate one lactose broth and one nutrient agar slant with a positive isolated *E. coli* colony obtained from each of the experimental water samples during the confirmed test.

3. Incubate all tubes for 24 hours at 37°C.

Procedure Lab Four

Completed test

1. Examine all lactose fermentation broth cultures for the presence or absence of acid and gas. Record your results in the Lab Report.

2. Prepare a Gram stain, using the nutrient agar slant cultures of the organisms that showed a positive result in the lactose fermentation broth.

3. Examine the slides microscopically for the presence of Gram -ve short bacilli, that are indicative of *E. coli* and thus nonpotable water. In the Lab Report, record your results for Gram stain reaction and morphology of the cells.

The MPN Index per 100 ml for Combinations of Positive and Negative Presumptive Test Results When Five 10-ml, Five 1-ml, and Five 0.1-ml Portions of Sample Are Used

NUMBER OF TUBES WITH POSITIVE RESULTS						NUMBER OF TUBES WITH POSITIVE RESULTS					
FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS		FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS	
				LOWER	UPPER					LOWER	UPPER
0	0	0	<2	0	6	4	2	1	26	7	67
0	0	1	2	<0.5	7	4	3	0	27	9	78
0	1	0	2	<0.5	7	4	3	1	33	9	78
0	2	0	4	<0.5	11	4	4	0	34	11	93
1	0	0	2	0.1	10	5	0	0	23	7	70
1	0	1	4	0.7	10	5	0	1	31	11	89
1	1	0	4	0.7	12	5	0	2	43	14	100
1	1	1	6	1.8	15	5	1	0	33	10	100
1	2	0	6	1.8	15	5	1	1	46	14	120
2	0	0	5	<0.5	13	5	1	2	63	22	150
2	0	1	7	1	17	5	2	0	49	15	150
2	1	0	7	1	17	5	2	1	70	22	170
2	1	1	9	2	21	5	2	2	94	34	230
2	2	0	9	2	21	5	3	0	79	22	220
2	3	0	12	3	28	5	3	1	110	34	250
3	0	0	8	2	22	5	3	2	140	52	400
3	0	1	11	4	23	5	3	3	180	70	400
3	1	0	11	5	35	5	4	0	130	36	400
3	1	1	14	6	36	5	4	1	170	58	400
3	2	0	14	6	36	5	4	2	220	70	440
3	2	1	17	7	40	5	4	3	280	100	710
3	3	0	17	7	40	5	4	4	350	100	710
4	0	0	13	4	35	5	5	0	240	70	710
4	0	1	17	6	36	5	5	1	350	100	1100
4	1	0	17	6	40	5	5	2	540	150	1700
4	1	1	21	7	42	5	5	3	920	220	2600
4	1	2	26	10	70	5	5	4	1600	400	4600
4	2	0	22	7	50	5	5	5	≥2400	700	– – –

Observations and results

Presumptive test

Using he above Table, determine and record the MPN.

Example: If gas appeared in all five tubes labeled LB2X–10, in two of the tubes labeled LB1X–1, and in one labeled LB1X–0.1, the series would be read as 5-2-1. From the MPN table, such a reading would indicate approximately 70 microorganisms per 100 ml of water, with a 95% probability that between 22 and 170 microorganisms are present.

	GAS																
	LB2X–10					LB1X–1					LB1X–0.1						
	Tube					Tube					Tube						
Water Sample	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	Reading	MPN	95% Probability Range
Sewage																		
Pond																		
Tap																		

Confirmed test

Water Sample	COLIFORMS		Potable	Nonpotable
	EMB Plate	Endo Agar Plate		
Sewage				
Pond				
Tap				

Completed test

		GRAM STAIN	POTABILITY	
Water Source	Lactose Broth A/G (+) or (–)	Reaction/ Morphology	Potable	Nonpotable
Sewage				
Pond				
Tap				